Interactions of the Oct-1 POU subdomains with specific DNA sequences and with the HSV α -trans-activator protein

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Trans-activation by the herpes simplex virus (HSV) protein, α TIF (VP16), is dependent on an inducible enhancer sequence that contains a homolog of the octamer element. An ordered series of multiprotein complexes can be assembled on this enhancer, requiring the interactions of Oct-1, α TIF, and two additional cellular factors (C1 and C2). Oct-1 binds to the octamer homolog, whereas α TIF, also a sequence-specific DNA-binding protein, recognizes sequences within the HSV enhancer core. The partially purified C1 factor interacts directly with α TIF in the absence of DNA and is required to form a stable Oct-1/ α TIF/C1 factor complex. The POU domain of Oct-1 is a bipartite sequence recognition structure, as both the POU-specific box and the POU homeo box contribute directly to the recognition of the octamer element. Surprisingly, the POU homeo box alone is sufficient to direct the cooperative binding of α TIF and to assemble the Oct-1/ α TIF/C1 factor complex.

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The diverse transcriptional regulatory events involved in the determination of cell type or development often appear to be mediated by the recognition of common DNA elements. Thus, the required specificities in such a program may rely on a combination of particular sites and the assembly of multiple factors. The regulation mediated by the common octamer element (5'-ATG-CAAAT-3') is a model system for the analysis of such interactions.

The octamer element is recognized by at least three sequence-specific DNA-binding proteins (Singh et al. 1986; Staudt et al. 1986; Schreiber et al. 1988; Lenardo et al. 1989; Scholer et al. 1989a). Oct-1 is constitutively expressed in many mammalian cell types, whereas Oct-2 and Oct-4 are expressed primarily in lymphoid and embryonic cells, respectively (Singh et al. 1986; Staudt et al. 1986; He et al. 1989; Scholer et al. 1989a,b). Members of the POU family, these *trans*-activators share a conserved domain (POU domain) that has been subdivided into two regions termed the POU-specific box and the POU homeo box (Clerc et al. 1988; Herr et al. 1988; Ko et al. 1988; Sturm et al. 1988). Collectively, these regions are responsible for the high-affinity, sequence-specific binding to the octamer element (Sturm and Herr 1988; Garcia-Blanco et al. 1989). The POU homeo box region contains the helix-turn-helix motif characteristic of the homeo box family and is hypothesized to be

the primary determinant in the recognition of the octamer element (Laughon and Scott 1984; McGinnis et al. 1984; Clerc et al. 1988; Jordan and Pabo 1988; Ko et al. 1988; Otting et al. 1988; Sturm et al. 1988; Qian et al. 1989; Scott et al. 1989). The most highly conserved region, the POU-specific box, appears to contribute to the DNA-binding specificity of the protein and may also play a role in the cooperative interactions that are exhibited by the intact POU domain (Sturm and Herr 1988; LeBowitz et al. 1989; Poellinger et al. 1989; Ingraham et al. 1990).

Although Oct-1 and Oct-2 recognize the octamer element in an apparently identical manner (Staudt et al. 1986), they have been implicated in a wide range of regulation, including the constitutive expression of snRNA genes (Ares et al. 1987; Bark et al. 1987; Carbon et al. 1987; Murphy et al. 1987), the cell cycle-dependent expression of the histone H2B (Sive et al. 1986; Fletcher et al. 1987; LaBella et al. 1988), the cell-specific expression of immunoglobulins (Mizushima-Sugano and Roeder 1986; Staudt et al. 1986; Scheidereit et al. 1987; Wirth et al. 1987; LeBowitz et al. 1988; Muller et al. 1988b; Gerster et al. 1990; Muller-Immergluck et al. 1990), and the stimulation of adenovirus DNA replication (Pruijn et al. 1986, 1987; O'Neill and Kelly 1988; Verrijzer et al. 1990). The regulation of such diverse processes by these proteins can be modeled by their interaction with other

specificity determinants such as the herpes simplex virus (HSV) α -trans-induction factor (α TIF).

α TIF (also referred to as VP16, ICP25, and VMW65) is a 53-kD protein, encoded by HSV and packaged within the tegument structure of the virion (Roizman and Furlong 1974; Batterson and Roizman 1983). Upon infection, the virion protein is released into the cell and induces the transcription of the five viral α or immediate early (IE) genes (Post et al. 1981; Campbell et al. 1984; Pellett et al. 1985). This regulation is dependent on specific enhancer elements in the regulatory domains of the α genes that contain a homolog of the octamer sequence (5'-GyATGnTAATGArATTCTTGGG-3') and a highly conserved core element (TAATGArAT) (Mackem and Roizman 1982a-c; Kristie and Roizman 1984; Gaffney et al. 1985; Bzik and Preston 1986; for review, see McKnight et al. 1986; Pruijn et al. 1986). Analysis by electrophoresis in native gels has shown that α TIF could be assembled into specific multiprotein-DNA complexes, dependent on both the octamer and the HSV-specific core sequences of the α /IE element (McKnight et al. 1987; Gerster and Roeder 1988; O'Hare and Goding 1988; Preston et al. 1988; Kristie et al. 1989; Stern et al. 1989). The assembly of α TIF into these complexes required either Oct-1 or Oct-2, in addition to several discrete cellular components (McKnight et al. 1987; Gerster and Roeder 1988; Kristie and Roizman 1988; Kristie et al. 1989; Stern et al. 1989). Although these interactions depend solely on the highly conserved POU domain, Oct-1 has a 100-fold greater potential than Oct-2 to form these complexes (Kristie et al. 1989). Thus, this system provides an excellent model for the assembly of specific multiprotein complexes involved in the regulation processes that are mediated by the Oct proteins.

Results

Cellular factors required for the assembly of the α TIF-dependent multiprotein-DNA complexes

Previous studies have demonstrated that a succession of protein-DNA complexes could be assembled in a step-wise manner on the α /IE element (Kristie et al. 1989). The simplest DNA-protein complex was formed by the binding of Oct-1 to the octamer homolog in the 5' domain of this site. This complex was assembled into the slower migrating C1 complex by the addition of a crude S100 extract from cells infected with a recombinant baculovirus that overexpressed the α TIF protein. However, partial purification of α TIF from these extracts suggested that the S100 extract supplied an additional factor, the C1 factor, which was required to assimilate α TIF into the C1 complex. Comparison of the metal ion cleavage patterns of the Oct-1 and C1 complexes showed that the former protected the octamer homolog while the latter protected the entire α /IE element. An additional complex, the C2 complex, was formed when a nuclear extract of HeLa cells was added to the reaction containing Oct-1 and α TIF. Therefore, a fourth component was hypothesized to bind to the C1 complex, resulting in the C2 complex. This complex did not alter the footprint of

the C1 complex, suggesting that the C2 factor associated with the C1 complex primarily via protein-protein interactions.

A definitive assay for the analysis and characterization of the cellular components in these multiprotein complexes required purified α TIF and Oct-1 POU domain proteins. Therefore, these proteins were expressed in *Escherichia coli*. Because the *trans*-activation domain of α TIF (carboxy-terminal, amino acids 413-490; Triezenberg et al. 1988) was not required for the formation of C1 and C2 complexes (Greaves and O'Hare 1990; Kristie et al. 1989; Werstuck and Capone 1989), only the interactive domain (amino acid 1-412) was produced as an in-frame fusion product with the *Staphylococcus aureus* protein A (PA- α TIF). The domain encoding the Oct-1 POU domain (amino acid 270-441) was similarly produced as a protein A fusion product (PA-POU). Bacteria containing the appropriate plasmids were induced to express the fusion proteins, lysates were prepared, and the proteins were purified by chromatography on IgG-Sepharose. Analysis by SDS-PAGE revealed the expected 74-kD (PA- α TIF) and 48-kD (PA-POU) purified fusion proteins (data not shown).

As anticipated, PA-POU readily bound the HSV α 0 α /IE element DNA probe, forming the PA-POU/DNA complex (Fig. 1A). Addition of either the bacterially produced PA- α TIF or an S100 extract (C1 factor) prepared from SF9 insect cells did not affect the PA-POU/DNA complex or result in the formation of additional DNA-protein complexes. However, addition of both PA- α TIF and the C1 factor extract to the reaction containing PA-POU generated the first α TIF-dependent complex (C1).

Addition of a HeLa cell nuclear extract, which had been depleted of Oct-1, to a reaction containing the C1 complex generated the slower mobility C2 complex. This suggests that the C2 complex contains a fourth component derived from the HeLa cell extract. Formation of both the C1 and C2 complexes could, alternatively, be generated by the addition of PA-POU and PA- α TIF to the the octamer-depleted HeLa cell nuclear extract (data not shown). Thus, the C1 factor is present in both SF9 insect cell and HeLa cell extracts, whereas the C2 factor is only present in the HeLa cell nuclear extracts.

Partial purification of the C1 factor

The C1 factor was partially purified from S100 extracts of SF9 cells, as these extracts do not contain octamer-binding and C2 factor activities. Extracts were chromatographed on a DEAE-Sepharose column, and the bound proteins were eluted by successive step washes of 200, 350, and 500 mM KCl. Column fractions were assayed in the presence of PA-POU and PA- α TIF for the ability to support the formation of the C1 complex (Fig. 1B). The C1 factor activity eluted from the DEAE column at 350 mM KCl (fractions 28-33, lanes 6-11), resulting in a 10-fold purification (DE-C1a). As evident with the original SF9 S100 extract, formation of the C1 complex was dependent on the addition of PA-POU,

Figure 1. Assembly of PA-POU and PA- α TIF into multiprotein complexes on the α /IE element: requirement for C1 and C2 factors. Protein-DNA-binding reactions were done as described in Materials and methods with 1000 ng poly[d(I-C)]/poly[d(I-C)]. The probe DNA, HSV α 0, was derived from pRB608 and contains the α /IE element from -168 to -142 of the α 0 promoter (Mackem and Roizman 1980). The positions of the PA-POU/DNA, C1, and C2 complexes are indicated with arrows. (A) The proteins included in each binding reaction are indicated at the top of the autoradiogram (+) and are as follows: 25 ng PA-POU; 50 ng PA- α TIF; C1 factor, 5 μ g SF9 cell S100 extract; C2 factor, 5 μ g of nuclear extract from HeLa cells, which was depleted of Oct-1 by affinity chromatography. (B) Chromatographic fractionation of the C1 factor on DEAE-Sepharose. Protein-DNA-binding reactions contained 25 ng PA-POU, 50 ng PA- α TIF, and 1 μ l of the column load (C1) or 2 μ l of the indicated fraction from the DEAE-Sepharose chromatography. (-, lane 1) A reaction in the absence of added C1 extract.

PA- α TIF, and the DEAE fractions. This activity was further purified sixfold by reapplication of the DE-C1a activity to a higher resolution DEAE-Sepharose column and elution with a linear gradient of 200–400 mM KCl. The C1 activity eluted at 270–290 mM KCl (DE-C1b). DE-C1b activity was subsequently applied to a heparin-agarose column and eluted with a linear gradient of 50–150 mM KCl, followed by successive steps to 250 and 350 mM KCl. With these conditions, the DE-C1b activity was resolved into two distinct C1 species (C1 and C1'), which elute at 125–200 and 250 mM KCl, respectively. Both species generated a C1-type complex that was dependent on the addition of α TIF and PA-POU. However, the C1 complex generated with the activity eluting at 125 mM KCl migrated slightly faster than the respective C1 complex generated with the activity eluting at 250 mM KCl. Treatment of the C1 activity eluting at 250 mM KCl with phosphatase resulted in the formation of a C1 complex with the faster mobility, suggesting that the two forms of C1 differ in their degree of phosphorylation (data not shown). The heparin-agarose chromatography resulted in an additional sixfold purification of the predominant C1 activity (H-C1).

Neither PA- α TIF nor the partially purified C1 factor had sequence-specific DNA-binding activity when assayed under the standard conditions. However, the C1 complex protects the sequences 3' to the octamer element in the HSV α 0 probe from metal ion cleavage (Kristie et al. 1989). It was therefore anticipated that the C1 factor would represent this second DNA-binding specificity. To address this, fractions containing the C1

activity (H-C1) were chromatographed on ds DNA-cellulose and denatured DNA-cellulose columns. Surprisingly, the H-C1 activity was recovered exclusively in the flowthrough fractions from these columns, suggesting that the C1 factor does not possess independent, high-affinity DNA-binding activity.

Interactions of α TIF, the Oct-1 POU domain, and the C1 factor in the absence of DNA

The C1 complex contains the Oct-1 protein, α TIF, and the C1 factor. To assess the interactions involved in this multiprotein complex, the C1 factor (DE-C1b) was chromatographed on a PA- α TIF or PA-POU protein-affinity column. Purified PA- α TIF or PA-POU was batch-adsorbed to IgG-Sepharose, resulting in affinity matrices containing 400 μ g of the appropriate fusion protein per milliliter of resin. DE-C1b activity was absorbed to each affinity matrix and the columns were successively washed with 50, 150, and 500 mM KCl. All of the DE-C1b activity bound to the α TIF column and was eluted by 500 mM KCl (Fig. 2, lanes 9–16). In contrast, 80% of the C1 activity did not bind the PA-POU affinity matrix and was found in the flowthrough fractions from this column. The remaining 20% was eluted from the PA-POU column in the 150 mM KCl wash (data not shown). Thus, the C1 factor binds α TIF with high affinity in the absence of the α /IE element and the Oct-1 protein. Conversely, this component has limited or no apparent affinity for the Oct-1 POU domain protein under these conditions. Similarly, the C1 factor but not the C2 factor, present in HeLa cell nuclear extracts, also

Figure 2. Affinity chromatography of the C1 factor on a PA- α TIF affinity matrix. Protein-DNA-binding reactions contained 750 ng poly[d(I-C)]/poly[d(I-C)] and 25 ng PA-POU, 50 ng PA- α TIF, and the following protein fractions as indicated: (lane 1) No additional protein extract; (lane 2) 2 μ g of SF9 cell S100 extract; (lane 3) 1.5 μ l of the DE-C1a column load; (lane 4) 2 μ l of the column flowthrough fraction; (lanes 5–16) 2 μ l of the indicated KCl step fractions from the column. The positions of the PA-POU/DNA and C1 complexes are indicated with arrows.

bound the α TIF matrix with high affinity and specificity. This factor was eluted from the α TIF column by 650 mM KCl, whereas control DNA-binding proteins were found exclusively in the flowthrough fractions (data not shown).

As the C1 factor did not exhibit high affinity for the POU domain fusion protein, α TIF (purified from recombinant baculovirus-infected SF9 cells) was chromatographed on the PA-POU and control PA affinity columns. Fractions from the columns were assayed by gel mobility shift for the formation of the C1 complex in the presence of PA-POU and the C1 factor. In both cases, all of the α TIF protein was found in the flowthrough and 150 mM KCl washes from these columns (data not shown). This suggests that any possible interactions between Oct-1 and α TIF are not of high affinity in the absence of the α /IE element and the C1 factor.

Purified α TIF specifically binds to the HSV α /IE element with low affinity

Several attempts have been made to detect the direct binding of α TIF, purified from HSV virions, to the α /IE element [Marsden et al. 1987; Preston et al. 1988]. However, we took advantage of the high level of α TIF production in the baculovirus expression system to assay the DNA-binding potential of this protein. At high concentrations of purified α TIF, several novel DNA-protein complexes were detected in a gel mobility shift assay. One of these complexes had a mobility that was expected of an α TIF/DNA complex. Due to the high protein concentration required for the formation of this

complex, it was possible that it represented the binding of a cofractionating protein in the α TIF protein fractions. Therefore, α TIF was further purified by preparative SDS-PAGE. The 53-kD doublet, representing the α TIF proteins was eluted from the gel, denatured in guanidine/HCl, and allowed to renature during dialysis. The gel-purified protein formed a novel doublet of complexes with the HSV α 0 probe (Fig. 3A, lane 1). This doublet of complexes was also formed, although less efficiently, with the wild-type probe (lane 4). This probe is several nucleotides shorter (3' end) than the HSV α 0 probe but maintains the highly conserved core TAATGARAT. However, a double point mutation in either the 5' or 3' region of the core sequence abolished the formation of the DNA-protein complexes (lanes 2 and 3). This sequence specificity is distinct from that of Oct-1, which is affected only by the 5' double point mutation. It is,

Figure 3. Independent binding of α TIF to the α /IE element and cooperative binding with the Oct-1 PA-POU domain protein. (A) Protein-DNA-binding reactions contained 30 ng poly[d(I-C)]/poly[d(I-C)] and the following proteins where indicated: [α TIF(gp)] 300 ng baculovirus-expressed and SDS-PAGE-purified α TIF; (C1) 2.5 μ l DE-C1a fraction; (POU) 25 ng PA-POU. (Left) Binding reactions contained the following DNA probes indicated at the top of the autoradiogram: (HSV α 0) 5'-CCGTGCATGCTAATGATATTCTTTGGGG-3'; (WT) wild-type 5'-CGTGCATGCTAATGATATTCTTG-3'; (5'MT) 5'-CGTGCATGCCGACTGATTCTTG-3'; (3'MT) 5'-CGTGCATGCTAATGCTGTTCTTG-3', where the point mutations are underlined. The positions of the α TIF/DNA, PA-POU/DNA, and C1 complexes are indicated with arrows. Both panels represent segments taken from the same autoradiogram. (B) Protein-DNA-binding and UV-cross-linking reactions were done as described in Materials and methods with 150 ng poly[d(I-C)]/poly[d(I-C)] and the following SDS-PAGE-purified proteins where indicated: 30 ng PA-POU, 100 ng PA- α TIF, or 60 ng α TIF. Lane 1 contains 14 C protein molecular weight markers whose sizes (in kD) are indicated at left. The positions of the PA- α TIF (74 kD), PA-POU (46 kD), and α TIF (53 kD) are indicated with arrows. The 27-kD radioactive protein species is a degradation product of PA-POU. The radioactive band at 17 kD represents the digested DNA and is present in control reactions in the absence of protein.

however, consistent with the formation of the C1 complex that is inhibited by either the 5' or 3' double point mutations. The gel-purified α TIF protein did not form an additional, stable complex in the presence of either the C1 factor (DE-C1b) or PA-POU but was functional to form the C1 complex in the presence of both proteins (Fig. 3A, right).

Equivalent assays using gel-purified PA- α TIF also exhibited a novel DNA-protein complex with the anticipated mobility of a PA- α TIF/DNA complex (74-kD protein). The DNA-binding specificity of this protein was identical to that of the baculovirus-expressed α TIF with respect to the wild-type, 5'MT and 3'MT probes. However, the binding efficiency of the renatured PA- α TIF was less than that of the renatured α TIF from the SF9 cells (data not shown).

The DNA-binding activity of α TIF was inhibited by the inclusion of MgCl_2 (0.1–2 mM) in the binding reaction but was stimulated by a cycle of denaturation and renaturation of the protein. Gel mobility assays, in which either the protein in the α TIF column fractions or the gel-purified protein was first denatured with guanidine/HCl and partially renatured by dilution, exhibited a 15-fold stimulation of DNA-binding activity over equivalent amounts of untreated protein (data not shown). One possible explanation for this phenomenon is that α TIF, consistent with its form and function in the tegument structure of the HSV virion, readily aggregates and precipitates from solution at high protein concentrations. The cycle of denaturation and renaturation at dilute concentrations may increase the percent of soluble monomeric protein. Alternatively, the protein may require a conformational change to expose its DNA-binding domain. Regardless of these possibilities, α TIF represents a second DNA-binding specificity in the assembly of the C1 and C2 complexes.

The DNA binding activity of α TIF is stimulated by the binding of the Oct-1 POU domain in the absence of the C1 factor

No apparent interactions could be detected between PA-POU and α TIF, either in the absence of DNA or in a gel mobility shift assay (see above). However, UV-cross-linking reactions revealed a potential cooperative DNA-binding interaction between these proteins. HSV α 0 DNA was uniformly labeled by the incorporation of [^{32}P]dNTP and was incubated with proteins purified and renatured with SDS-PAGE. The binding reactions were UV-irradiated, digested with nucleases, and resolved on an SDS-denaturing gel. Reactions containing high concentrations of PA- α TIF or α TIF revealed unique radioactive protein products of 74 and 53 kD, respectively (data not shown). This supports the conclusion that these two forms of the α TIF protein have inherent, independent, DNA-binding potential. More significantly, as illustrated in Figure 3B, the binding PA-POU to the octamer element in the HSV α 0 probe stimulated the efficiency of UV cross-linking of the α TIF proteins significantly. Reactions containing gel-purified PA-POU exhibited the

anticipated 48-kD radioactive product (Fig. 3B, lane 2). In this set of experiments, no significant UV cross-linking of α TIF-related proteins was detected in reactions containing reduced concentrations of PA- α TIF or α TIF alone (lanes 3 and 4). However, addition of PA-POU to identical reactions containing either PA- α TIF (lane 5) or α TIF (lane 6), stimulated the efficiency of UV cross-linking by 15-fold of the respective 74- or 53-kD proteins. No stimulation of the cross-linking of α TIF was recorded in control reactions containing the α TIF proteins and PA. In addition, in cross-linking reactions with the wild-type, 5'MT, and 3'MT probes, PA-POU did not bind to the 5'MT probe DNA and did not stimulate the efficiency of cross-linking of α TIF. In contrast, the cross-linking of α TIF was stimulated by the binding of PA-POU to the wild-type and 3'MT probes. As α TIF does not bind the 3'MT probe independently, it is likely that the stimulation observed with this probe represents the importance of the cooperative interaction between PA-POU and α TIF (data not shown). Thus, the cooperative DNA binding of the α TIF/PA-POU proteins, as indicated by the increase in the efficiency of UV cross-linking, required the binding of PA-POU to the octamer element. To demonstrate definitively that the novel 74- and 53-kD species represented PA- α TIF and α TIF, respectively, the SDS-PAGE-resolved reaction products were blotted with an anti- α TIF sera. This sera reacted specifically with the anticipated radioactive bands (data not shown).

Similar UV cross-linking reactions with fractions containing the C1 factor (H-C1) exhibited minor DNA-binding species of 45 and 55 kD. However, no stimulation of the DNA-binding activity of α TIF was observed in reactions containing this protein and the H-C1 fractions. Additionally, no alteration in the cross-linking pattern was seen in reactions containing PA-POU/C1 or PA-POU/PA- α TIF/C1 as compared to equivalent reactions in the absence of the C1 activity (data not shown).

The homeo box of Oct-1 binds the HSV α 0 site cooperatively and interacts with α TIF/C1

The POU domain of Oct-1 contains all of the determinants required for the high-affinity binding to the octamer element and for the specific interaction with α TIF/C1. This domain consists of the highly conserved POU-specific box and the POU homeo box. To investigate the roles of these subdomains in the assembly of the C1 complex, PA fusion proteins were produced consisting of either the POU-specific box (PA-Poubox, amino acids 270–368) or the POU homeo box (PA-homeo box, amino acids 368–441). The purified proteins were compared to the intact POU domain (PA-POU) for the ability to bind to the HSV α 0 DNA probe and to interact with the α TIF/C1 proteins. Titration of PA-POU in DNA-binding reactions determined that this protein bound the octamer homolog in the HSV α 0 probe with a K_d of $\sim 3.5 \times 10^{-10}$ M. This affinity was not significantly different than that exhibited by the protein in re-

actions containing a consensus octamer element probe ($kD = \sim 2.2 \times 10^{-10}$ M). Similarly, in reactions containing a constant concentration of PA- α TIF and C1 factor, formation of the multiprotein PA-POU/ α TIF/C1 complex was only twofold more efficient than formation of the PA-POU/DNA complex. Therefore, the primary determinant in the affinity of the multiprotein C1 complex for the α /IE site is the recognition of this element by the Oct-1 POU domain.

The POU-specific box subdomain (PA-Poubox) did not exhibit significant binding to the consensus octamer or the HSV α 0 probes and did not support the formation of a C1 complex in the presence of α TIF and C1 factor. In contrast, the homeo box subdomain (PA-homeo box) bound the consensus octamer element but had a significantly lower affinity than PA-POU ($kD = \sim 2.3 \times 10^{-5}$ M). Additionally, as shown in Figure 4 (lanes 1 and 2), PA-homeo box formed a protein/HSV α 0 DNA complex with an electrophoretic mobility expected of a PA-homeo box homodimer/DNA complex [cf. the migration of PA-POU/DNA (lane 5) with PA-homeo box/DNA (lanes 1 and 2)]. The mobility of this complex was slower than that of the PA-homeo box/consensus octamer element complex and equivalent to that of a dimeric complex formed by the binding of PA-homeo box to a dimeric consensus octamer element. Additional results also support the dimeric nature of the PA-homeo box/HSV α 0 DNA complex. As shown below, the PA-homeo

box protected a dimeric binding site in the HSV α 0 element from Cu/phenanthroline cleavage. Titration of PA-homeo box did not exhibit any indication of a monomer PA-homeo box/DNA complex, suggesting that the protein bound the HSV α 0 probe only via a highly cooperative interaction. Furthermore, binding of the PA-homeo box dimer to the HSV α 0 probe required 10-fold less protein than the equivalent monomeric binding to the consensus octamer element. In contrast, titration of the intact POU domain protein (PA-POU) to high concentrations showed no evidence of cooperative, dimeric binding to this probe (data not shown), although it is clear that the POU domain of the Oct proteins can exhibit cooperative binding to appropriately spaced and oriented octamer elements (LeBowitz et al. 1989). The binding of PA-homeo box to the HSV α 0 probe suggests that this 60-amino-acid DNA-binding domain has the potential for cooperative interactions.

As PA-homeo box bound the HSV α 0 probe, it was tested directly for its ability to form a C1 complex in the presence of PA- α TIF and the C1 factor. As shown in Figure 4 (lanes 3 and 4), this protein was fully capable of forming the C1 complex. In parallel binding reactions containing equivalent concentrations of α TIF/C1 factor, however, the formation of equivalent amounts of the C1-type complexes occurred at a 10^2 lower concentration of PA-POU than PA-homeo box. This lower efficiency of formation of the C1 complex is probably accounted for by the 10^4 -fold difference in DNA-binding affinity between the PA-homeo box and PA-POU proteins. Thus, the interactions between either the PA-homeo box or the PA-POU and the PA- α TIF/C1 factor may be of comparable affinity.

Because the Oct-1 POU-specific box (PA-Poubox) did not bind the HSV α 0 DNA under these conditions, it could not be directly assessed for interactions with the α TIF and C1 factors. Therefore, this protein was used as a competitor in a C1 complex formation assay. Increasing molar amounts of PA-Poubox, PA-homeo box, or PA proteins were added to a C1 formation reaction consisting of PA-POU/PA- α TIF/C1 factor. Inclusion of either the control PA or the PA-Poubox proteins in these reactions did not affect the formation of the PA-POU/DNA and C1 complexes at a 100-fold molar excess over the concentration of the PA-POU protein. However, inclusion of PA-homeo box specifically competed for a component (PA- α TIF or the C1 factor) of the C1 complex under conditions where the DNA binding of PA-POU was unaffected (data not shown).

Consistent with its interactions with a component of the C1 complex and its ability to support the formation of the C1 complex, PA-homeo box bound the HSV α 0 probe and specifically stimulated the binding of either PA- α TIF or α TIF in the UV-cross-linking assays (data not shown). As anticipated, PA-Poubox did not exhibit either significant DNA binding or cooperative interactions with the α TIF proteins in equivalent assays. Thus, the homeo box of the Oct-1 protein appears to contain most, if not all, of the determinants required for the interaction with α TIF/C1 factor.

Figure 4. The Oct-1 homeo box (PA-homeo box) binds the α /IE element cooperatively and forms an α TIF-dependent C1 complex. Protein-DNA-binding reactions contained 200 ng poly[d(I-C)]/poly[d(I-C)] and the following proteins: (Lanes 1 and 3) 350 ng PA-homeo box; (lanes 2 and 4) 700 ng PA-homeo box; (lane 5) 25 ng PA-POU. Lanes 3–5 contained, in addition to the Oct-1 fusion protein, 100 ng PA- α TIF and 2 μ l of the DE-C1b fraction, as indicated at top (+). Each panel is derived from the same gel autoradiogram. The positions of the PA-homeo box/DNA, PA-POU/DNA, and C1 complexes are indicated with arrows.

Protection of the HSV α 0 DNA from orthophenanthroline/Cu cleavage by the PA-homeo box dimer, PA-POU, and PA-homeo box-C1 complex

The HeLa cell Oct-1 protein protects the octamer homolog in the HSV α 0 probe from cleavage by orthophenanthroline/Cu (Kristie et al. 1989). Furthermore, the Oct-1/ α TIF/C1 complex extends this protection to encompass the entire HSV α /IE element. As shown in Figure 5, A and B, PA-POU and the PA-POU-C1 complex afforded a protection equivalent to that of the intact Oct-1 and Oct-1-C1 complex, respectively. In contrast, the PA-homeo box-C1 complex protected only a portion of the octamer homolog but protected the remainder of the α /IE site in an identical fashion to that of the PA-POU-C1 complex. Of significant note is the lack of protection of the first several nucleotides of the octamer homolog, which was protected by the intact POU domain protein [Fig. 5A, cf. all lanes and Fig. 5B, 5'-ATGC-3' (top strand) and 5'-TGC-3' (bottom strand)]. Assuming that the homeo box subdomain occupies the same position in the PA-homeo box-C1 complex and the PA-POU-C1 complex, this lack of protection by PA-homeo box indicates that the POU-specific box subdomain contributes directly to the recognition of the octamer element. The octamer sequence therefore appears to be a bipartite element that is recognized by the two subregions of the POU domain.

As illustrated in Figure 5, A (left) and B, the apparent PA-homeo box homodimer also did not protect the 5' part of the octamer homolog but did protect sequences 3' to the octamer element in the α /IE site. This additional protection probably reflects the binding of the second PA-homeo box protein.

Discussion

A wide variety of regulatory processes are dependent on an octamer DNA element. Even the existence of discrete octamer-binding proteins such as Oct-1 and Oct-2 cannot solely account for the role of this sequence in the distinct regulation of different cellular genes. Differen-

tial regulation, however, can be explained by the interaction of Oct-1 and Oct-2 with other proteins to form complexes resulting from the combined specificities of multiple factors. These hypothetical regulatory assemblies are modeled by the interaction of the HSV *trans*-activator, α TIF, with the Oct-1, C1, and C2 proteins.

Interactions and specificities in the assembly of the α TIF-dependent complexes

The assembly of complexes on the α /IE element is initiated by the binding of Oct-1 to the octamer homolog

Figure 5. Protection from orthophenanthroline/Cu cleavage of the HSV α 0 DNA sequences by PA-homeo box, PA-POU, and the PA-homeo box-C1 complex. (A) Protein-DNA-binding and cleavage reactions of electrophoretically resolved protein-DNA complexes were done as described in Materials and methods and the legend to Fig. 4. Purified DNA cleavage products were resolved in a 10% sequencing gel. (Free) The cleavage products of the unbound DNA. (GA) A guanine plus adenine sequencing reaction ladder (Maxam and Gilbert 1980). The nucleotide sequence of the noncoding coding strand of the HSV α 0 DNA probe is (5' to 3', top to bottom) at left and right. (B) The bars schematically delineate the boundaries of the protection from orthophenanthroline/Cu for the noncoding (top) and coding (bottom) strands of the HSV α 0 probe. (●) Nucleotides that are partially protected. The consensus octamer (Falkner and Zachau 1984) sequence is aligned above. (POU and homeo box) PA-POU and PA-homeo box, respectively. The protection limits of the POU-C1 complex were published previously (Kristie et al. 1989).

Figure 6. Interactions and sequence specificities involved in the assembly of the multiprotein C1 complex. (A) A schematic representation of the proteins that assemble on the α /IE element. (PSB and PHB) The Oct-1 POU-specific box and POU homeo box, respectively. α TIF is positioned so as to make contact with the POU-homeo box and the HSV core sequence of the α /IE element. The C1 factor is arbitrarily represented as a modulator of the α TIF activity. The DNA specificity of each of these components is shown below the diagram for the noncoding strand (5' to 3'). The 3' boundary of the binding site for the POU homeo box monomer was deduced from the 3' boundary of the sequences protected by the intact POU domain. The sequences recognized by the

POU-specific box are those nucleotides that are protected in the C1-type complexes by the intact POU domain, but not by the POU homeo box. The DNA-binding specificity of the α TIF/C1 factor is based on the sequences protected by the C1 complex and the positions of the point mutations that interfere with the binding of α TIF. The positions of the double point mutants in the α /IE element (5' and 3') are shown and are described in the text and in the legend to Fig. 3. (B) The α /IE element is illustrated as a planar representation of the cylindrical projection of the DNA surface (Siebenlist et al. 1980; 10.5 bp per turn). The nucleotide sequence of the noncoding (top) and coding (bottom) DNA strand is written alongside the phosphate backbone (●). The major and minor grooves of the DNA helix are indicated. (Faces A and B) The front and back faces of the helix, respectively. (H1–H3) The three helices of the Oct-1 POU homeo box are arranged according to Qian et al. (1989) and oriented as described in the Discussion. The nucleotides protected from Cu/phenanthroline cleavage are enclosed by a dashed line. (C) The α /IE element and the Oct-1 homeo box are represented as in B with the deduced Cu/phenanthroline cleavage protections of the Oct-1 POU-specific box (PSB) and α TIF/C1 factor enclosed by the indicated dashed lines.

(Fig. 6A). This provides the basis for the subsequent binding of α TIF/C1 factor (C1 complex), followed by the association of the HeLa cell nuclear C2 factor (C2 complex; Kristie et al. 1989). A second sequence specificity is contributed by α TIF whose DNA binding is abolished by point mutations in the highly conserved TAATGARAT core of the α /IE element. The independent DNA-binding affinity of this protein is low and would, in vivo, undoubtedly require a cooperative interaction with Oct-1. Although α TIF interacts directly with the Oct-1 POU domain, no interaction was detected in the absence of the α /IE element, suggesting that this interaction is of low affinity. Thus, α TIF clearly represents a second DNA-binding specificity that is involved in the selection and activation of an α -gene enhancer site.

The C1 factor, present in both SF9 insect cell extracts and HeLa cell nuclear extracts, represents a third determinant. Direct evidence for the presence of the C1 factor in the C1 complex derives from the chromatographic resolution of two modified forms of the C1 factor. These forms differ in their state of phosphorylation and generate C1 complexes of distinct mobilities. The association of the C1 factor with α TIF, in the absence of the α /IE element, suggests that an α TIF/C1 factor complex may be the configuration with the highest affinity for DNA binding or interactions with Oct-1. The C1 factor may either induce a conformational change in α TIF, interact with both α TIF and Oct-1, or directly contribute

to the recognition of the α /IE site core sequences. As a cycle of denaturation–partial renaturation of α TIF increased its DNA-binding efficiency, a conformational alteration may be required for significant DNA recognition by this protein. The C1 factor may function by inducing such a conformational change. Alternatively, although the partially purified C1 factor does not possess detectable DNA-binding activity, this protein may be partially responsible for some sequence-specific binding in the C1 complex. In spite of this uncertainty with regard to its function, it is clear that the C1 factor is a chromatographically distinct component that interacts directly with α TIF in the absence of DNA and is required to form a stable C1 complex.

In the biology of an HSV infection, the association of α TIF and the C1 factor is probably critical for the initiation of infection. Productive infection of cells by HSV can be blocked by the expression of a mutant α TIF that lacks the *trans*-activation domain (Freidman et al. 1988; Triezenberg et al. 1988). It is possible that this inhibition resulted from the sequestering of the C1 factor by the mutant protein or by the formation of nonproductive C1-type complexes on the α /IE elements.

The fourth factor, C2, is present in nuclear extracts of HeLa cells and is required to assemble the larger C2 complex. This factor did not alter the metal ion cleavage protection of the α /IE site by the C1 complex, nor was it retained on an α TIF protein matrix (data not shown).

Therefore, the association of the C2 factor is mediated primarily by protein–protein interactions with Oct-1, the C1 factor, or a combination of the C1 complex components. The assembly of the C1 and C2 complexes thus depends on the interaction of multiple components that contribute both DNA–protein and protein–protein specificities to the selection and activation of particular octamer sites.

The role of the Oct-1 homeo box in the interaction with α TIF/C1

Oct-1 and Oct-2 are members of the POU family, which includes a large number of tissue-specific transcription factors (Herr et al. 1988; Thali et al. 1988; He et al. 1989; Johnson and Hirsch 1990). The conserved POU domain of these proteins is subdivided into the POU-specific box and the POU homeo box, which contains the helix–turn–helix motif characteristic of the DNA-binding domains of the homeo box family (Laughon and Scott 1984; McGinnis et al. 1984; Clerc et al. 1988; Jordan and Pabo 1988; Ko et al. 1988; Otting et al. 1988; Sturm et al. 1988; Qian et al. 1989; Scott et al. 1989).

The fusion protein, containing only the POU–homeo box, bound the consensus octamer and the α /IE elements specifically but with a significantly lower affinity than the intact POU domain. In contrast to its binding to the consensus octamer, PA–homeo box bound the α /IE element as a homodimer (PA–homeo box/PA–homeo box/DNA). A monomeric PA–homeo box/DNA complex was not detected, indicating that the homeo box bound the α /IE element in a cooperative manner. This cooperative binding of PA–homeo box is probably due to the inverted repeat (TAATGAT/ATTCTTT) in the α /IE element that is related to the consensus homeo box element (TCAATTAAAT; Desplan et al. 1988; Hoey and Levine 1988; Muller et al. 1988a). Nonetheless, this suggests that the Oct-1 homeo box is capable of cooperative DNA-binding interactions.

Surprisingly, the Oct-1 homeo box protein (PA–homeo box) readily interacted with α TIF/C1 to form a C1-type complex. In contrast, the POU-specific box protein (PA–Poubox) did not form a C1 complex with α TIF/C1 factor, nor was it able to compete for these proteins. Additionally, UV-cross-linking reactions with PA–homeo box and α TIF exhibited a specific interaction between these two proteins in a manner equivalent to that of the intact Oct-1 POU domain and α TIF. The 100-fold difference in the efficiencies of formation of the C1 complex with the homeo box and the intact POU domain proteins can be accounted for by their difference in relative DNA-binding affinities. It is therefore likely that the homeo box contains most, if not all, of the Oct-1 determinants that mediate its protein–protein interactions in the C1 complex.

Although both the Oct-1 and Oct-2 POU domains may interact with α TIF/C1, Oct-1 has a 100-fold greater affinity for this interaction (Kristie et al. 1989). A major part of this specificity appears to be due to differences in the amino acid sequence of the Oct-1 and Oct-2 homeo

box subdomains (Stern et al. 1989). These regions differ in 8 of 63 positions, whereas the POU-specific boxes of these proteins are nearly identical (1 of 75 amino acids; Clerc et al. 1988; Sturm et al. 1988). More directly, Stern and colleagues have shown that conversion of two amino acids in helix 2 of the Oct-1 homeo box subdomain to those found in Oct-2 eliminated its ability to form the C1-type complex efficiently (Stern et al. 1989).

The role of the Oct-1 POU-specific box and POU homeo box in the recognition of an octamer element

The high-resolution footprints of the C1-type complexes formed with the POU and homeo box proteins on the α /IE element differ at the outer boundary of the octamer homolog. Several nucleotides [5′-ATGC-3′ (top strand) and 5′-TGC-3′ (bottom strand)] at the 5′ terminus of the octamer homolog are protected by the intact POU domain but not by the homeo box subregion (refer to Fig. 5B), while the remainder of the α /IE element is identically protected by the C1 complexes formed with either PA–POU or PA–homeo box. The same bases are specifically not protected by the complex formed with the homeo box protein as compared to the POU domain protein. This suggests that the POU-specific box of the POU domain functions to increase the affinity and specificity of the Oct-1 DNA-binding activity by direct interaction with the 5′ part of the octamer element. The recognition of specific DNA sequences by the POU-specific box is consistent with previous studies in which the POU-specific box was switched between the Oct-1 and Pit-1 proteins, leading to a change in the DNA-binding specificities of these proteins (Ingraham et al. 1990). The concept that the POU domain binds DNA with a bipartite specificity is additionally supported by the direct DNA-binding activity of a fusion protein that contains only the isolated Oct-1 POU-specific box (data not shown). Thus, the Oct-1 POU domain consists of two semi-independent DNA recognition domains that act synergistically. It is likely that all of the members of the POU domain family contain two binding specificities: one conferred by the POU–homeo box and another conferred by the POU-specific box. The sum of these yields the total sequence recognition specificity.

The relative orientation of the POU domain on the octamer site can be deduced from both the DNA sequences whose protection is assigned to the POU-specific box and the requirement that helix 2 of the homeo box subdomain be accessible for specific interactions with other proteins in the C1 complex. Only one orientation of the helix–turn–helix (helix 2–helix 3) of the homeo box is consistent with these two results (Fig. 6B,C; for the structure of a homeo box protein, see Qian et al. 1989). In this arrangement, the DNA recognition helix 3 is positioned in the major groove, extending from helix 2, which must occupy the interface between the octamer site and the hypothetical α TIF-binding site. The POU-specific box at the amino terminus of helix 1 of the homeo box would be positioned near the 5′-ATGC-3′ sequences that are protected by the binding of this subdo-

main. This assigned orientation is consistent with the recently determined crystal structure of a homeo box/DNA complex [C. Pabo, pers. comm.].

The homeo box of Oct-1 must be a finely evolved structure. It must accommodate the DNA binding of the POU-specific box on one side and the α TIF/C1 factor on the other side. In addition, the homeo box can interact cooperatively in a homodimeric configuration upon binding to a site with inverted symmetry. This cooperativity is probably mediated by similar interactions to those that are involved in the cooperative interaction with α TIF. This range of possible protein-protein interactions probably accounts for the high degree of conservation in homeo box structures and may also explain the observation that all POU domain proteins share a conserved homeo box sequence that is a distinct subgroup of the large number of homeo box proteins.

Materials and methods

Production and purification of PA fusion proteins in E. coli

DNA fragments encoding the Oct-1 POU domain [amino acids 270–441, [Sturm et al. 1988]], the Oct-1 POU-specific box [amino acids 270–368], the Oct-1 homeo box [amino acids 368–441], and the α TIF interactive domain [amino acids 1–412 [Pellett et al. 1985]] were isolated and cloned to generate in-frame fusions with the PA gene in pRIT2T (Pharmacia). *E. coli* N4830 strains, harboring the appropriate plasmids, were grown at 30°C to $OD_{600} = 0.8$ and induced to express the fusion products at 42°C for 1.5 hr. Extracts were prepared by freezing the harvested cells in 50 mM Tris at pH 8.0/10% (wt/vol) sucrose and thawing them in the presence of 0.5 mg/ml lysozyme. The cells were heat-shocked at 37°C for 10 min, brought to 500 mM NaCl, and centrifuged at 200,000g for 1 hr. The supernatants were brought to 50 mM Tris at pH 7.6/150 mM NaCl/0.05% Tween-20, and the fusion products were purified on IgG–Sepharose (Pharmacia) according to the manufacturer's protocol for low pH elution. The purified fusion proteins were lyophilized briefly and brought to buffer A + 100 mM KCl [40 mM HEPES at pH 7.9/0.5 mM EDTA/0.5 mM DTT/20% (vol/vol) glycerol]. Protein concentrations were determined by Bio-Rad protein assay and by densitometric scanning of Coomassie-stained SDS-PAGE-resolved proteins. The fusion proteins were judged to be >70% homogeneous.

Purification of baculovirus-expressed α TIF

The expression and production of S100 extracts from AcNPV– α TIF-infected SF9 cells was as described [Kristie et al. 1989]. S100 extracts were applied to a DEAE–Sepharose CL6B column at 8 mg/ml in buffer A + 100 mM KCl. The column was washed with 10 column volumes of buffer A + 100 mM KCl, followed by successive steps of 5 column volumes each of buffer A containing 200 mM KCl, 350 mM KCl, and 500 mM KCl. The 350 mM KCl step fractions were pooled, dialyzed to buffer A + 100 mM KCl, and reappplied to a high-resolution DEAE–Sepharose column. Adsorbed proteins were eluted with a linear KCl gradient of 200–400 mM. Protein concentrations were determined by Bio-Rad protein assay. Fractions were assayed for α TIF via SDS-PAGE and by gel mobility shift in the presence of HeLa nuclear extract. This α TIF preparation was judged to be >90% homogeneous and contained no detectable C1 activity as determined by gel mobility shift assays in the presence of PA–POU protein.

SDS-PAGE purification and denaturation–renaturation of Oct-1 and α TIF proteins

Where indicated, proteins were further purified by preparative SDS-PAGE. IgG–Sepharose-purified PA fusion products or DEAE-purified α TIF protein fractions were resuspended in SDS-PAGE loading buffer, heated to 68°C for 10 min, and resolved in a 12.5% polyacrylamide SDS-denaturing gel. The proteins were visualized by staining the gel with 0.25 M KCl as described [Hager and Burgess 1980]. Gel slices containing the appropriate protein bands were excised from the gel, crushed in silated Dounce homogenizers, and incubated with elution buffer [Hager and Burgess 1980] + 100 μ g/ml insulin for 12 hr at 25°C. The eluted proteins were precipitated with acetone, resuspended in 100 μ l HCl-guanidine denaturation buffer [Hager and Burgess 1980] + 100 μ g/ml insulin, and dialyzed against buffer A + 100 mM KCl at 4°C. Alternatively, the denatured proteins were diluted 100-fold in buffer A + 100 mM KCl. The yield and purity of the renatured proteins were determined by SDS-PAGE. Renatured proteins were judged to be homogeneous preparations containing the purified protein and carrier insulin protein.

Protein–DNA-binding assays

HSV α 0, wild-type, 5' MT, and 3' MT DNA probes are equivalent to pRB608, α OH1, α OH1–5' MT, and α OH1–3' MT, respectively, and have been described [Kristie and Roizman 1987; Kristie et al. 1989]. SF9 cell S100 extracts and octamer-depleted HeLa nuclear extracts were prepared as described previously [Kristie et al. 1989]. Unless otherwise noted, DNA/protein-binding reactions contained 0.5 ng [8.0 fmol] HSV α 0 DNA probe, 50–1000 ng poly[d(I-C)]/poly[d(I-C)], 10 mM HEPES (pH 7.9), 0.5 mM EDTA, 25–75 mM KCl, 0.75 mM DTT, 4% Ficoll 400, 300 μ g/ml bovine serum albumin, and the appropriate purified protein or chromatographic fraction in a total volume of 10 μ l. Reactions were incubated at 30°C for 30 min and resolved in 4% nondenaturing polyacrylamide gels using 0.5 $\times$ Tris-glycine electrophoresis buffer as described [Fried and Crothers 1981; Garner and Revzin 1981]. Autoradiograms of dried gels were scanned, where appropriate, on an LKB XL ultradensitometer. For the determination of molecular mass values for the PA–POU and PA–homeo box proteins, increasing amounts of these proteins were added to a binding reaction containing either the HSV α 0 or the consensus octamer [GCATGCAAAT (Falkner and Zachau 1984)] DNA probes and 30 ng poly[d(I-C)]/poly[d(I-C)]. The protein–DNA complexes and free DNA were quantitated after electrophoresis by scanning the dried gel mobility shift gel using a Molecular Dynamics phosphorimager. Molecular mass values for PA–POU were obtained at 50% bound DNA using the equation $kD = (\text{protein concentration at 50\% saturation}) - (\text{concentration of bound DNA})$, where the DNA concentration was 6.7×10^{-10} M. Values for PA–homeo box were extrapolated from those obtained at protein concentrations below 50% saturation. Values for the C1-type complexes containing either PA–POU or PA–homeo box complexes were determined similarly by titration of the respective proteins in reactions containing 50 ng PA– α TIF and 2 μ l DE–C1b fraction.

Chromatographic fractionation of the C1 factor

S100 extracts were prepared from SF9 cells and applied to a DEAE–Sepharose CL6B column in buffer A + 100 mM KCl. The column was washed with 10 column volumes of buffer A + 100 mM KCl, and the adsorbed proteins were eluted with successive steps of 5 column volumes each of buffer A + 200 mM KCl, 350 mM KCl, and 500 mM KCl. The 350 mM KCl step

fractions were pooled (DE-C1a) and reapplied to a high-resolution DEAE-Sephacrose column. Bound proteins were eluted with a linear KCl gradient of 200–400 mM. Fractions containing C1 activity (DE-C1b) were diluted to buffer A + 50 mM KCl and applied to a heparin-agarose column, equilibrated in buffer A + 50 mM KCl. The column was washed as above. The adsorbed proteins were eluted with a linear gradient of 50–150 mM KCl in buffer A, followed by successive steps to 250 mM KCl and 350 mM KCl. DE-C1b activity was loaded onto a single-stranded DNA-cellulose and a double-stranded DNA-cellulose column, equilibrated in buffer A + 50 mM KCl. The columns were washed with 10 column volumes of buffer A + 50 mM KCl, and the proteins were eluted with successive steps of 100, 250, 350, and 500 mM KCl. All fractions were assayed for C1 activity by gel mobility shift in the presence of PA-POU and PA- α TIF. Protein concentrations were determined by Bio-Rad protein assays. KCl concentrations were assayed by conductivity assays. Phosphatase treatment of C1 activity was done by incubation of the appropriate fractions from the heparin-agarose chromatography with potato acid phosphatase (Sigma) or alkaline phosphatase (Boehringer Mannheim) for 0.25 hr at 30°C prior to the addition to the DNA-binding reaction.

Protein affinity chromatography

PA-q-POU, PA- α TIF, and PA were purified on IgG-Sephacrose as described above. Then, 1.5 mg of each purified protein was diluted to 10 ml in 50 mM Tris at pH 7.6/150 mM NaCl/0.05% Tween-20. IgG-Sephacrose (2 ml) was added, and the slurry was mixed on a rotator at 4°C for 3 hr. The matrices were washed successively with buffer A + 50 mM KCl, buffer A + 50 mM KCl + 100 μ g/ml insulin, and buffer A + 50 mM KCl. C1 fractions (DE-C1b) were batch-absorbed with 1 ml of PA-POU or 1 ml PA- α TIF matrix in buffer A + 50 mM KCl at 4°C for 2 hr. The matrices were poured into columns and washed with 3 column volumes each of buffer A + 50 mM KCl, 150 mM KCl, and 500 mM KCl. C1 activity was assayed by gel mobility shift in the presence of PA-POU and PA- α TIF. Then 5 μ g baculovirus-expressed α TIF was chromatographed similarly on 1-ml PA-POU and 1-ml PA columns. The elution of α TIF was monitored by gel mobility shift in the presence of PA-POU and 2 μ l DE-C1a fraction or, alternatively, in the presence of 10 μ g of complete HeLa nuclear extract.

Orthophenanthroline/Cu footprinting reactions

Orthophenanthroline/Cu footprinting reactions were done as described (Kuwabara and Sigman 1987; Kristie et al. 1989) except that the cleavage reactions were done at 4°C. Autoradiographs of the dried gels were analyzed by densitometry, and the limits of protection were determined in several independent experiments.

UV-cross-linking reactions

Body-labeled probes were prepared by polymerase chain reaction (PCR) amplification of 50 ng *PvuII*-cut pRB608 (HSV α 0) in the presence of 200 μ M [α -³²P]dATP, 200 μ M [γ -³²P]dCTP, 5 μ M dATP, 5 μ M dCTP, 200 μ M dGTP, 200 μ M dTTP or 200 μ M BrdU · triphosphate, 0.5 μ M M13 universal primer, and 0.5 μ M M13 reverse sequencing primer. PCR reaction products were digested with *EcoRI* and *HindIII*, and the DNA probe was isolated from a 6% polyacrylamide gel as described (Maniatis et al. 1982). DNA-protein-binding reactions, containing SDS-PAGE-purified proteins, were scaled up threefold, incubated at 30°C

for 30 min, and irradiated with a fotodyne UV lamp (254 nm) at 4000 μ W/cm² for 30 min in an ice-water bath. The reactions were brought to 7.5 mM CaCl₂ and digested with 10 μ g DNase I and 5 units micrococcal nuclease for 30 min at 37°C. Nuclease-digested reaction products were resolved in a 12.5% SDS-denaturing gel and transferred to nitrocellulose in the presence of 0.1% SDS. For Western blot analysis of radiolabeled proteins, the nitrocellulose was cut into the appropriate strips after autoradiography and blotted with anti- α TIF sera (gifts of J.L.C. McKnight and S. McKnight).

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Note added in proof

We would like to note that C.P. Verrijzer, A.J. Kal, and C. van der Vliet have come to similar conclusions with regard to the sequence-specific interactions of the POU-specific box of the Oct-1 protein [Verrijzer et al., *Genes & Dev.* **4**: 1964–1974, 1990].

Interactions of the Oct-1 POU subdomains with specific DNA sequences and with the HSV alpha-trans-activator protein.

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