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The octamer-binding proteins form multi-protein-DNA complexes with the HSV αTIF regulatory protein

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The herpes simplex virus transactivator, αTIF, stimulates transcription of the α/immediate early genes via a *cis*-acting site containing an octamer element and a conserved flanking sequence. The αTIF protein, produced in a baculovirus expression system, nucleates the formation of at least two DNA-protein complexes on this regulatory element. Both of these complexes contain the ubiquitous Oct-1 protein, whose POU domain alone is sufficient to allow assembly of the αTIF-dependent complexes. A second member of the POU domain family, the lymphoid specific Oct-2 protein, can also be assembled into similar complexes at high concentrations of αTIF protein. These complexes contain at least two cellular proteins in addition to Oct-1. One of these proteins is present in both insect and HeLa cells and probably recognizes sequences in the *cis* element. The second cellular protein, only present in HeLa cells, probably binds by protein-protein interactions.

Key words: Baculovirus/DNA-protein complex/octamer-binding proteins/POU domain/protein-protein interactions

Introduction

The promoters and enhancers of eukaryotic genes are composed of arrangements of *cis* elements whose potential for transcriptional stimulation is mediated by sequence specific DNA binding proteins (Jones *et al.*, 1988; Maniatis *et al.*, 1987). However, in many cases, promoters with distinct patterns of regulation share an apparently identical regulatory sequence. The octamer element (5'-ATGCAAAT-3') is one such site, being represented in the promoters and enhancers of such divergently regulated genes as those encoding histones, snRNAs, viral regulatory proteins and immunoglobulins (Gilles *et al.*, 1983; Falkner and Zachau, 1984; Parslow *et al.*, 1984; Augereau and Chambon, 1986; Sive and Roeder, 1986; Ares *et al.*, 1987). Some of this distinct regulation is accomplished by cell type specific proteins which recognize the common octamer element (Landolfi *et al.*, 1986; Staudt *et al.*, 1986; Scheidereit *et al.*, 1987; Wang *et al.*, 1987).

Oct-1, an octamer binding protein which is constitutively expressed in many mammalian cell types, is probably responsible for the activation of several cell type independent promoters (Singh *et al.*, 1986; Sive *et al.*, 1986; Bark *et al.*, 1987; Carbon *et al.*, 1987; Flecher *et al.*, 1987; Murphy *et al.*, 1987). Oct-2, expressed primarily in lymphoid cells, is most likely responsible for the cell specific activation of immunoglobulin gene transcription (Mizushima-Sugano and Roeder, 1986; Staudt, *et al.*, 1986; Dreyfuss *et al.*, 1987; Wirth *et al.*, 1987; LeBowitz *et al.*, 1988; Müller *et al.*, 1988). Although products of distinct genes, these proteins share a highly conserved domain (POU domain) which has been subdivided into two regions termed the POU specific box and the POU homeobox (Clerc *et al.*, 1988; Herr *et al.*, 1988; Ko *et al.*, 1988; Sturm *et al.*, 1988). Collectively, these two regions are responsible for the high affinity, sequence specific binding of the Oct proteins to the octamer element (Sturm and Herr, 1988; Garcia-Blanco *et al.*, 1989). Therefore, although the expression of the Oct-2 protein in a tissue specific manner may partially account for the selective high level transcription of the immunoglobulin genes, the high degree of homology between the Oct-1 and Oct-2 DNA binding domains and the lack of any indication that the two proteins bind the octamer element with different affinities (Staudt *et al.*, 1986), suggests that additional components are important in the transcriptional regulation of octamer-containing genes.

The α-*trans*-induction factor (αTIF, also referred to as VP16, ICP25 and VMW65) is a 64 000 dalton protein, encoded by herpes simplex virus (HSV), and packaged within the tegument structure of the virion (Roizman and Furlong, 1974; Batterson and Roizman, 1983). A potent transactivator, αTIF induces the transcription of the five viral α/immediate early genes (Post *et al.*, 1981; Campbell *et al.*, 1984; Pellet *et al.*, 1985). Although not a DNA binding protein (Marsden *et al.*, 1987), the selective induction of transcription by αTIF is dependent upon reiterated elements in the promoter regulatory domains of α genes which contain a homolog of the octamer element (GyATGnTAATGArATTCyTTGnGGG, TAATGArAT site) (Mackem and Roizman, 1982a-c; Kristie and Roizman, 1984; reviewed in McKnight *et al.*, 1986; Pruijn *et al.*, 1986). This homolog binds a cellular protein, originally identified as α-H1, which was proposed to be required for the assembly of the αTIF protein into a specific protein-DNA complex (Kristie and Roizman, 1987, 1988; McKnight *et al.*, 1987). Recently, the α-H1 factor has been tentatively identified as the ubiquitous Oct-1 protein (Gerster and Roeder, 1988; O'Hare and Goding, 1988). Thus, the HSV α genes provide an ideal system for the study of the differential regulation mediated by specific octamer sites.

We have purified the αTIF protein from a recombinant baculovirus system and have used this protein to assay for interactions with cellular proteins. The results demonstrate that multiple cellular factors interact with αTIF to generate successively more complicated DNA-protein complexes.

Results

Production and fractionation of αTIF

A baculovirus−αTIF gene recombinant virus was constructed by *in vivo* recombination of wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and a baculovirus expression vector (pVL941) containing the entire αTIF coding sequences under the control of the late polyhedrin promoter. One recombinant, AcNPV−αTIF was isolated and the expression of αTIF was characterized after infection of the SF9 insect cell line. Mock infected, AcNPV-αTIF infected and control recombinant virus (Vler) infected cells were harvested, and total cell, cytoplasmic (S100) and nuclear extracts were prepared. Aliquots of the extracts were resolved in SDS−polyacrylamide gels and stained with Coomassie Brilliant Blue. A novel protein product of ~60 kd was clearly visible in the total cell and S100 AcNPV-αTIF infected cell extracts. To clearly identify this protein as αTIF, identical SDS−polyacrylamide gels were transferred to nitrocellulose and blotted with an anti-peptide−αTIF rabbit sera. As anticipated, the sera specifically reacted with the novel 60 kd protein (data not shown).

S100 extracts prepared from production-scale infections with AcNPV-αTIF were compared with a titration of bovine serum albumin standard by densitometric scanning of Coomassie stained SDS−polyacrylamide gels. Under these conditions, αTIF represented ~3% of the total soluble S100 protein (20 mg of αTIF/5 × 10^9 infected SF9 cells). These S100 extracts were used for many of the protein−DNA binding studies detailed below. However, for some experiments, αTIF was purified by chromatography of the S100 extract on DEAE−Sepharose CL6B (fraction TIF-D26). The recombinant protein eluted from the column at 0.35 M KCl and was judged to be ~70% pure by analysis in SDS−polyacrylamide gels (data not shown).

αTIF-dependent formation of multiple DNA−protein complexes

The S100 extract of AcNPV-αTIF infected cells (TIFS100) generated several αTIF-dependent DNA−protein complexes in a gel mobility assay. The formation of these complexes was dependent upon the successive addition of discrete cellular extracts and chromatographic fractions. As exemplified in Figure 1A, the HSVα0 regulatory element (refer to Figure 2B) forms one major DNA−protein complex (designated Oct-1) when mixed with a nuclear extract of HeLa cells. Competition assays with HSV and octamer DNA fragments demonstrated that this was the anticipated Oct-1−DNA complex (data not shown). No complex was detected in reactions containing the TIFS100 extract, indicating that infected SF9 cell extracts do not contain an appreciable level of an octamer binding protein (data not shown). When the TIFS100, control infected (Vler), or mock infected extracts were mixed with the nuclear extract of HeLa cells, several novel DNA−protein complexes were generated specifically in reactions containing the TIFS100 extract (see complexes C1 and C2 in Figure 1A and data not shown). To begin to identify the protein components required for the formation of the C1 and C2 complexes, HeLa nuclear extract was chromatographed on an octamer specific oligonucleotide column to generate Oct-1 depleted (ΔHeLa) and Oct-1 enriched (Oct1bd) fractions

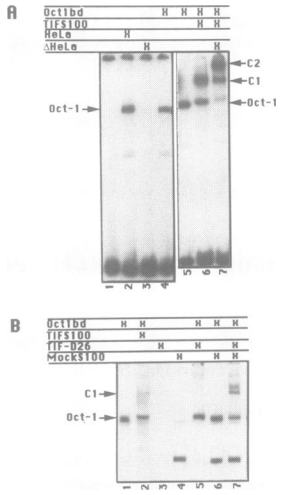

Fig. 1. Multiple αTIF-dependent DNA−protein complexes are formed in the presence of distinct protein extracts. Protein−DNA binding assays were carried out essentially as described in Materials and methods. The probe DNA, HSVα0 was derived from pRB608 and contains the αTIF response element from −168 to −142 of the α0 promoter (Mackem and Roizman, 1980). The protein extracts included in each binding reaction are indicated with an x at the top of the autoradiogram, and are as follows: Oct1bd, affinity-selected Oct-1 protein from HeLa cells; TIFS100, S100 extract prepared from SF9 cells infected with AcNPV-αTIF virus; HeLa, nuclear extract from HeLa cells; ΔHeLa, nuclear extract from HeLa cells which was depleted of Oct-1 protein by affinity chromatography; TIF-D26, purified αTIF protein resulting from chromatography of TIFS100 extract on DEAE−Sepharose CL6B; MockS100, S100 extract prepared from mock-infected SF9 cells. All reactions were brought to an equivalent protein concentration with the addition of bovine serum albumin. The position of the Oct-1 protein−DNA complex (Oct-1) and the αTIF dependent C1 and C2 complexes are indicated with arrows. (**A**) **Lane 1** contains the HSVα0 probe in the absence of protein extracts. Reactions 1−4 and 5−7 were electrophoresed on separate but parallel nondenaturing gels.

(Figure 1, lanes 3 and 4). Addition of TIFS100 extract to the Oct1bd fraction results in the generation of both the Oct-1−DNA complex and the slower mobility C1 complex (lane 6). Addition of higher concentrations of either the Oct1bd or TIFS100 fractions did not generate new complexes. To generate the C2 complex, evident in reactions containing complete HeLa nuclear and TIFS100 extracts, addition of the ΔHeLa fraction was required (lane 7). This implies that a minimum of three distinct components (Oct-1, αTIF and ΔHeLa-C2 factor) are required for the formation of the C2 protein−DNA complex.

In contrast to the results obtained with the TIFS100 extract, addition of αTIF, purified by chromatography on DEAE−Sepharose (TIF-D26), to the Oct1bd fraction did not generate the C1 complex (Figure 1B, lanes 1, 2, 3 and 5). Generation of the C1 complex required the addition of a third component(s), which could be supplied by an S100 extract of mock-infected insect cells (lanes 4, 6 and 7). Thus, the C1 complex consists of a minimum of three distinct components: Oct-1, αTIF and C1 factor. If the C1 complex is a precursor to the novel C2 complex, the latter must consist of a minimum of four distinct components: Oct-1, αTIF, C1 factor and ΔHeLa-C2 factor. While both C1 and C2 factors are present in the HeLa cell extract, only C1 factor can be supplied by the insect cell extract.

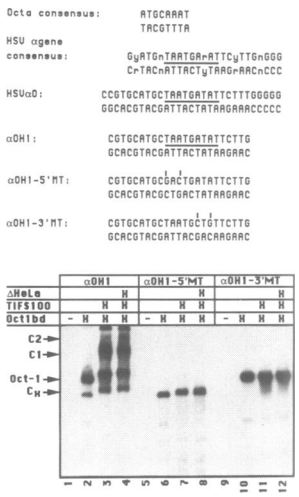

Fig. 2. Formation of C1 and C2 complexes depends upon the octamer and HSV specific sequence elements. The DNA sequence of the octamer and HSV α gene (Mackem and Roizman, 1982b) consensus elements are aligned above the various wild type and mutant DNA probes used in this study. HSVα0 has been described (Kristie and Roizman, 1984). αOH1 consists of sequences from −167 to −145 of the HSV α0 gene. The highly conserved core TAATGArAT element is underlined. αOH1−5′MT and αOH1−3′MT contain a double point mutation, indicated with a |, in the octamer element or the HSV specific 3′ sequences respectively of the αOH1 element. The DNA binding assay, shown above, was carried out as described in Figure 1 and Materials and methods. The DNA probe used in each set of four reactions is indicated at the top of the gel. (−) reactions contained DNA probe in the absence of protein extracts (**lanes 1, 5** and **9**). All other binding reactions contained the affinity selected Oct-1 protein (Oct1bd) either alone (**lanes 2, 6** and **10**) or in addition to the protein extracts indicated above the gel lane. The relevant DNA−protein complexes are noted with arrows.

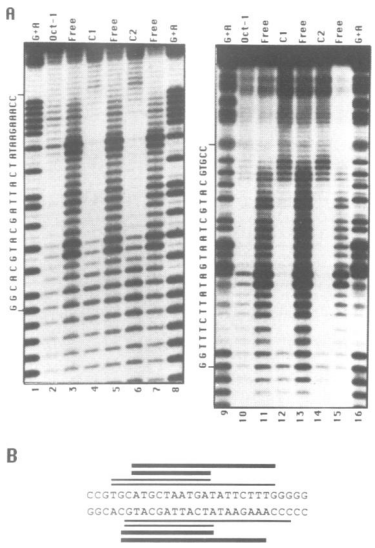

Fig. 3. Orthophenanthroline·Cu protection of Oct-1−DNA, C1 and C2 complexes. (**A**) Orthophenanthroline·Cu cleavage reactions of electrophoretically resolved Oct-1−DNA, C1, and C2 complexes were carried out as described in Materials and methods. Affinity selected Oct1bd fraction was used as a source of the Oct-1 protein. C1 and C2 were generated by the addition of Oct1bd and TIFS100 or Oct1bd, TIFS100 and ΔHeLa extracts respectively. The purified DNA cleavage products were resolved in a 10% sequencing gel. Free represents the cleavage products of the unbound DNA from the appropriate binding reaction. G+A is a guanine plus adenine sequencing ladder (Maxam and Gilbert, 1980). The nucleotide sequence of the HSVα0 DNA probe is written (5′−3′, top to bottom) at the left of each reaction set for the coding (left panel, lanes 1−8) and noncoding (right panel, lanes 9−16) strands. (**B**) Thin and thick bars schematically delineate the boundaries of the protection from DNase I and orthophenanthroline·Cu cleavage respectively. Short bars represent protection by the Oct-1 protein while the longer bars represent the protection by the components of the C1 and C2 complexes.

DNA sequence specificity of the C1 and C2 complexes

The consensus DNA sequence for HSV αTIF-dependent transactivation is compared to the octamer sequence in Figure 2. The HSV consensus (Mackem and Roizman, 1982b) was derived from *in vivo* analysis of multiple elements in the regulatory domains of the five α/IE genes. These elements can be considered bipartite recognition sites composed of a 7 of 8 nucleotide homology to the octamer element, adjacent to a second set of sequences common to the α/IE genes of HSV.

The αOH1 DNA probe, which contains the most highly conserved core TAATGArAT sequence, bound the Oct-1 protein (Figure 2, lane 2) and formed the C1 complex and C1 and C2 complexes when TIFS100 or TIFS100 + ΔHeLa extracts were also added to the reactions respectively (lanes 3 and 4). A double point mutant in the octamer element of this sequence, αOH1-5′MT, did not bind the Oct-1 protein and, upon addition of TIFS100 or TIFS100 + ΔHeLa fractions to the reaction, did not generate the C1 or C2 complex (lanes 5−8). However, similar to the wild-type element, this probe did form a DNA−protein complex (designated C_x) which migrated faster than the Oct-1−DNA complex. Conversely, αOH1-3′MT, which contains a double point mutation in the conserved sequences immediately flanking the octamer site, retained the ability to bind Oct-1 with high affinity but did not form the C_x complex. However, similar to the 5′ point mutant, the 3′ point mutant also was incapable of forming the C1 and C2 complexes upon addition of the TIFS100 or TIFS100 + ΔHeLa fractions (lanes 9−12). Thus formation of these αTIF-dependent complexes strictly requires both the octanucleotide homolog and the flanking HSV specific element.

It is interesting that the C_x complex exhibits a specificity for the 3′ HSV specific sequences and is present in fractions eluted from the octa-specific affinity column. The nature of the protein generating this complex has not been further studied but the fact that the C_x complex is not formed with the probe αOH1-3′MT suggests it is a sequence specific binding protein.

Further evidence for the specificity of the C1 and C2 complexes as well as their strong dependence upon the octamer element of the HSV sequences was derived from competition studies. HSVα27 and HSVα0 fragments contain the HSV αTIF responsive element from −183 to −134 of the α27 gene and −168 to −142 of the α0 gene respectively. These competitor DNAs efficiently abolished the formation of the Oct-1−DNA, C1 and C2 complexes when added to binding reactions at a 10-fold molar excess over the HSVα0 probe. Similarly, competitor DNAs containing the octa site (ATGCAAAT) including one derived from the immunoglobulin heavy chain promoter (CATGAGTATGCAAAT CATGTG, μprom+) effectively blocked formation of the Oct-1−DNA, C1 and C2 complexes at a 10- to 30-fold molar excess over the HSVα0 probe. Addition of similar

ratios of control DNAs, containing the NFκB/H2TF1 (Baldwin and Sharp, 1987) or NF1 binding sites, had no effect. Interestingly, when α0H1-5′MT DNA was added as a competitor at a molecular ratio of 30:1, no competition was evident even though this DNA retains the HSV specific 3′ sequences. Finally, the specificity of the C1 and C2 complexes, with respect to the HSV specific 3′ sequence, was also evident by the inability of the μprom+ probe DNA to form these complexes under similar assay conditions (data not shown).

Protection from both DNase I and orthophenanthroline·Cu cleavage was used to define the DNA sequences recognized by proteins in the C1 and C2 complexes. Only the latter results are presented in detail while results with DNase I are summarized in Figure 3B. The HSVα0 probe was incubated with Oct1bd fraction, Oct1bd and TIFS100 fractions, or Oct1bd, TIFS100 and ΔHeLa fractions to assemble the Oct-1−DNA, C1 and C2 complexes respectively. The reactions were subjected to gel electrophoresis and the resolved complexes were cleaved with orthophenanthroline·Cu *in situ*. DNA was eluted from the individual bands and analyzed on a sequencing gel. The protection patterns of the orthophenanthroline·Cu reactions are shown in Figure 3A. As previously noted (Singh *et al.*, 1986; Kristie and Roizman, 1988), the Oct-1 protein protected the sequence 5′-ATGCTAATGA-3′ and 5′-TCATTAGCATG-3′ on the two strands (Figure 3A, lanes 2 and 10). This sequence contains the homolog of the octamer element in the 5′ part of the HSV sequence (Figure 3B and refer to Figure 2). Complexes C1 and C2 both exhibited protection patterns which encompassed the sequences protected in the Oct-1 complex and extended over the flanking sequences conserved in the HSV α/immediate early promoters (5′-ATGCTAATGATATTCTTT-3′ and 5′-AAGAATATCATTAGCATGC-3′ for the two strands) (lanes 4, 6, 12 and lanes 14). The results of both DNase I and orthophenanthroline·Cu cleavage assays are schematically summarized in Figure 3B.

Both types of cleavage analyses produced very similar results. Most significantly, the cleavage patterns of the C1 and C2 complexes were indistinguishable. This suggests that the C1 factor, αTIF, and Oct-1 that form the C1 complex are retained in the C2 complex. It further suggests that the C2 factor binds the C1 complex through protein−protein rather than protein−DNA interactions.

The Oct-1 protein is assembled into the αTIF-dependent C1 and C2 complexes

Several lines of evidence, including competition studies and the use of partially or affinity purified Oct-1 protein from extracts of HeLa cells, have indicated that the Oct-1 protein is a component of the Cl and C2 complexes (McKnight *et al.*, 1987; Gerster and Roeder, 1988; Kristie and Roizman, 1988; O'Hare and Goding, 1988). However, definitive identification of this sequence specific DNA binding factor as Oct-1 only recently became possible with the isolation of cDNAs encoding the 90 kd HeLa cell Oct-1 and the lymphoid specific 60 kd Oct-2 proteins (Staudt *et al.*, 1988; Sturm *et al.*, 1988).

Polyclonal antisera, specifically reactive to the Oct-1 and Oct-2 proteins, was generated by immunization of New Zealand White rabbits with a *Staphylococcus* protein A−Oct-2 fusion protein containing the highly conserved

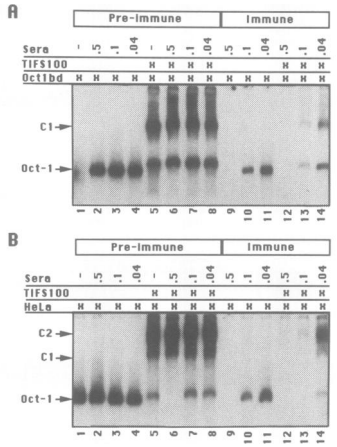

Fig. 4. Oct-2 antisera specifically inhibits the formation of Oct-1−DNA, C1 and C2 complexes. Protein−DNA binding assays were carried out as stated in Materials and methods and contained either Oct1bd fraction (**A**) or nuclear extract from HeLa cells (**B**) as a source of the Oct-1 protein. Reactions contained either the Oct-1 protein extract alone (**lanes 1−4** and **9−11**) or with the addition of TIFS100 extract (**lanes 5−8** and **12−14**) as indicated by x. Fifteen minutes after the onset of the reaction, BSA (**lanes 1** and **5**), pre-immune serum (**lanes 2−4** and **6−8**) or immune serum (**lanes 9−14**) was added for 10 min (Kristie and Roizman, 1986). The Oct-1−HSVα0 DNA, C1 and C2 complexes are indicated with arrows. − reactions represent control assays in the absence of sera while 0.5, 0.1 and 0.04 indicate the μl amount of the appropriate added sera. Note that the absences of the Oct-1−DNA complex in (**A**) lane 1 and (**B**) lane 6 are abberances of those particular reactions.

POU domain sequences (LeBowitz *et al.*, 1989). The specificity of this antisera for Oct-1 and Oct-2 was analyzed by Western blot analysis. The antisera specifically reacted with a 90 kd protein (Oct-1) in a blot of total nuclear extract of HeLa cells and with additional proteins of 60 and 68 kd [Oct-2 and Oct-2′; also referred to as Oct-2B (Schreiber *et al.*, 1988)] in nuclear extracts of BJA-B cells (data not shown).

As the immune sera, produced against the protein A−Oct-2 protein antigen, clearly also reacted specifically with the Oct-1 protein; it was used to determine directly that the Oct-1 protein is a component of the Oct-1−HSVα0, C1 and C2 complexes. DNA binding reactions containing either Oct1bd protein fraction alone or with TIFS100 extract were prepared and serial dilutions of either pre-immune or anti-Oct sera were added after the Oct-1−DNA and C1 complexes were formed. As shown in Figure 4A, 0.5, 0.1 or 0.04 μl of pre-immune sera had no effect upon the Oct-1−DNA complex (lanes 1−4) or the C1 complex (lanes 5−8). In direct contrast, addition of equivalent amounts of immune sera to parallel reactions dramatically inhibited the formation of the Oct-1−DNA (lanes 9−11) and C1 complexes (lanes 12−14). The detection of Oct-1−DNA and C1 complexes showed a clear inverse relationship to the amount of the added immune sera. In a similar experiment, designed to detect the Oct-1 protein in the C2 complex, complete HeLa nuclear extract was used as a source of Oct-1, C1 and C2 factors in place of the Oct1bd affinity fraction. Figure 4B demonstrates that identical results were obtained for the C2 complex as for the C1 complex, with the anti-Oct immune sera inhibiting the formation of Oct-1−DNA, C1 and C2 complexes. It should be noted that the inhibition of complex formation by the anti-Oct-2 sera, rather than a

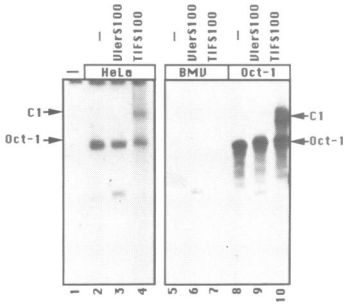

Fig. 5. The translated product of the Oct-1 cDNA is required for the formation of C1 complex. Protein–DNA binding assays contained the following protein extracts as indicated at the top of the autoradiogram: (–), no added protein extract (**lane 1**); HeLa, nuclear extract from HeLa cells (**lanes 2–4**); BMV, rabbit reticulocyte lysate programmed with bromo mosaic virus mRNA (**lanes 5–7**); Oct-1, rabbit reticulocyte lysate programmed with the transcribed mRNA of *Hin*dIII digested pBS$^+$Oct-1 plasmid DNA (**lanes 8–10**); –, bovine serum albumin (**lanes 2, 5** and **8**); VlerS100, S100 extract from control recombinant baculovirus infected SF9 cells (**lanes 3, 6** and **9**) and TIFS100, S100 extract from AcNPV–αTIF recombinant baculovirus-infected SF9 cells (**lanes 4, 7** and **10**). The Oct-1–HSVα0 DNA and C1 complexes are indicated with arrows. Reactions 1–4 and 5–10 were electrophoresed on separate but parallel nondenaturing gels.

shift in the mobility of these complexes, is consistent with the expectation that the highly conserved POU domain is the antigenic determinant shared by Oct-1 and Oct-2. As this region contains the DNA binding domain of these proteins (Clerc *et al.*, 1988; Sturm *et al.*, 1988), it is likely that the bound antibodies interfere with DNA binding of Oct-1. However, to further control for nonspecific inhibition of DNA binding in the gel shift assay, the antiserum was also added to control DNA binding reactions. Under the conditions of this binding reaction, a predominant DNA–protein complex (HSVα27–αH2/3; Kristie and Roizman, 1988) is formed which is unaffected by the pre-immune or immune sera (data not shown).

As a second approach to demonstrate that the recently isolated Oct-1 cDNA encodes a protein which forms the core of the C1 and C2 complexes, Oct-1 protein was translated *in vitro* from RNA transcribed from this cDNA (pBS + Oct-1, gift of R.Stern and W.Herr). The Oct-1 translation product, a control bromo mosaic virus (BMV) translation product, and HeLa cell nuclear extract were then assayed for the ability to generate a C1 complex upon addition of the TIFS100 fraction (Figure 5). In reactions containing HeLa cell nuclear extract, addition of control infected baculovirus S100 extract (VlerS100) did not generate complexes in addition to the Oct-1–HSVα0 DNA complex (lane 3). As anticipated, addition of TIFS100 extract to the HeLa nuclear extract resulted in the formation of the C1 complex (lane 4). It should be noted that under the conditions of these particular reactions, the C2 complex is not generated. Reactions containing the control BMV-mRNA translation product yielded a low level of Oct-1–DNA complex, due to endogenous Oct-1 protein in the reticulocyte lysates. Consequently, addition of the control VlerS100 or TIFS100 extract to the BMV-mRNA translation product did not result in the formation of additional Oct-1–DNA or C1 complexes (lanes 5–7). Reactions containing the translation product of the Oct-1 cDNA yielded the anticipated Oct-1–DNA complex. The trial of faster migrating complexes is probably due to fragments of Oct-1 generated

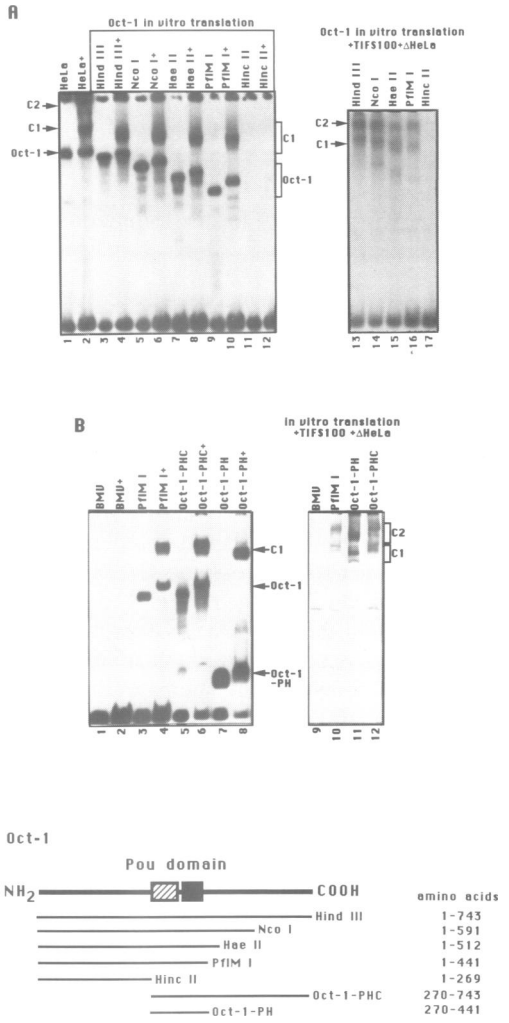

Fig. 6. Deletion mapping of the Oct-1 domain required for C1 and C2 complex formation. Protein–DNA binding assays were carried out using HeLa nuclear extract (**lanes 1** and **2**) or reticulocyte lysate programmed with mRNAs transcribed from the Oct-1 cDNA constructs pBS$^+$Oct-1 and pBSATG$^+$Oct-1-PHC. The positions of the Oct-1–α0H1 DNA, C1 and C2 complexes are noted with arrows or brackets. (**A**) The restriction enzymes used to produce the full length (*Hin*dIII, **lanes 3, 4** and **13**) and C-terminal truncated Oct-1 mRNAs (*Nco*I, *Hae*II, *Pfl*MI and *Hinc*II , **lanes 3–12** and **14–17**) from pBS$^+$Oct-1 are indicated at the top of the autoradiogram. In **lanes 1–12**, + denotes the addition of TIFS100 extract to generate the C1 complex. In **lanes 13–17**, the DNA binding reactions contained the indicated Oct-1 translation product, TIFS100 extract, and ΔHeLa extract to generate the C1 and C2 complexes. (**B**) Protein–DNA binding reactions contained reticulocyte lysate programmed with BMV control RNA (**lanes 1, 2** and **9**) or mRNAs transcribed from the appropriate Oct-1 cDNA construct as follows: *Pfl*MI, *Pfl*MI digested pBS$^+$Oct-1 (**lanes 3, 4** and **10**); Oct-1–PHC, *Sac*I digested pBSATG$^+$Oct-1–PHC (**lanes 5, 6** and **12**); Oct-1–PH, *Pfl*MI digested pBSATG$^+$Oct-1–PHC (**lanes 7, 8** and **11**). + reactions contained the Oct-1 extract and TIFS100 extract. In **lanes 9–12**, the DNA binding reactions contained the indicated Oct-1 translation product, TIFS100 extract, and ΔHeLa extract to generate the C1 and C2 complexes. (**C**) The wild type Oct-1 is represented as a thick bar above the appropriate series of polypeptides used in this study. The location of the POU domain is shown as a striped box and filled box representing the POU specific box and the POU homeobox respectively. Where indicated, the restriction enzyme name designates the site used to terminate *in vitro* transcription of mRNAs from the Oct-1 cDNA construct (pBS$^+$Oct-1; Sturm *et al.*, 1988). The number of the N-terminal and C-terminal amino acid of each protein, relative to the sequence of the wild type Oct-1 protein is shown at the right.

by premature translation termination. Addition of the control VlerS100 extract to the reaction containing the translated Oct-1 did not generate slower migrating complexes. However, addition of the TIFS100 extract specifically generated a novel complex with the same mobility as the previously described C1 complex (lanes 8–10). This strongly suggests that the Oct-1 protein encoded by the isolated cDNA is necessary for the formation of complexes with the αTIF protein.

The Oct-1 POU domain is sufficient for the formation of the C1 and C2 complex

Truncations of the Oct-1 cDNA were used in the transcription–translation assay to identify the protein domain which is essential for the formation of the αTIF complexes (Figure 6A–C). Wild type (743 aa, *Hind*III) and progressive C-terminal deletions of 152 (*Nco*I), 231 (*Hae*II), 302 (*Pfl*MI) and 474 amino acids (*Hinc* II) were translated *in vitro* and assayed for the formation of the C1 complex in the presence of TIFS100 extract (Figure 6A and C). As anticipated, all deletion mutants which retained the POU domain were fully capable of binding the HSVα0 DNA probe (lanes 3, 5, 7 and 9). Deletions which removed this domain, *Hinc*II (lane 11, and data not shown), eliminated specific DNA binding. Addition of TIFS100 extract to these reactions generated a C1 type complex with all of the DNA binding competent mutants (lanes 4, 6, 8, 10 and 12). As would be expected, the mobility of the C1 type complex increased slightly with decreases in the mol. wt of the Oct-1 derivatives. Similarly, all mutants which were capable of forming the C1 complex were also fully competent to generate the C1 and C2 complexes in binding reactions containing TIFS100 and Oct-1 depleted HeLa fractions (ΔHeLa; lanes 13–17).

High affinity binding to the octamer site requires only the POU domain of the Oct-1 protein. To test whether this region of Oct-1 was sufficient for the formation of αTIF-dependent complexes, N-terminal deletion mutants of the Oct-1 cDNA were inserted into the transcription–translation vector pBSATG$^+$ (Clerc *et al.*, 1988). Two mutant proteins were tested. Oct-1-PHC contained the entire POU domain to the C-terminus of the wild type Oct-1 protein while Oct-1-PH retained only the POU domain (Figure 6C). As illustrated in Figure 6B, both the Oct-1-PHC and Oct-1-PH proteins were fully capable of binding the HSVα0 probe (lanes 5 and 7) and of forming the C1 and C2 type complexes (lanes 6, 8, 11 and 12). This indicates that all of the chemical specificity for formation of the αTIF-dependent complexes is contained within the Oct-1 POU domain.

The lymphoid specific Oct-2 protein also forms a αTIF-dependent C1 type complex

The ubiquitous Oct-1 and the lymphoid specific Oct-2 proteins share >86% amino acid identity in the POU domain region, with much of the divergence centered primarily within the linker peptide between the POU specific and the POU homeo subdomains (Clerc *et al.*, 1988; Herr *et al.*, 1988; Sturm *et al.*, 1988). In view of this similarity, it was of interest to test whether the Oct-2 protein would form a C1 type complex. As shown in Figure 7A, a nuclear extract of the B cell line, BJA-B, generated several DNA–protein complexes with the HSVα0 probe, representing the Oct-1, Oct-2′ and Oct-2 protein species. Addition of TIFS100 extract to this reaction specifically generates a smear of

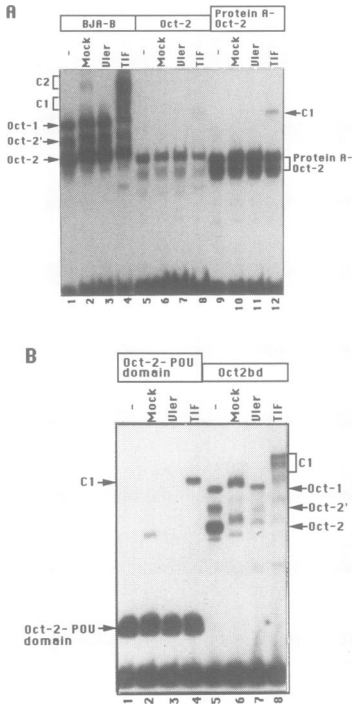

Fig. 7. The POU domain of the cell specific Oct-2 protein forms an αTIF-dependent C1 complex. Protein–DNA binding assays contained the Oct-2 protein extracts indicated at the top of the autoradiogram. In addition, the appropriately noted reactions contained: –, no additional protein extract; MockS100, S100 extract prepared from mock-infected SF9 cells; VlerS100, S100 extract prepared from Vler-infected SF9 cells; or TIFS100, S100 extract prepared from AcNPV-αTIF infected SF9 cells. The positions of the Oct-2–HSVα0 DNA, C1 and C2 complexes are indicated with arrows or brackets. (**A**) BJA-B, nuclear extract from BJA-B cells (**lanes 1–4**); Oct-2, partially purified *in vitro* translation product of the Oct-2 cDNA (gift of R.Clerc, **lanes 5–8**); protein A–Oct-2, partially purified protein A–Oct-2 chimeric fusion protein (**lanes 9–12**). (**B**) Oct-2 POU domain, reticulocyte lysate programmed with the transcribed mRNA of *Pst*I digested pBSATG$^+$Oct-2-POU (**lanes 1–4**); Oct2bd, affinity-selected octamer binding proteins from BJA–B cells (**lanes 5–8**). The DNA–protein complex appearing upon addition of the MockS100 control extract represents a MockS100–DNA protein complex, and does not depend upon the octamer binding proteins. This host cell protein is reduced or eliminated in the Vler and AcNPV–αTIF infected cell extracts.

slower mobility complexes in the region of the gel where C1 and C2 complexes migrate (lanes 1–4). The presence of the Oct-1 protein in the BJA-B extract made detection of a potential Oct-2–C1 or Oct-2–C2 complex difficult. Therefore, Oct-2 was synthesized by *in vitro* transcription–translation of an Oct-2 cDNA and partially purified by affinity chromatography (Figure 7A, lanes 5–8). Upon addition of TIFS100 extract to a binding reaction containing this protein, a low level of Oct-2–C1 complex was specifically generated (lane 8). Although inefficient, this suggested that the Oct-2 protein could generate an αTIF-dependent complex.

Two variants of the Oct-2 protein were assayed with control or TIFS100 extracts to further demonstrate that the Oct-2 POU domain was capable of forming C1 type complexes. A fusion protein containing the *Staphylococcus* protein A fused to the Oct-2 protein at the amino terminus of the Oct-2 POU domain was purified from bacterial lysates. The second Oct-2 derivative consisted solely of the Oct-2 POU domain; produced by *in vitro* transcription–translation

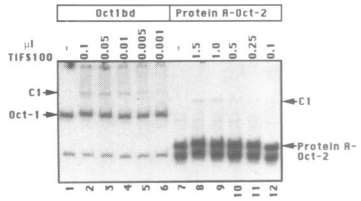

Fig. 8. Relative affinity of Oct-1 and Oct-2 for the components of the C1 complex. Protein–DNA binding reactions contained S100 extract from mock-infected SF9 cells and either affinity-selected HeLa cell Oct-1 protein (**lanes 1–6**) or partially purified protein A–Oct-2 fusion protein (**lanes 7–12**). The μl amount of AcNPV–αTIF infected cell S100 extract (TIFS100) added to each reaction is indicated at the top of each lane. The Oct-1–HSVα0 DNA, protein A–Oct-2–HSVα0 DNA, and their respective C1 complexes are noted with arrows.

of an Oct-2 cDNA segment in a reticulocyte lysate (Clerc *et al.*, 1988). As evident in Figure 7A (lanes 9–12) and 7B (lanes 1–4), both of these Oct-2 proteins specifically formed an αTIF-dependent C1 complex. As a control for the efficiency of forming αTIF complexes, protein was affinity purified from BJA-B cell extracts on an octamer specific column. This protein fraction contained Oct-1, Oct-2 and Oct-2′ and efficiently generated the C1 complex upon addition of the TIFS100 extract (Figure 7B, lane 8).

Further evidence that the αTIF-dependent Oct-2–DNA complex represents a C1 type complex results from ortho-phenanthroline·Cu cleavage patterns of the Staph A–Oct-2 DNA complex, the Oct-2–Pou domain DNA complex and their respective C1 type complexes. In both cases, the octamer element in the HSVα0 probe DNA was protected in a manner indistinguishable from that afforded by the Oct-1 protein. Similarly, the protection patterns of the C1 type complexes generated with the Oct-2 related proteins were identical to that of the C1 type complex generated by Oct-1 protein (data not shown; refer to Figure 3B). Therefore, both the Oct-1 and Oct-2 POU domains are capable of interacting with αTIF and a C1 factor to form a C1 type complex.

As observed in these experiments, the ability to generate an αTIF-dependent C1 complex with the Oct-1 protein was substantially greater than with the Oct-2 protein. This probably reflected the relative difference in affinity of these proteins for the components of the C1 complex. To quantitate this difference, equivalent DNA binding activities of the Oct-1, Oct-2, or the derivatives of these proteins described above, were mixed with an S100 extract from mock-infected insect cells to supply a constant concentration of the C1 component. TIFS100 extract was then titrated into these reactions (Figure 8). The level of C1 type complex which was generated at each titration point was determined by densimetric scanning of the appropriate autoradiograph. All of the Oct-2 related proteins (Oct-2, protein A–Oct-2 and Oct-2–POU domain proteins) generated a C1 type complex at similar levels of TIFS100 extract (data not shown). In the titration shown in Figure 8, a C1 type complex was readily detected after 0.001 μl of TIFS100 extract was added to the Oct1bd fraction (lanes 1–6). In contrast, detection of equivalent levels of the C1 type complex with the protein A–Oct-2 fusion required a $2-5 \times 10^2$ higher concentration of TIFS100 (lanes 7–12). Therefore, the Oct-1 protein has an affinity that is at least two orders of magnitude higher than the Oct-2 protein for the components in the C1 complex.

The C-terminal transactivation domain of the αTIF protein is not required for the formation of the C1 or C2 complexes

The αTIF contains a highly acidic carboxy-terminus which is necessary and sufficient for transactivation of a target gene *in vivo* (Campbell *et al.*, 1984; Sadowski *et al.*, 1988; Trienzenberg *et al.*, 1988). To assess the role of this domain in the *in vitro* formation of C1 and C2 complexes, the coding domain of the αTIF gene was inserted into the *in vitro* transcription vector pSP65 to generate SP6-αTIF. A series of C-terminal deletion mutants were produced by transcription of truncated DNA templates and translation of the resultant mRNAs in reticulocyte lysates. The wild type and mutant polypeptides were assayed for the ability to generate a C2 complex in the presence of a nuclear extract of HeLa cells. Deletion of the C-terminal 88 amino acids, previously defined as the transactivation domain, did not affect formation of the αTIF-dependent C2 complex. However, deletion of an additional 96 or 159 amino acids abolished complex formation (data not shown).

Discussion

The Oct-1 and Oct-2 proteins are members of a family of sequence specific DNA binding proteins that contain the POU domain. Currently, seven members of this family have been identified in mammalian cells (He *et al.*, 1989). The two subregions of the POU domain, the POU specific box and the POU homeobox, are highly conserved, and thus must reflect critical functions that depend upon the integrity of the domain. The homeobox is probably responsible for sequence specific DNA binding while the adjacent POU specific box may contribute to the stability and specificity of the binding (Sturm and Herr, 1988; Garcia-Blanco *et al.*, 1989). As most sequence specific DNA binding proteins with homeoboxes do not contain a POU specific box, this latter region must have other critical functions.

The α/IE promoters of HSV are transactivated by the virion protein, αTIF, through a specific sequence element. Since purified Oct-1 binds to this element with high affinity, it was proposed to be involved in the αTIF-dependent transcriptional stimulation (Gerster and Roeder, 1988). Therefore, this viral system provides a paradigm for the role of Oct-1 in the regulation of transcription.

An HSV probe, containing the αTIF-responsive site, formed three DNA–protein complexes (Oct-1–DNA, C1 and C2) with different mobilities in a native gel. Formation of these complexes depended upon the presence of Oct-1, αTIF and, at a minimum, two additional cellular factors. The Oct-1–DNA complex was the fastest migrating band and only the octamer related sequences were protected from cleavage in this complex. The second fastest migrating complex, C1, was only formed if Oct-1, αTIF and a third, cellular component were present. Purified Oct-1 and αTIF would not form this complex but yielded only the Oct-1–DNA complex. This third component, tentatively named the C1 factor, is present in both insect (SF9) and mammalian (HeLa) cells, and thus must represent a highly conserved and ubiquitous protein. As both the octamer-related and immediately flanking sequences are protected from cleavage in the C1 complex, this second binding specificity must be contributed by the C1 factor(s) alone, or in conjunction with the αTIF protein. Consistent with this

conclusion, mutation of the HSV specific sequences immediately flanking the octamer site precluded the formation of the C1 complex.

Formation of the C2 complex required a component, C2 factor, which is found in HeLa cells but not in insect cells. Again, generation of the C2 complex depended upon the addition of both αTIF and Oct-1 proteins. Since the footprint patterns of complexes C1 and C2 were identical, it is likely that the C1 factor is also present in the C2 complex. Thus, the C2 factor probably represents a fourth protein which binds through protein–protein interactions to the C1 complex. There may also be other components which bind to the C1 complex, and the potential role for this type of factor in gene regulation is intriguing.

It is important to note that the transactivation domain of αTIF is not required for the formation of the C1 and C2 complexes. This provides a mechanistic interpretation of the dominant mutant experiments of Friedman *et al.* (1988). These authors demonstrated that a cell line expressing a mutant αTIF gene, lacking the transactivation domain, was resistant to HSV infection. The mutant gene, when expressed at a sufficient level, blocked transcription of the α/IE HSV promoters. This dominance probably reflects the formation of C1 and C2 type complexes with the mutant αTIF protein. Such complexes would lack an activation domain (Greaves and O'Hare, 1989; this paper) and their formation would compete with the formation of similar complexes with a wild type αTIF protein.

The determinants of the Oct-1 protein, required for the formation of both the C1 and C2 complexes, are contained within the POU domain. Thus, this domain probably binds DNA, αTIF, C1 factor and perhaps, the C2 factor. The homeobox subdomain of the POU domain is undoubtedly in contact with the octamer element and is likely to contact the C1 factor, as this factor probably binds to the immediately adjacent sequences. The αTIF protein may interact with the POU specific box, the POU homeobox, and also with the C1 factor. It is interesting that the POU domain has also been shown to be sufficient for interactions leading to cooperative DNA binding of Octa proteins (LeBowitz *et al.*, 1989). Thus, the POU domains of these proteins have an ability to bind specifically a number of other proteins. Part of this ability may be the critical function of the POU specific box, accounting for its evolutionary conservation.

The POU domains of Oct-1 and Oct-2 differ in their potential to form the αTIF-dependent complex, C1. Approximately 100-fold higher concentration of αTIF is required to drive the Oct-2 protein into a C1 type complex under conditions of excess C1 factor. As mentioned above, seven different mammalian proteins are known to contain POU domains. Many of these proteins are expressed exclusively in nerve cells. It is interesting to speculate on the potential importance of these POU domain transcription factors in the regulation of an HSV infection. All of these proteins may interact with the αTIF protein with varying efficiencies and consequences. Such complexes could, in theory, competitively inhibit the formation of the C1 and C2 complexes and block transcriptional stimulation or even actively suppress transcription. Since αTIF controls the critical α/IE family of HSV genes, the result could be that the virus enters a latent state.

Protein–protein interactions obviously are important for the functions of Oct-1 in an HSV infection. Similar interactions may also be important for this protein's function in the regulation of cellular processes such as the expression of snRNA genes and the regulated expression of histone genes during the cell cycle. The role of the Oct-1 protein in promoting adenovirus DNA replication probably also depends upon interactions with other proteins in the replication complex. In the latter case, Oct-1 binds viral DNA sequences immediately adjacent to the NF-1 factor (Pruijn *et al.*, 1986; O'Neill and Kelly, 1988). Thus, the POU domain of the Oct-1 protein probably interacts with a structure common to a number of transcription factors. As in the case of the regulation of the α/IE promoters of HSV, these interactions probably dictate the selection of Oct-1 or Oct-2 in the regulation of specific genes.

Materials and methods

Baculovirus expression and fractionation of αTIF

The entire αTIF coding sequence from −28, relative to the translation initiation ATG (Pellett *et al.*, 1985), was cloned into the *Bam*HI site of the baculovirus expression vector pVL941 (gift of M.Summers) to generate pVL–αTIF. Recombinant viruses were produced and isolated as described (Summers and Smith, 1987). For large scale production of TIFS100 or control S100 extract, 2×10^9 cells were infected or mock infected, in a 6000 cm^2 cell culture factory (Nunc), with AcNPV–αTIF (20–50 p.f.u./cell) for 72 h. The cells were harvested and an S100 extract was prepared as described (Dignam *et al.*, 1983) except that, due to extensive lysis of the SF9 cells during the buffer A washes, the cells were resuspended directly in two packed cell volumes of buffer A and disrupted by Dounce homogenization. The final extract was dialyzed extensively against buffer D + 0.025% Nonidet P-40 (Dignam *et al.*, 1983). The average αTIF concentration was 1 mg/ml in the S100 extract. VlerS100 extract (gift of M.Brown) was prepared in an equivalent manner from SF9 cells infected with a recombinant virus expressing the estrogen receptor (M.Brown and P.A.Sharp, unpublished data).

TIFS100 extract was fractionated by chromatography of 10 mg of protein extract on a 1.0 ml DEAE–Sepharose CL6B column equilibrated in buffer A [40 mM HEPES pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 20% (v/v) glycerol] + 100 mM KCl. The column was washed with 8 column volumes of buffer A + 100 mM KCl. Absorbed proteins were eluted by successive steps of 3 column volumes each of buffer A containing 200 mM KCl, 350 mM KCl, 500 mM KCl, 650 mM KCl and 1000 mM KCl. Protein concentrations were determined using the BioRad protein assay. Elution of αTIF was monitored by SDS–PAGE of alternate fractions.

DNA–protein gel shift assays

Unless otherwise stated, DNA–protein binding reactions contained 0.5 ng (~8.0 fmol) DNA probe fragment, 2.25 μg poly(dIdC)·poly(dIdC), 10 mM HEPES pH 7.9, 0.75 mM DTT, 0.5 mM EDTA, 60 mM KCl, 4% Ficol 400 (w/v), 200 μg/ml bovine serum albumin and 10–20 μg protein extract in 15 μl total volume. Binding reactions were incubated at 30°C for 15 min and resolved in a 4% (29:1 acrylamide to bisacrylamide ratio) nondenaturing gel using ×0.5 Tris-borate electrophoresis buffer (Fried and Crothers, 1981; Garner and Revzin, 1981). Probe DNA fragments were prepared as described (Maniatis *et al.*, 1982). The activity was generally 100 000 c.p.m./ng DNA fragment. HSVα0 and HSVα27 are equivalent to pRB608 and pRB606 respectively (Kristie and Roizman, 1984). All other DNAs were derived by cloning complementary oligonucleotides in the *Eco*RI–*Bam*HI sites of pUC19 (refer to Figure 1). MHC competitor DNA (gift of A.Baldwin) contains the H2TF1 binding site from the MHC class I gene (Baldwin and Sharp, 1987). All competitor DNAs were purified from polyacrylamide gels and were quantitated by densitometric scanning of a Kodak +/− film negative of ethidium bromide stained agarose gels.

Protein extracts

HeLa and BJA-B nuclear extracts (20 mg/ml) were prepared essentially as described (Dignam *et al.*, 1983). ΔHeLa or ΔBJA-B and Oct1bd or Oct2bd extracts were prepared by absorption of 0.5 ml HeLa or BJA-B nuclear extract equilibrated in 20 mM Tris–HCl (pH 7.6), 50 mM KCl, 0.2 mM DTT, 0.5 mM EDTA, 10% (v/v) glycerol, 0.025% Nonidet P-40, 50 μg/ml poly(dIdC)·poly(dIdC) to a 0.4 ml octamer–DNA column (gift of L.Staudt) at 25°C. Δ extracts represent the flowthrough fractions while Oct1bd or Oct2bd are those 500 mM KCl eluate fractions which are enriched for

octamer binding proteins. All fractions were assayed for octamer binding activities in gel shift assays. Protein A–Oct-2 fusion protein was constructed and purified as described (LeBowitz *et al.*, 1989). *In vitro* translation extracts of Oct-1, Oct-2, αTIF and mutant polypeptides were prepared by *in vitro* transcription of the appropriate plasmid DNA and translation of the purified mRNAs in rabbit reticulocyte extract (Promega Biotech) according to the manufacturer's recommendations. Translation efficiencies were monitored by inclusion of 50 μCi [^{35}S]methionine (Amersham 1134 Ci/mM) for 30 min, followed by the addition to 50 μM unlabeled methionine for 30 min (McKnight *et al.*, 1987). The Oct-2 protein translation product was partially purified and concentrated as described (Clerc *et al.*, 1988).

DNase I and orthophenanthroline · Cu footprinting

DNA–protein binding reactions were as described except that the reactions were scaled up 3-fold. For DNase I cleavage reactions (Siebenlist and Gilbert, 1980), $MgCl_2$ and DNase I were added to 2.5 mM and 20 μg/ml respectively, for 2 min at 25°C. The reactions were terminated by the addition of EDTA to 5 mM and immediately electrophoresed in a 4% nondenaturing gel. For orthophenanthroline·Cu footprinting, equivalent binding reactions were first separated in a 4% nondenaturing gel prior to treatment of the gel essentially as described (Kuwabara and Sigman, 1987). In both cases, the DNA was then transferred to NA45 paper in ×0.25 Tris-borate electrophoresis buffer at 4°C for 1.5 h at 90 V (BioRad Transblot). The DNAs were recovered from the NA45 paper (Schleicher and Schuell) and purified as described (Kristie and Roizman, 1988). The DNA pellets were resuspended in 2.5 μl 90% formamide, 10 mM EDTA and equivalent amounts of radioactivity were separated in 10% sequencing gels (Maxam and Gilbert, 1980).

Antibodies and Western blots

Rabbit anti-αTIF antiserum was a gift of J.L.C.McKnight (McKnight *et al.*, 1987). The anti-Oct serum was generated by s.c. injection of SDS–PAGE purified protein A–Oct-2 in RIBI adjuvant into New Zealand white rabbits (0.5 mg protein/rabbit) according to a standard immunization protocol.

For Western blot analysis of αTIF, Oct-1 and Oct-2 extracts, protein extracts were subjected to SDS–PAGE and transferred to nitrocellulose in the presence of 0.1% SDS. The blots were developed by absorption of the appropriate antisera or preimmune sera, followed by a secondary anti-rabbit alkaline phosphatase conjugated antibody (Promega Biotech) according to the manufacturer's recommendations.

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