Recruitment of the priming protein pTP and DNA binding occur by overlapping Oct-1 POU homeodomain surfaces

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The human transcription factor Oct-1 can stimulate transcription from a variety of promoters by interacting with the coactivators OBF-1/OCA-B/BOB-1, SNAP190 and VP16. These proteins contact Oct-1 regions different from the DNA binding surface. Oct-1 also stimulates the DNA replication of adenovirus through its DNA binding site in the origin. The Oct-1 POU homeodomain (POUhd) binds the adenovirus precursor terminal protein pTP, which serves as the protein primer of DNA replication and recruits pTP to the origin. To map the interaction with pTP at the POUhd surface, we screened a library of randomly mutated POU domains and identified mutations that interfered with pTP interaction and DNA replication stimulation. These mutants clustered at a surface different from those recognized by OBF-1, SNAP190 and VP16. Unexpectedly, the pTP binding region largely overlapped with the DNA binding surface of POUhd. In agreement with this, pTP binding and DNA binding were mutually exclusive. We propose a model to reconcile pTP recruitment and DNA binding by Oct-1.

Keywords: adenovirus precursor terminal protein/DNA polymerase/eukaryotic DNA replication/human transcription factor Oct-1/NFIII

Introduction

Oct-1 is a ubiquitously expressed human transcription factor that can stimulate RNA pol II and pol III transcription from a variety of promoters (Herr and Cleary, 1995; Phillips and Luisi, 2000). It consists of a central bipartite POU DNA binding domain (Sturm and Herr, 1988) surrounded by regions responsible for transcription activation. POU domains consist of a POU-specific domain (POUs), which binds DNA with high specificity but low affinity and a POU homeodomain (POUhd), which binds DNA with low specificity but high affinity (Verrijzer *et al.*, 1992a). POU domains show a remarkable flexibility in recognition of promoter DNA sequences, which is caused partly by their bipartite structure and partly by their ability to dimerize in different conformations and compositions (van Leeuwen *et al.*, 1997a; Botquin *et al.*, 1998; Tomilin *et al.*, 2000).

The interactions between Oct-1 and other proteins have been studied in the presence and absence of DNA. The Oct-1 POU domain interacts with the accessory protein HMG-2 (Zwilling *et al.*, 1995), with the cellular coactivators OBF-1/OCA-B/BOB-1 and SNAP190 and with the viral transactivator VP16. The co-crystal structure of an OBF-1 peptide complexed to DNA-bound Oct-1 POU domain revealed that OBF-1 contacts a hydrophobic pocket in the POU domains and that it has additional contacts with DNA and the C-terminal amino acids of the POUhd DNA recognition helix (Chasman *et al.*, 1999). Using these three interaction points, OBF-1 clamps the Oct-1 POU subdomains to their DNA recognition sites, which confirmed mutagenesis data and the model proposed by Sauter and Matthias (1998). VP16 contacts amino acids at the surface of the POUhd completely opposite to the DNA binding surface, as determined by studies using Oct-1/Oct-2 chimeras and Oct-1 point mutants (Lai *et al.*, 1992; Pomerantz *et al.*, 1992). None of these coactivators contact the DNA binding surfaces of Oct-1.

Although the primary function of Oct-1 is sequence-dependent transcription stimulation, it is also capable of stimulating adenovirus (Ad) DNA replication (Prujn *et al.*, 1986; Rosenfeld *et al.*, 1987). For this function, the transcription activation domains are dispensable. Human adenoviruses contain a linear, double-stranded DNA genome of ~36 kb with origins of DNA replication located in the inverted terminal repeats. Replication of viral DNA can be efficiently reconstituted *in vitro* using the three viral proteins DNA polymerase (pol), precursor terminal protein (pTP) and DNA binding protein (DBP) and the three cellular proteins nuclear factor I (NFI), Oct-1 and nuclear factor II (NFII) (reviewed in de Jong and van der Vliet, 1999). The viral protein primer pTP and the viral pol form a pTP-pol heterodimer in solution, which can be recruited to the origins of Ad DNA replication by the cellular transcription factors Oct-1 and NFI. This stimulates replication initiation up to 200-fold depending on the pTP-pol concentration. The Oct-1 POUhd has been demonstrated to interact with pTP directly (van Leeuwen *et al.*, 1997b; Botting and Hay, 1999) and the presence of an Oct-1 binding site in the origin stimulates viral replication *in vivo* (Hay, 1985; Hatfield and Hearing, 1993). The sequence and exact position of the Oct-1 binding site in the origin are essential for the stimulation of Ad DNA replication (Wides *et al.*, 1987; Pruijn *et al.*, 1988). Oct-1 enhances the DNA binding of pTP-pol by lowering its dissociation rate (van Leeuwen *et al.*, 1997b). These observations support a model in which Oct-1 tethers pTP-pol to the replication origin.

Ad DNA replication could be stimulated both by different POU family members and by POUhd point mutants incapable of interacting with VP16, suggesting that conserved Oct-1 residues interact with pTP (Verrijzer

General strategy

To map the site of interaction, we generated a library of Oct-1 POUhd mutants using random mutagenesis PCR, linked these mutants to a GST fusion of wild-type Oct-1 POU_s (Figure 1A) and isolated bacterial protein extracts as described in Materials and methods. Protein extracts were screened for expression of full-length GST–Oct-1 POU protein and DNA binding to an Oct-1 binding site-containing probe. Selected mutants were sequenced and screened for interaction with pTP using a GST-pulldown

assay. Finally, the effects of all mutations were related to the co-crystal structure of the Oct-1 POUhd-DNA complex (Klemm *et al.*, 1994).

We optimized the PCR conditions to generate single or double point mutations in each homeodomain (Materials and methods). We noted that under these conditions, AT to GC base pair transitions occurred much more frequently than vice-versa. Since the GC content of the mutated DNA sequence is 46% and GC base pairs are evenly distributed throughout the sequence, we feel confident that the mutant library obtained represents a non-biased portion of the homeodomain DNA sequence.

To ensure the structural integrity of the mutant proteins, we screened for mutants capable of DNA binding. We isolated 225 protein extracts and sequenced the 83 full-length mutants displaying at least 50% of the wild-type expression level. The mutation distribution, illustrated in Figure 1B, shows that mutations are scattered throughout the DNA sequence, resulting in 22 single amino acid

Fig. 1. Identification of Oct-1 POUhd mutants. **(A)** Domain organization of Oct-1. The DNA binding POU domain is surrounded by two transcription activation domains. A GST-tag was fused to a wild-type Oct-1 POU domain and a randomly mutated library of Oct-1 POUhds. The first amino acid of POU is GenBank amino acid 280 (DDBJ/EMBL/GenBank accession No. P14859) and Klemm PDB amino acid 1 (accession No. 1OCT); the first amino acid of POUhd SRERK is GenBank amino acid 380 and Klemm PDB amino acid 101. We named our mutants after the Klemm PDB amino acid definition. The mutated amino acid sequence (95–160) is shown in detail below with the secondary structure (H = α -helix) deduced from the crystal structure (Klemm *et al.*, 1994). **(B)** Mutation distribution of soluble expressed Oct-1 homeodomain library mutants. Nucleotides in bold were hit during mutagenesis and the substitutions are indicated above the nucleotide sequence. 0 indicates every tenth nucleotide. See Table I for details on sequence and phenotypic behavior of individual POUhd mutants.

Table I. Oct-1 POUhd mutants: summary of sequence and experimental data

Mutations		DNA binding	pTP interaction	Replication stimulation (%POUwt)
amino acids	nucleotides			
R102W	A304T	+/-	-	1 ± 8
K104T	A311C	+	+/-	21 ± 17
R105S	C313A	+/-	+/-	1 ± 14
T106P	A316C	+/-	-	7 ± 16
T106S	A316T	+	+/-	68 ± 22
E109G	A326G	+	+	173 ± 58
I112N	T335A	+	+	97 ± 28
E122V (2)	A365T	+	+	105 ± 19
Q124L	A372T	+/-	+	13 ± 8
K125E	A373G	+/-	+/-	13 ± 8
K125M	A374T	+	-	41 ± 10
E129G	A386G	+	+	134 ± 15
E130G	A389G	+	+/-	80 ± 11
T132S	A394T	+	+/-	94 ± 35
V144E	T431A	+/-	+	20 ± 8
V147G	T440G	+	-	27 ± 5
F149Y	T446A	+	-	60 ± 22
C150Y	G449A	+	-	24 ± 3
N151D	A451G	-	-	11 ± 9
R153S	C457A	-	+/-	5 ± 7
K157E (2)	A469G	-	+	7 ± 6
K157I	A470T	+/-	+	4 ± 7
I95T/V147A	T284C/T440C	+	+/-	nd
S99G/K142E	A295G/A424G	+	-	nd
K103M/Q154L	A308T/A441T	-	+	nd
K104E/L116S	A110G/T347C	+	+/-	nd
S107I/S119G	G320T/A355G	+	+	nd
E109G/T132S	A326G/A394T	+	+	nd
T110A/I145N	A328G/T434A	-	+	nd
N111S/F149L	A332G/T445C	-	+	nd
E117A/I145V	A350C/A433G	+/-	+	nd
E122G/T127S	A365G/A379T	+	+	nd
E122G/M140L	A365G/A418T	+	+/-	nd
K125Q/R146S	A373C/C436A	-	+/-	nd
E130G/E141D (2)	A389G/A423C	+	+	nd
I134F/V147D(4)	A400T/T440A	-	+/-	nd
D136G/V147G	A407G/T440G	+	-	nd
Q137L/C150S	A410T/T448A	+	+/-	nd
M140R/R146P	T419G/G437C	-	+	nd
E141G/K155E	A422G/A443G	-	+/-	nd

For each POUhd amino acid mutation, the nucleotide transition, DNA binding efficiency, pTP binding efficiency, functional behavior in replication assays and clone number(s) are indicated. Scales are defined as follows. DNA binding: (-) <20%, (+/-) 20–50% and (+) 50–100%. DNA classification was verified by titration of purified proteins for all DNA (+) single point mutants and a representative set of DNA (-) mutants. pTP interaction: (-) <20%; (+/-) 20–50%; (+) 50–100%. pTP binding efficiency was estimated by comparing with input lanes. Replication stimulation (%): percentage stimulation of replication by mutant compared with stimulation by wild-type POU (100%) and basal replication level (0%). All stimulation values are averaged over four determinations. nd, not determined.

changes and 18 double changes. Some DNA stretches are hit less frequently, which could be caused by the effects of such mutations on the solubility or stability of the protein. The DNA binding capacity of all library proteins was analyzed using a DNA probe consisting of the first 50 bp of the Ad origin of DNA replication, which contains a (T)ATGATAAT(G) Oct-1 binding site (data not shown; Materials and methods). The 40 mutants identified showed levels of DNA binding varying from 2 to 150% compared with wild type (Table I).

Fig. 2. Analysis of the interaction between pTP and Oct-1 POUhd mutants capable of DNA binding. GST–POU mutant extracts were coupled to GA beads and tested for their capacity to pull down pTP. After stringent washing, bound pTP and GST–POU were eluted with 20 mM glutathione and analyzed on western blot for pTP (upper bands) and POU (lower bands). Upper (lanes 1–11) and lower panels (12–36) are independent experiments with different POU blot exposure times but equal POU loading amounts. Lanes 21 and 23 correspond to two independently isolated clones; 10% input: 60 ng pTP. Data are summarized in Table I.

Point mutants interfering with pTP binding cluster at the DNA binding surface

We assayed the 25 POU mutants that displayed >30% of wild-type DNA binding efficiency for their ability to recruit Ad pTP using a GST–pulldown experiment. Ad DNA polymerase can be recruited via pTP by GST–POU (van Leeuwen *et al.*, 1997b), but we minimized background binding to GST by assaying for pTP binding directly. GST–POU proteins were coupled to glutathione–agarose (GA) beads, washed extensively and incubated with purified bacterial pTP. After several washing steps, both proteins were eluted from the beads using 20 mM glutathione and analyzed using western blotting for Oct-1 POU and pTP. Figure 2 shows the capacity of the GST–POU mutants to bind pTP. Comparison with wild-type and GST control lanes allowed us to classify the mutants as non-binding (-), reduced binding (+/-) or wild-type-like binding (+) to pTP. Loading was analyzed by blotting of the eluted GST–POU. Of the single mutants displaying >30% DNA binding, five failed to bind pTP: R102W, K125M, V147G, F149Y and C150Y (lanes 35, 10, 8, 34 and 7). Mutation F149Y forms part of the hydrophobic core, where mutations could easily lead to unfolding of the homeodomain and was therefore not used for the interaction mapping. Mutants K104T and T106S (lanes 16 and 25) displayed reduced (20–50%) pTP

Fig. 3. pTP binds to the DNA binding surface of Oct-1 POUhd. The pTP binding behavior of individual mutants was mapped to the POUhd surface in the DNA-POUhd co-crystal structure (Klemm *et al.*, 1994). The POU is not shown. Amino acids in (C) and (D) were color coded as follows: red, mutation strongly reduced pTP binding; orange, mutation partly reduced pTP binding; green, mutation did not effect pTP binding; yellow, not hit during mutagenesis. (A) Schematic representation of the Oct-1 POUhd-DNA co-crystal structure. The DNA binding helix is indicated in red, the structural helices in light blue and the POUhd half-site DNA is indicated in dark blue. (B) POUhd amino acids that interact with DNA in the crystal structure (Klemm *et al.*, 1994) were colored red to allow comparison of the overlap between the pTP and DNA binding surface of Oct-1. (C) Surface map of pTP binding by Oct-1 POUhd mutants capable of DNA binding. Amino acids that interfere with pTP binding when they are mutated, are indicated by arrows. (D) Surface map of pTP binding by all identified Oct-1 POUhd mutants. Mutation of amino acids R105, T106, N151 and R153 interfered with both pTP binding and DNA binding. Mutation of amino acids R146, Q154 and K157 interfered with DNA binding but not with pTP binding (green).

binding. Mutants E130G and T132S (lanes 17 and 33) showed reduced binding as a single mutant, but not as part of a double mutant (lanes 30 and 36) and were therefore considered not to contribute to pTP binding. Some double mutants (e.g. K104E/L116S; lane 12) were impaired in pTP binding, but these were excluded from our analysis, since we cannot ascribe the phenotype to either of the side chains. Some mutants showed lower expression (lanes 20–24), but the reduced pTP binding efficiency that could result from lower POU mutant loading (lanes 23 and 24) did not contribute to pTP interaction surface mapping since they were double mutants.

The crystal structure of the POUhd complexed to DNA (Klemm *et al.*, 1994) is shown schematically in Figure 3A. The DNA binding helix is indicated in red, the two structural helices in light blue. The POU domain is not shown. In Figure 3B, a POUhd surface map in the same orientation is shown, with residues interacting with DNA in the crystal structure indicated in red. The pTP binding efficiency by POU mutant proteins is summarized for all

DNA binding positive POUhd mutants by color coding in Figure 3C. Amino acid mutations that caused loss of pTP binding (<20%) are indicated in red, those causing weak pTP binding (20–50%) in orange and mutations not inhibitory to pTP binding (50–100%) in green. Double mutants were included in the figure only if they were able to bind pTP (green) to limit the interaction surface. The single point mutants showing loss of pTP interaction clustered at the DNA binding surface of Oct-1 (compare Figure 3B and C) and not at a surface freely accessible from solution in the POU-DNA complex structure.

These results are surprising, since DNA binding by Oct-1 to the replication origin is essential for its stimulation of Ad DNA replication (Wides *et al.*, 1987). Before we can conclude that pTP binds to the Oct-1 DNA binding surface, we have to exclude two possible caveats. First, since pTP is also capable of DNA binding (Temperley and Hay, 1992), contaminating DNA in the bacterial protein extracts could be essential for indirect, DNA-mediated pulldown of pTP. Therefore homeodomain mutants that are partially affected in the DNA binding site, could be unable to recruit pTP efficiently. Second, mutations at the positions identified might lead to partial unfolding of the homeodomain, causing an indirect loss of pTP interaction.

DNA binding is not required for pTP binding

To exclude the possibility of DNA-mediated pulldown, we performed incubations in the presence of ethidium bromide, which has been shown to interfere strongly with the DNA binding of proteins (Lai and Herr, 1992). Binding of pTP by GST-POU wild type was not inhibited by the addition of 50 µg/ml ethidium bromide as shown in Figure 4A, although it effectively competed for POU DNA binding (not shown). Rather, a moderate stimulation of pTP binding by GST-POU was observed. High-salt (500 mM NaCl) washing after coupling of GST-POU to remove nucleic acids did not affect the subsequent pTP pulldown efficiency. Next, GST-POU wild-type extracts were pretreated with benzonase, a nuclease capable of degrading RNA, ssDNA and dsDNA without detectable protease activity, followed by binding to glutathione beads and high-salt washing steps. This purified, nucleic acid-free GST-POU was still able to bind pTP with the same efficiency as untreated GST-POU (Figure 4A).

Finally, if DNA binding of GST-POU is essential during DNA-mediated GST pulldown of pTP, we would expect all GST-POU mutants incapable of DNA binding to be incapable of pTP binding as well. Therefore we analyzed 15 POU mutants showing <30% DNA binding for their capacity to bind pTP, as shown in Figure 4B. A number of POUhd mutants defective in DNA binding could still bind pTP, e.g. T110A/I145N and K157E (lanes 5 and 13). This confirmed that POUhd DNA binding is not a prerequisite for pTP binding.

Assuming that the mutants with changes in solvent-directed side chains are properly folded, we used their positions to further define the pTP binding site on the POUhd surface. In Figure 3D, the surface mapping of the pTP interaction data of both DNA binding negative and positive mutants is combined. Of the DNA negative mutants, T106P and N151D were incapable of pTP binding (–, red), while mutants R105S, K125E and R153S showed reduced pTP binding (+/–, orange). The

Fig. 4. DNA does not mediate the pulldown of pTP by GST-POU (mutant) extracts. (A) GST-POU pulldown assay of pTP performed as described (Figure 2) with the following modifications: EthBr, complete assay was performed in the presence of 50 μ g/ml ethidium bromide; 500 mM, GST(-POU) extracts were batch-purified before pTP incubation using three 500 mM NaCl washing steps to remove nucleic acids; benzon., GST(-POU) extracts were treated with benzonase and 4 mM Mg^{2+} and batch-purified (500 mM NaCl) before incubating them with pTP. POU refers to GST-POUwt. (B) GST-POU pulldown of pTP performed as described (Figure 2). GST-POUhd mutants incapable of binding DNA were tested for their capacity to bind pTP. Eluted proteins were analyzed on western blot for pTP (upper bands) and POU (lower bands); 2%, 10% input: 12 ng, 60 ng pTP. Data are summarized in Table I.

positions of all mutants affected in pTP binding converged to the same protein surface as that where DNA is bound in the crystal structure (Figure 3B). Mutants incapable of DNA binding, but capable of pTP binding (e.g. K157E), mapped alongside the pTP binding surface, at both termini of the DNA recognition helix. Summarizing the results for all mutants, we can define the pTP binding surface on Oct-1 by amino acids R102, K104, R105, T106, K125, V147, C150, N151 and R153. With the exception of V147, these amino acids all contain hydrophilic or positively charged side chains.

DNA binding and pTP binding are mutually exclusive

The overlap between the interaction surfaces suggested that pTP and DNA would compete for the same region on Oct-1. We tested this in a pulldown assay by preincubating 1 μ g highly purified GST-POU with increasing amounts of competitor DNAs that do not bind pTP for 15 min before adding pTP (Figure 5A). Addition of 2.5 μ g of competitor DNA containing an Oct-1 binding site reduced pTP binding by GST-POU to approximately background level (Oct), whereas a non-specific DNA did not interfere with pTP binding (random). This confirms that the POU amino acids forming the DNA binding surface participate

Fig. 5. DNA competes for direct binding of pTP to GST-POU.

GST-POU pulldowns of pTP were performed in the presence of competitor DNAs; 2%, 5% input: 12 ng, 30 ng pTP. (A) Competition with DNA not recognized by pTP. ds competitor DNA 2.5, 5 or 10 μ g, with an Oct-1 binding site (Oct) or without one (random), were preincubated with 1 μ g purified GST-POU and GA beads for 15 min before adding pTP. (B) Competition with DNA that can bind pTP. Five and 10 μ g competitor containing the first 20 bp of the Ad5 genome (TD1-20) or the 50 3' terminal bases of the template strand read by the DNA polymerase (T1-50) were preincubated with GST-POU as described (A).

in pTP binding as well. Simultaneous pTP binding and DNA binding by Oct-1 POUhd therefore seem mutually exclusive.

We also tested the pTP binding efficiency of GST-POU in the presence of DNA competitors that only bind pTP, but not Oct-1 POU. Intriguingly, pTP-specific competitors consisting of ds DNA (TD1-20) or ssDNA (T1-50) also inhibited pTP binding to GST-POU (Figure 5B). Since the DNA competitors used in Figure 5A do not bind pTP (data not shown), this indicates that DNA binding of both Oct-1 and pTP inhibits their capability to interact directly with each other.

POUhd mutants impaired in pTP binding are partially defective in Ad DNA replication stimulation

The pulldown assays shown were performed with pTP only, but during DNA replication, pTP is present as a heterodimer with the DNA polymerase. To analyze the functional behavior of Oct-1 POUhd mutants in Ad DNA replication, we purified GST, GST-POUwt and all 22 GST-POUhd single point mutants in parallel using a three-step disposable column purification procedure. This rapid procedure allowed the purification to homogeneity of 3 mg each of these 24 individual proteins in 2 days (Materials and methods). Ad DNA replication can be stimulated by Oct-1 POU depending on the pTP-pol concentration (Mul *et al.*, 1990). Using digested viral TP-DNA as a template, replication of the two origin fragments results in the labeling of two ds fragments and in the formation of labeled ss fragments displaced in subsequent rounds of replication (Figure 6A).

The GST-tag had to be removed using thrombin for full replication stimulation (data not shown), but 3-4 pmol

Fig. 6. POUhd mutants incapable of interacting with pTP show decreased stimulation of Ad DNA replication. **(A)** DNA replication assay. Digested Ad TP-DNA was incubated with pTP-pol, DBP and POU mutants as indicated, resulting in labeling of the two ds origin fragments (B/C bands) and single strands displaced during subsequent rounds of replication (ss). None, no additional protein added (basal replication level); His-POUwt, 2 pmol purified bacterial His-tagged POUwt; NFI DNABD, purified baculovirally expressed DNA binding domain of NFI. In the experiment shown, GST and GST-POUwt were digested with a different thrombin batch from that used with the mutants, resulting in inhibited replication, but similar stimulation. On average, thrombin-treated GST and GST-POUwt showed activities comparable to the 'none' and His-POUwt controls that were used to index the replication stimulations from 0 to 100%. **(B)** Average replication stimulations of all single point POUhd mutants. Replication stimulations were determined for addition of 3 or 4 pmol POU mutant in two experiments (Materials and methods).

GST-POU and 2 pmol His-POU displayed similar maximal stimulation levels (Figure 6B). POUwt stimulated replication 4- to 6-fold, while the DNA binding domain of NFI stimulated up to 30-fold. The replication stimulation of POUhd point mutants varied from 1 to 170% of the POUwt stimulation (Figure 6A and B) and was quantified as the percentage of stimulation compared with His-POU (100%) and basal replication (0%) as summarized in Table I (see Materials and methods for details).

Mutants E109G, I112N, E122V and E129G, essentially wild type in both DNA binding and pTP binding, were all indeed able to stimulate DNA replication to wild-type levels or better. The DNA binding (+) mutants K104T, K125M, V147G and C150Y were deficient in pTP binding and stimulated replication from 21 to 41% of the maximal stimulation by POUwt. This confirmed that these residues are important determinants of the pTP-POU interaction. Mutants with weak (+/–) DNA binding stimulated DNA replication to <20% of wild type regardless of their pTP binding efficiency. Mutants with low DNA binding affinity retained <11% of wild-type DNA replication stimulation. Disruption of DNA binding crippled replication stimula-

tion in all cases more severely than disruption of pTP binding, illustrating that for full stimulation of replication, both DNA binding and the interaction with pTP are essential.

Mutants E130G and T132S stimulated DNA replication to wild-type levels, arguing against a role of these two residues in the pTP-POU interaction. This was in agreement with the wild-type pTP binding efficiency of double mutants E109G/T132S and E130G/E141D. The observed intermediate pTP binding efficiency of E130G and T132S (Table I) could be due to experimental variation.

None of the Oct-1 point mutants deficient in pTP binding lost all stimulation of DNA replication. In contrast, interfering with the DNA binding capacity of Oct-1 did inhibit replication stimulation to <11% of wild type. This argues for an additional role of Oct-1 during Ad DNA replication that is not mediated by the POUhd contacts with pTP. Oct-1 could stabilize pTP-pol on DNA through assisting in changes of the origin DNA structure just by binding its recognition site (see Discussion). Alternatively, replication could be stimulated by additional contacts between Oct-1 and pTP once both are bound to origin DNA.

Fig. 7. POU hydrophobic pocket mutants are not affected in replication stimulation. (A) Alignment of the POU hydrophobic pocket-interacting regions of OBF-1 and SNAP190 with human Ad5 pTP. (B) DNA replication assay with POU mutants. See Figure 6 for detailed information. (C) Quantification of replication stimulations by POU hydrophobic pocket mutants.

The POU hydrophobic pocket is not involved in replication stimulation

Since the POUhd mutants always contained a wild-type POU domain in our assays, we hypothesized that POU might contribute to pTP binding, even though a direct interaction between POU and pTP can not be detected under pulldown conditions (van Leeuwen *et al.*, 1997b; Botting and Hay, 1999). The Oct-1 POU contains a hydrophobic pocket that interacts with a common motif in cofactors SNAP190 and OBF-1 (Ford *et al.*, 1998) and that is important for dimerization of Oct-1 on MORE elements (Tomilin *et al.*, 2000). When we aligned this motif with pTP, one homologous pTP region was identified (Figure 7A). To test whether the POU domain residues in the hydrophobic pocket contribute to pTP-pol complex stability on the origin during DNA replication, we changed three Oct-1 amino acids that interact with or are close to this OBF motif in the Oct1-OBF crystal structure (Chasman *et al.*, 1999).

Using site-directed mutagenesis, amino acids L6, E7 and F57 were changed to an alanine or an arginine to

Table II. Comparison of Oct-1 amino acids involved in pTP and DNA binding

Amino acid	Potential pTP contact	DNA contact
R102	X	–
K103	–	X
K104	X	–
R105	X	X
T106	X	X
R113	?	X
K125	X	X
S128	?	X
R146	–	X
V147	X	X
C150	X	X
N151	X	X
R153	X	X

Potential pTP contact: amino acids that cause loss of pTP binding when mutated (this study). DNA contact: POUhd contact with DNA identified by Klemm *et al.* (1994). X, contact; – no contact; ? mutation not identified in our screening.

evoke charge repulsion, since the OBF motif is highly positively charged (Figure 7A). When POU mutants L6A, L6R, E7A, E7R, F57A and F57R were tested in DNA replication stimulation assays, all mutants stimulated DNA replication to wild-type levels with the exception of F57A (Figure 7B and C). This residue is probably not involved in replication stimulation, since F57R displayed wild-type stimulation. The hydrophobic pocket of the POU domain apparently does not contribute to stabilization of the pTP-pol complex on the origin. This rules out one candidate surface recognized by multiple proteins interacting with Oct-1.

Discussion

Since structural data on the pTP-POU interaction are not available, we used random mutagenesis to map the Oct-1 POUhd surface that interacts with Ad pTP in detail. Our results show that most Oct-1 amino acids essential for the interaction with pTP are located within the DNA binding surface of the POUhd (Figures 2 and 4B), but these binding sites are not identical. This result was not biased by the presence of DNA during pulldown, since addition of ethidium bromide (Lai and Herr, 1992) and degradation of nucleic acids with benzonase did not interfere with pTP binding efficiency (Figure 4A).

The extent of the overlap between the pTP and DNA binding surfaces becomes very clear by comparing Figure 3D with the crystal structure of amino acids that contact DNA indicated in Figure 3B. Of the nine amino acids important for pTP binding, seven contact DNA in the co-crystal (Table II), while K104 and R102 are close to the minor groove (Figure 3A). Although the structural overlap is striking, there are subtle differences. While amino acids K103 and R146 contact DNA in the crystal, their mutation did not interfere with pTP binding. In contrast, mutants V147G and C150Y had retained their capacity to bind DNA, but were unable to bind pTP. Residues R113 and S128 contribute to DNA binding in the crystal, but mutations of these residues were not identified in our selection procedure.

Fig. 8. Model of Ad DNA replication stimulation by consecutive Oct-1 POUhd binding to pTP and DNA. Indicated schematically are Ad origin DNA (double lines), the heterodimer of pTP and DNA polymerase (pol) and the Oct-1 POUss and POUhd domains connected by their linker. The DNA binding surfaces of both POU subdomains are colored black. See text for details.

The extensive overlap between the two binding sites is surprising, because previous data (see below) indicated that even after recruitment of pTP-pol to the origin, Oct-1 and the pTP-pol complex continue to interact. Since DNA binding and direct pTP binding by POUhd appear to be mutually exclusive (Figures 3D and 5), we have to adapt the simple recruitment model of Ad DNA replication stimulation by Oct-1. After binding pTP, Oct-1 might first recognize its DNA binding site in the viral replication origin by virtue of the POUss, followed by competition for the homeodomain surface between the DNA recognition site and pTP (Figure 8). This could result in the release of pTP at the viral replication origin, after which the pTP-homeodomain interaction would be lost.

In this model proposing consecutive binding of pTP and DNA to POUhd, DNA recognition by POUss plays an essential role. Indeed, POU proteins with point mutations in the POUss domain that strongly reduced DNA binding, were incapable of stimulating Ad DNA replication (van Leeuwen *et al.*, 1995). Moreover, the Oct-1 POUhd alone inhibits Ad DNA replication (Verrijzer *et al.*, 1990). This was attributed to the low specificity of Oct-1 POUhd DNA binding, but might now be explained by a failure to recognize origin DNA when pTP interacts with the POUhd DNA binding surface. The model has to be reconciled with two observations: first, pTP binding-deficient mutants did not lose all replication stimulation (Figure 6) and secondly, Oct-1 can stabilize pTP-pol after binding its recognition site (van Leeuwen *et al.*, 1997b). We can

imagine two explanations: additional contacts between Oct-1 and pTP-pol in the DNA-bound context or DNA-mediated stabilization of pTP-pol on the origin through Oct-1 DNA binding.

POUss residues for example could stabilize the pTP-pol complex on the origin in the DNA-bound context, although a solution interaction between pTP and POUss was not detected in pull-down assays (Coenjaerts *et al.*, 1994; van Leeuwen *et al.*, 1997b; Botting and Hay, 1999). One such POUss candidate region was the hydrophobic pocket contacted by OBF-1 in the Oct-1-DNA-OBF-1 crystal structure by a motif shared with SNAP190 (Ford *et al.*, 1998; Chasman *et al.*, 1999). Although this motif appeared to be present in pTP (Figure 7A), mutations in the POUss pocket did not affect replication stimulation (Figure 7B).

Alternatively, pTP-pol might be stabilized on origin DNA when Oct-1 binding to its recognition site changes the DNA structure. Evidence for such DNA structural changes caused by Oct-1 comes from DNA bending studies (Verrijzer *et al.*, 1991) and was confirmed by the Oct-1-DNA co-crystal structure, which displayed a bend in the DNA of $\sim 30^\circ$ (Klemm *et al.*, 1994). This could explain why all DNA binding POU mutants that did not interact with pTP retained low levels of replication stimulation, while interfering with POUhd DNA binding was even more detrimental to replication stimulation (Figure 6).

Consistent with the observed overlap between the pTP and DNA interaction surfaces on Oct-1, binding of pTP and DNA were mutually exclusive (Figure 5A). We also tested whether pTP could inhibit Oct-1 DNA binding to its recognition site in an electrophoretic mobility shift assay, but found no detectable inhibition, indicating that Oct-1 binds with higher affinity to DNA than to pTP (data not shown). Interestingly, when we assayed the interaction between pTP and Oct-1 in the presence of competitor DNA capable of binding pTP, the direct interaction was also inhibited (Figure 5B). This suggests that the direct interaction with the POUhd might lose its purpose once pTP has been loaded on origin DNA. pTP-pol and Oct-1 can bind simultaneously to DNA (van Leeuwen *et al.*, 1997b), but the short length of competitor DNAs used in Figure 5 prevented binding of both proteins adjacent to each other.

When we correlate the data on double mutants affected in pTP binding with the pTP interaction surface identified with single point mutants, we find consistent results. Mutants I95T/V147A, K104E/L116S, K125Q/R146S, I134F/V147D, D136G/V147G and Q137L/C150S show decreased pTP binding and all of these mutants contain a mutation in one of the essential pTP-interacting amino acids. Mutant S99G/K142E also shows reduced pTP binding. It is unlikely that K142 is involved in pTP binding, since it is surrounded by amino acids not contributing to pTP binding, which indicates that the pTP interaction surface might include S99 in the linker region (not visible in Figure 3). The reduced pTP binding of E141G/K155E could be explained by mutation of K155, since this amino acid maps right next to the pTP interaction surface identified. However, the reduced binding of E122G/M140L was not consistent with the wild-type binding by E122G/T127S and M140R/R146P and

might be explained by lower expression (Figure 2, lanes 21 and 23).

The herpes simplex virus transcription activator VP16 contacts Oct-1 homeodomain residues in the two structural α -helices supporting the DNA binding helix (Lai *et al.*, 1992; Pomerantz *et al.*, 1992; Chasman *et al.*, 1999). Mutation of these VP16 interacting amino acids at the homeodomain solution surface directed away from the DNA binding site did not interfere with Ad DNA replication stimulation (Coenjaerts *et al.*, 1994). We can now also explain previous observations, that different Oct-1 family members (Oct-2, Oct-4, Oct-6, Brn-3A) were all capable of stimulating Ad DNA replication, although the conservation between these proteins is limited (Verrijzer *et al.*, 1992b). The residues we identified as essential for the direct pTP-POUhd interaction are highly conserved in all of these different proteins. From an evolutionary standpoint, the use of one of the most abundant and conserved protein surfaces by the Ad replication priming protein could prove advantageous for optimization of viral replication.

There are other examples of proteins interacting with the DNA binding surface of another protein. TAF_{II}230 contacts the concave DNA binding surface of TBP and thereby inhibits TBP from binding the TATA box (Liu *et al.*, 1998). The TBP binding surface of TAF_{II}230 mimics the (partially unwound) TATA box bound by TBP. The overlap between the DNA and pTP binding surface on POUhd raises the intriguing possibility that the Ad pTP might mimic DNA to interact with the Oct-1 homeodomain surface. Whereas TAF_{II}230 recognizes mainly hydrophobic residues on the surface of TBP, all but one of the Oct-1 amino acids essential for pTP recruitment are highly hydrophilic.

Recently, comparison of the DNA-bound and -free crystal structures of the eukaryotic DNA replication protein RPA70 revealed a related strategy. In the absence of DNA, two consecutive OB-fold DNA binding domains in RPA can contact each other by binding a basic flexible loop with their acidic ssDNA binding grooves. In the DNA-bound mode, this basic loop folds over unwound DNA contacting the acidic phosphate groups, while the ssDNA binding cleft contacts the unwound DNA bases (Bochkareva *et al.*, 2001). Since no protein interactions with the Oct-1 DNA binding surface have been reported, we provide here the first evidence for such a contact with Oct-1.

Materials and methods

Construction of GST-POU mutant library

The multiple cloning site of pRP265 (Coenjaerts *et al.*, 1994) was modified to accept *NdeI*–*BamHI* fragments in frame with the N-terminal GST-tag creating pRP265NB (S. Werten, unpublished). The Oct-1 POU open reading frame was digested from pET15b-POUwt (van Leeuwen *et al.*, 1995) with *NdeI* and *BamHI* and inserted C-terminally to the GST-tag and thrombin site in pRP265NB creating pRP-GST-POUwt. Mutagenesis PCR of the Oct-1 POUhd was performed using forward primer TGCCCTGAATTCTCCAGGAA and reverse primer TAGCAGCCGGATCCTTAGTT with 1 μ l *Taq* DNA polymerase (Pharmacia), 1 $\times$ *Taq* buffer, 0.5 mM MnCl₂, 10 mM β -mercaptoethanol, 10% dimethylsulfoxide (DMSO), 1 mM dATP, 1 mM dCTP, 0.2 mM dTTP and 0.2 mM dGTP. Total reaction volumes of 200 μ l were split into 10 separate PCRs to prevent early mutations from appearing in all mutants. PCR products were ligated into pGEM-T (Promega) following the

protocol supplied by the manufacturer. The total number of individual colonies harvested was 4E+03. pGEM-T POUhd mutant DNA was digested with *EcoRI* and *BamHI* and introduced into pRP-GST-POUwt replacing the wild-type homeodomain with a mutant homeodomain, generating a GST-POUs(wt)–POUhd (mutant) library with a multiplicity of 3E+03. Thirty-six of the 225 isolated extracts contained GST-POU wild type.

POUs mutants were made by site-specific mutagenesis using pRP265NB-POUwt as template and single PCRs with suitable primers for L6A, L6R, E7A and E7R. F57 mutants were made by a two-step megaprimer PCR strategy (Barik, 1995), with a *NdeI* forward primer and ACATGTTCTTAGCGCTGAGGTTCAA (F57A) or ACATGTTCTTACGGCTGAGGTTCAA (F57R) to create the upstream megaprimer and with a *BamHI* reverse primer and TTGAACCTCAGCGCTAAGAA-CATGT (F57A) or TTGAACCTCAGCCGTAAAGAACATGT (F57R) to create the downstream megaprimer PCRs. Corresponding megaprimers were hybridized and amplified twice according to the protocol. POU PCR products were inserted into the *NdeI*–*EcoRI* sites of pRP265NB-POUwt.

Expression and isolation of GST-POU protein extracts

pRP265NB-POUwt and the POU-mutant library were transformed in BL21 DE3 cells. Protein expression was induced by adding 2 mM of isopropyl- β -D-thiogalactopyranoside (IPTG) to 3 ml liquid shaker cultures at OD 0.5. After 2 h of induction, 1 ml of cells was pelleted by centrifugation (4.6E+03 g, 1 min, 20°C) and lysed by adding 200 μ l lysis buffer L [50 mM Na-phosphate pH 8, 300 mM NaCl, 5 mM β -mercaptoethanol, 10 mM Na₂S₂O₅, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml lysozyme, 10 μ g/ml RNase and Complete protease inhibitor cocktail (Roche)] and vortexing. The cells were left on ice for 10 min, frozen–thawed once and centrifuged (2.5E+04 g, 15 min, 4°C). The supernatant was transferred to a fresh tube and stored at –20°C. In total, 225 individual mutant GST-POU colonies were grown in liquid culture and harvested. Mutant expression was used to select for levels of 50–100% of wild-type expression (Coomassie).

Cloning and purification of bacterial pTP

The N-terminal 183 amino acids of Ad5 pTP were amplified by PCR using primers ATAAATAGAATACATGTCTTGAGCGTCAAC and TGGCCGTAACATGTGGTACCTTCCTCCCATG and pVapTP (Stunnenberg *et al.*, 1988) as a template, introducing a unique *KpnI* site in front of the C-terminus, digested with *AflIII* and ligated in the *NcoI* site of pET15B (Novagen) N-terminal to the His-tag. This construct pETpTP1S2-183 contained an Ala to Ser mutation at amino acid 2 inherent to the cloning strategy, that did not interfere with Oct-1 binding, DNA binding or initiation activity (data not shown). The stop codon of pTP was removed by ligating suitable primers over amino acids 657–671 in the *BspEI* sites of *BspEI*-digested pVapTP, replacing the stop codon with a unique *KpnI* site after amino acid 671, creating pVapTP(-stop). The C-terminal amino acids 98–671 were digested from this vector using *SnaBI* and *KpnI* and ligated in *SnaBI*/*KpnI*-digested pET15BpTP1S2-183, resulting in the full-length expression construct pTP1S2-671 with a C-terminal His-tag. Low pTP binding to Ni-NTA forced us to purify this His-pTP1S2-671 using an affinity chromatography procedure.

A 12 l BL21 expression culture transformed with pETpTP1S2-671 was induced to produce pTP with 1 mM IPTG at OD 1 at 37°C. Cells were harvested (3.2E+03 g, 10 min, 4°C) and lysed with 20 ml lysis buffer per liter of culture (see above). After clearing the lysate by ultracentrifugation (2.74E+05 g, 60 min, 4°C), the supernatant was diluted with 50 mM NaCl lysis buffer to 150 mM NaCl, and purified over a SP-Sepharose (150–400 mM NaCl), a Q-Sepharose (200–450 mM NaCl), a phenyl-Superose [1000 to 0 mM (NH₄)₂SO₄ in the presence of 300 mM NaCl] and a 1 ml MonoQ (Pharmacia; 200–450 mM NaCl) column. The buffer conditions during purification were 20 mM HEPES pH 8, 20% glycerol, 1 mM EDTA, 2 mM dithiothreitol (DTT) and 0.5 mM PMSF with variable salt concentrations as indicated. The yield varied from 1 to 1.5 mg purified pTP per 12 l culture. Bacterial pTP was active in replication initiation (not shown) and had significantly lower background binding to GA beads (not shown) than baculovirally expressed pTP.

Baculovirally expressed pTP (King *et al.*, 1997) was purified as described above, but using a heparin cartridge (Bio-Rad; 300–650 mM NaCl) instead of the phenyl-superose step.

DNA binding studies

DNA binding studies were performed as described (van Leeuwen *et al.*, 1997b) using a dsDNA probe containing the first 50 bp of the Ad5 origin of DNA replication. The DNA binding of 0.1 μ l GST-POU mutant and

wild-type bacterial lysates (~250 ng/ μ l) was quantified by phosphorimaging using a STORM820, and classified as + (>50% of wt), +/- (20–50%) or – (<20%).

GST pulldown

GA beads (20 μ l) were washed with HIP1000 (20 mM HEPES–KOH pH 8, 10% glycerol, 0.5 mM β -mercaptoethanol, 0.1% NP-40, 0.5 mM PMSF, 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ and NaCl to the concentration indicated: HIP1000 contains 1000 mM NaCl), HIP0 and three times with HIP300 buffer containing 1% bovine serum albumin (BSA). Approximately 2.5 μ g of GST–POU in bacterial lysates was added to 400 μ l HIP300/1% BSA and 20 μ l of GA beads, which were tumbled for 1 h at 4°C. The beads were washed three times with 400 μ l HIP300/1% BSA and three times with 400 μ l HIP50/0.2% BSA. Purified bacterial pTP (600 ng) was added and tumbled in a volume of 400 μ l HIP50/0.2% BSA for 1 h at 4°C. Non-bound pTP was removed by washing once with 400 μ l of HIP100/0.2% BSA and twice with the same volume of HIP100/no BSA. Both GST–POU and bound pTP were eluted from the beads with 30 μ l of 20 mM glutathione (pH 8) in HIP50/no BSA for 10 min while vortexing every 2 min. The eluate was mixed with 15 μ l 3 $\times$ Laemmli sample buffer and 30 μ l (67%) was separated on SDS–PAGE and analyzed by western blotting using rabbit antisera directed against Oct-1 POU and Ad5 pTP in the same blot, but separately, by cutting it in half. Estimated pTP pulldown efficiency was classified as (–) <20%, (+/–) 20–50% or (+) 50–100% of wild-type efficiency by comparing with input control lanes.

When mentioned in the text, benzonase (Merck) was added to remove nucleic acids following the protocol supplied by the manufacturer. Competitions were performed with 1 μ g purified GST or GST–POU (see below) and ds DNA Oct (GTATGCAAATAAGG), random (5'-CCGCTGCCGCGCGGCACAGGTAC-3'; 5'-CTGGTGCGCGCGGCAGCGGTAC-3'), TD1–20 or ss T1–50 (Kenny and Hurwitz, 1988).

Parallel purification of GST proteins

All column handlings were performed at 4°C by gravity flow and use of caps to prevent columns from running dry. A 1 ml DEAE–Sephacrose column and a 1 ml GA column were poured in disposable Bio-Rad Poly-Prep chromatography columns for each of the 22 GST–POU point mutants, GST and GST–POUwt. Liquid cultures (100 ml) of all single mutants were induced and lysed with 10 ml buffer L with 150 mM NaCl, centrifuged (2.74E+05 g, 60 min, 4°C) and loaded on the DEAE columns in parallel. These were set up above GA columns to load the POU-containing DEAE flow-through on GA simultaneously. DEAE columns were washed with 10 ml buffer L to the underlying GA columns, which subsequently were washed with 10 ml of buffer A500 (20 mM HEPES–KOH pH 7.5, 1 mM DTT, 5 mM $\text{Na}_2\text{S}_2\text{O}_5$, 0.5 mM PMSF, 15% glycerol and NaCl to the concentration indicated: A500 contains 500 mM NaCl), 5 ml A100 and block eluted with 4 ml A100 with 5 mM glutathione. These glutathione elutions were loaded onto 1 ml SP–Sephacrose columns, washed with 10 ml A100 without glutathione and block eluted with 4 ml A500. While GST–POU proteins bound to SP–Sephacrose, GST was present in the flow-through. When purified GST was used in pulldowns, glutathione was removed by dialysis against A500 (see above). Protein yields varied from 1.6 to 4 mg/mutant. The six POU mutants were purified using the same strategy.

In vitro Ad TP–DNA replication

In vitro Ad DNA replication was performed essentially as described (van Leeuwen *et al.*, 1997b) using 15 ng DNA polymerase, 20 ng baculovirally expressed pTP, 30 ng *Xho*I-digested Ad5 TP–DNA, 1 μ g DBP, 58 mM NaCl and 3 or 4 pmol purified and thrombin-digested GST–POUwt, GST–POU mutant or GST as indicated. Replication B/C bands were quantified using a Storm 820 phosphorImager and calculated by comparing with 2 pmol His–POUwt (100%) purified as described (van Leeuwen *et al.*, 1997b) and with the negative (0%, no extra protein added) indexes on each gel allowing comparison of multiple gels. Calculation: stimulation (%) = $100 \times [(A_{\text{mut}} - A_{\text{neg}}) / (A_{\text{wt}} - A_{\text{neg}})]$ with A = B/C band activity of mut(–ant), neg(–ative) or wt (His–POU wild type). The replication stimulation values were averaged over two experiments for both concentrations (four values in total), because stimulation can be inhibited slightly by saturating POU concentrations.

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