The Oct-1 POU Homeodomain Stabilizes the Adenovirus Preinitiation Complex via a Direct Interaction with the Priming Protein and Is Displaced when the Replication Fork Passes*

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Initiation of adenovirus DNA replication is strongly enhanced by two cellular transcription factors, NFI and Oct-1, which bind to the auxiliary origin and tether the viral precursor terminal protein-DNA polymerase (pTP-pol) complex to the core origin. NFI acts through a direct contact with the DNA polymerase, but the mode of action of Oct-1 is unknown.

Employing glutathione S-transferase-POU pull-down assays and protein affinity chromatography, we have established that the POU domain contacts pTP rather than pol. The POU homeodomain is responsible for this interaction. The protein-protein contacts lead to increased binding of pTP-pol to the core origin, which is caused by a reduced off-rate. The enhanced formation of a pTP-pol-POU complex on the origin correlates with stimulation of replication.

Using an immobilized replication system, we have studied the kinetics of dissociation of the Oct-1 POU domain during replication. In contrast to NFI, which dissociates very early in initiation, Oct-1 dissociates only when the binding site is rendered single-stranded upon translocation of the replication fork. Our data indicate that NFI and Oct-1 enhance initiation synergistically by targeting different targets in the preinitiation complex and dissociate independently after initiation.

The adenovirus genome is a linear double-stranded DNA molecule of 36 kbp, which replicates very efficiently, producing high amounts of progeny DNA (10^5–10^6) in infected cells. Origins of replication are located at both ends of the genome, within the inverted terminal repeats, and encompass approximately 50 bp. The main proteins required for efficient replication are encoded by the virus itself and are expressed early in infection. These are the DNA polymerase (pol); the precursor terminal protein (pTP), which is required for correct initiation; and the DNA-binding protein (DBP), which is essential for elongation. The first two are present in infected cells as a stable heterodimer (pTP-pol).

Initiation of DNA replication occurs by a protein-priming mechanism in which a serine residue in pTP covalently binds a deoxycytidine residue, via a phosphodiester bond (reviewed in Ref. 1). A pTP-trinucleotide intermediate is formed, guided by base pairing with an internal triplet and then jumping back to the very end of the DNA (2). Subsequently, this intermediate is used as a primer for further chain elongation via a strand displacement mechanism requiring the viral DNA-binding protein. The pTP remains bound to the 5’ terminus and probably serves several functions, such as protection from exonucleases, attachment to the nuclear matrix, and stabilizing the incoming pTP-pol complex (3, 4). pTP-pol binding to the origin requires base pairs 8–18 for correct positioning of the complex (5).

In addition to the three viral proteins, at least two host proteins are recruited by the virus for maximal origin function. Both proteins, nuclear factor I (NFI) and the octamer-binding protein (Oct-1), are cellular transcription factors that bind independently to a region adjacent to the 18-bp core origin. Together, these two factors stimulate replication up to 200-fold. Their mechanism of stimulation has been studied both in vitro and in vivo (6–9).

NFI binds as a dimer to a partially symmetric sequence, 5’-TGGA(N)_2GCCAA-3’, located between residues 25 and 38 of the Ad5 origin. The position of this NFI site relative to the core origin is critical for efficient replication. In vitro, NFI stimulates initiation in a pTP-pol concentration-dependent fashion (7), suggesting that the proteins interact. A direct, DNA-independent contact can indeed be detected with the polymerase in the pTP-pol complex (7, 10, 11). This interaction is functional because mutations in NFI that fail to bind pTP-pol are also defective in stimulation of replication (12). Template commitment studies showed a 10-fold increased stability of the pTP-pol complex on the origin in the presence of NFI, explaining most of the stimulatory action of NFI (13).

Oct-1 binds immediately next to the NFI site to the sequence 5’-TATGATAATGA-3’, which is located between residues 39 and 49. Deletion of the Oct-1 binding site in the adenovirus type 5 origin results in poorly growing viruses, with a yield 50-fold lower than that of the wild type (9). As for NFI, the stimulatory activity of Oct-1 resides within the DNA binding domain. This DNA binding domain, the POU domain, consist of two combined helix-turn-helix DNA binding elements, the POU-specific domain (POUs), and the POU homeodomain (POUhd). POUs binds the sequence 3’-TATGAG-5’, and POUhd recognizes 3’-TATGAG-5’ (14, 15). In vitro Oct-1 stimulates replication 3–7-fold, also depending on the pTP-pol concentration and a DNA independent interaction between the pTP-pol complex and the POU homeodomain was observed (16). The target in the pTP-pol complex and the mechanism of stimulation were not established; these discoveries are the purpose of the present study.

Together with the viral proteins, NFI and Oct-1 assemble a
preinitiation complex leading to optimal initiation. After initiation, this complex presumably disintegrates, but the details and kinetics of dissociation are still largely unknown. Employing an immobilized replication system, we previously showed that NFI disembarrows from the preinitiation complex already at an early stage (17). Using the same technology, we have now obtained evidence that Oct-1 dissociates later, upon passage of the replication fork through the Oct-1 binding site.

**EXPERIMENTAL PROCEDURES**

*Adenovirus DNA Replication in Vitro—Replication reactions were performed in 15 μl of buffer A (25 mM HEPES, pH 7.5, 50 mM NaCl, 1.5 mM MgCl₂, 1 mM dithiothreitol), 40 μM of dATP, dTTP, dGTP, 500 mM [α-32P]dCTP (400 Ci/mmol), 15 ng of pTP-pol complex, 0.9 μg of DBP. These mixtures were incubated with either 30 ng of XhoI-digested Ad5 terminal protein (TP)-DNA or 1 ng of 110-bp EcoRI-XbaI fragment of Ad5 plasmid pHRI (6). All replication proteins were purified as described previously (18). Reactions were incubated for 45 min at 37 °C and stopped by the addition of 1.5 μl of stop mix (40% sucrose, 1% sodium dodecyl sulfate (SDS), 0.1% bromphenol blue, and 0.1% xylene cyanol). Replication products were analyzed on a 1% agarose gel (0.5 × Tris borate-EDTA, 0.1% SDS) for TP-DNA templates or a 7.5% SDS-polyacrylamide gel for plasmid templates. Replication products were visualized by phosphorimaging (Molecular Dynamics).

Oct-1 POU Domain Dissociation Assay—Recombinant GST-POU and GST-NFI were purified from bacterial lysates by successive applications of DEAE anion exchange, glutathione agarose affinity, and fast flow Q anion exchange chromatography essentially as described previously (16).

100 ng of GST-POU were incubated with 0.05 pmol of a 817-bp end-labeled Ad5 origin fragment (pHR1 digested with EcoRI and ApaLI) in 20 μl of buffer B (25 mM Hepes-KOH, pH 7.5, 5 mM MgCl₂, 55 mM NaCl, 0.5 mM dithiothreitol, 5% glycerol, 5% dimethyl sulfoxide). Subsequently, 5 μl of glutathione agarose (GA) beads (50% v/v) were added and the suspension was placed on a tumbling wheel at 4 °C for 45 min, beads were spun in a Microfuge (15 s) and washed twice in 150 μl of buffer B. Under these conditions, more than 80% of the input DNA was bound. The beads were resuspended in 20 μl of buffer C containing 0.9 μg of DBP and 15 ng of pTP-pol. Replication was started by adding nucleotides at 40 μM and placing the reaction tube at 37 °C on a tumbler wheel. After 30 min, the beads were spun down. To the supernatant, 4 μl of stop mix was added, and the released products were analyzed on 7.5% polyacrylamide gel.

DNA Binding Studies—The Ad5 origin probe used for band shift assays consisted of partially duplex oligonucleotides that lacked the first 14 nucleotides from the 5′ end of the non-template (TD15; Ref. 19). The DNA was end-labeled with T4 polynucleotide kinase and purified by preparative polyacrylamide gel electrophoresis. Binding reactions were incubated for 80 min on ice in 20 μl of binding buffer (20 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.025% Nonidet P-40, 4% Ficoll). The concentration of input DNA was 1 mM. Free DNA and protein-DNA complexes were separated on a 7% polyacrylamide gel (37:5:1) run in 0.5 × Tris borate-EDTA at 4 °C for 15 h at 100 volts. For the dissociation experiments, after 1 h, a 200-fold excess of unlabelled single-stranded DNA (derived from the template strand of the origin) was added, and samples were loaded onto a running polyacrylamide gel at the indicated time points.

**GST-Oct-1 POU Domain Coprecipitation—**To determine whether the Oct-1 POU domain and NFI associate with the different replication components, 0.25 μg of GST, GST-POU, or GST-NFI fusion proteins were coupled to casein-coated GA beads (10% v/v) by incubation for 2 h at 4 °C in 60 μl of buffer C (20 mM HEPES-KOH, pH 7.5, 10% glycerol, 100 mM NaCl, 0.5 mM β-mercaptoethanol, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na₃S₂O₄) and 50 μg/ml ethidium bromide on a tumbler wheel. The beads were spun down and washed three times with 400 μl of buffer C. Resin-bound proteins were separated on SDS-polyacrylamide gels and detected by immunoblotting (16).

**GST Chromatography—**2 mg of cytoplasmic extracts from Sf9 cells infected with a recombinant baculovirus expressing pTP (11) were applied to a 0.25-m1 column of GA beads coupled with 100 μg of GST-POU or GST equilibrated with buffer containing 200 μg/ml ethidium bromide. After washing with 5 ml of the same buffer, a 10-ml linear gradient from 75 to 1000 mM NaCl in this buffer was applied. 0.5 ml fractions were collected and 5 μl of each were run on an SDS-polyacrylamide gel. pTP was detected by immunoblotting with polyclonal anti-pTP serum (16).

**RESULTS**

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Cytosplastic extracts from Sf9 cells infected with recombinant baculovirus expressing pTP were applied to a 0.25-ml column of glutathione agarose coupled with GST-POU (top panel) or GST (bottom panel). After being washed, bound proteins were eluted with a 10-ml linear gradient from 75 to 1000 mM NaCl. 0.5-ml fractions were collected and 5 μl run on SDS-polyacrylamide gel electrophoresis. pTP was visualized by immunoblotting with polyclonal anti-pTP-pol antisemur. L, load; FT, flow-through; numbers indicate fraction number. 10 ng of purified pTP were added as a marker. The bands below the pTP position are presumably degradation products.

**DNA Binding Studies—**Using the same technology, we have now shown an interaction between the Oct-1 POU domain and the pTP-pol complex (16). To study which of the two proteins in the complex is involved in this interaction, we separately expressed pTP and pol using recombinant baculoviruses. Protein-protein interactions were assessed by affinity chromatography with the Oct-1 GST-POU domain fusion protein bound to glutathione agarose beads. A cytosplastic extract of insect cells expressing pTP was passed over the resin, and bound proteins were eluted by applying a linear gradient from 75 to 1000 mM NaCl. The presence of pTP in eluted fractions was determined by Western blotting using polyclonal anti-pTP-pol antiserum (Fig. 1). Although some of the pTP ran through the column, a significant amount was specifically retained on the GST-POU column and eluted at approximately 225 mM NaCl. A control column in which only GST was bound to the matrix did not retain any pTP, thus excluding nonspecific binding (Fig. 1). Moreover, the column was equilibrated in a buffer containing ethidium bromide to prevent a DNA-mediated interaction. In analogous experiments using crude extracts, we could not detect an interaction between the Oct-1 POU domain and the DNA polymerase.

**NFI and the POU Domain Bind Different Subunits in the pTP-pol Complex—**Because we could not exclude the presence of inhibitors in the crude extracts, we purified pTP and pol separately, as well as the pTP-pol complex, and studied binding in a pull-down assay employing GST-POU or GST-NFI. Fig. 2A shows that pTP specifically binds to GST-POU beads (lane 3) but not to GST-NFI beads (lane 4), whereas pol is only retained by GST-NFI (lane 8). A slightly smaller product, presumably a degradation product of pTP, is also bound. The pTP-pol complex bound both POU and NFI, as expected (lanes 11 and 12). The levels of pTP-pol retained by NFI were slightly higher than for the POU domain, possibly indicating stronger binding, in

**FIG. 1. pTP interacts with the Oct-1 POU domain.** Cytosplastic extracts from Sf9 cells infected with recombinant baculovirus expressing pTP were applied to a 0.25-ml column of glutathione agarose coupled with GST-POU (top panel) or GST (bottom panel). After being washed, bound proteins were eluted with a 10-ml linear gradient from 75 to 1000 mM NaCl. 0.5-ml fractions were collected and 5 μl run on SDS-polyacrylamide gel electrophoresis. pTP was visualized by immunoblotting with polyclonal anti-pTP-pol antisemur. L, load; FT, flow-through; numbers indicate fraction number. 10 ng of purified pTP were added as a marker. The bands below the pTP position are presumably degradation products.
The POU Domain Enhances Binding of pTP to the Origin. Enhance binding of pTP to the origin could be due to a increased association or a decreased dissociation of the complex or both. We assayed the on-rate of the pTP-pol complex to TD15 DNA but could not detect a difference between the presence and the absence of the POU domain (data not shown). A stabilizing effect of the POU domain was observed when dissociation was measured (Fig. 4). A pTP-pol-origin complex was allowed to form with or without the presence of the POU domain. After equilibrium was reached, a 200-fold excess of unlabeled template strand DNA was added. This single-stranded competitor can be bound efficiently by the pTP-pol complex, whereas it is not recognized by the POU domain, thus allowing an accurate measurement of the stabilizing effect. Dissociation was measured as a function of time by analyzing samples on a nondenaturing gel (Fig. 4). Without the POU domain, dissociation of the pTP-pol complex was observed within 1 min (lane 10), whereas in the presence of the POU domain, a pTP-pol complex is still present after 10 min (lane 5).
We conclude that the enhanced equilibrium binding of the pTP-pol complex in the presence of the POU domain is caused by increased stability of the complex rather than by an increased rate of assembly.

The POU Domain Dissociates when the Replication Fork Passes—In order to establish the moment at which Oct-1 dissociates from the template, we used a modified immobilized replication system developed previously in our laboratory (17). In this system, a functional initiation complex was assembled on GA beads employing GST-NFI. As for NFI, the system enables the study of dissociation of the POU domain during the early stages of replication. A labeled Ad5 origin fragment bound by GST-POU was immobilized on GA beads (Fig. 5A). Dissociation of the POU domain from the origin can be monitored by the release of labeled DNA from the beads because the GST-GA interaction remains. When formation of a pTP-dCMP complex is allowed to take place by adding only dCTP, the template is not released (Fig. 5B). This is in contrast to NFI, which already dissociates when the polymerase binds the first nucleotide (17). Addition of dATP and dTTP allows progression of the polymerase up to 26 nucleotides but does not result in release of the origin from the POU domain. Only when all four nucleotides are added, allowing the polymerase to proceed beyond position 26, is the origin released. We conclude that the POU domain dissociates only when the polymerase passes the POU domain recognition sequence, presumably because this site is rendered single-stranded upon translocation of the replication fork. We did not detect a release of all bound POU domains from the origin, which was probably due to the fact that not all templates are efficiently replicated (17). Reassociation of the released POU domains might also lead to lower release levels. In parallel reactions, we tested the replication products formed under the same conditions using α<sup>32</sup>P-dCTP and unlabeled template. As can be seen in Fig. 5C, replication proceeded in a way similar to a soluble system.

POUhd Stimulates Replication Only on a Small Origin-containing Fragment—Because POUhd stabilizes the binding of pTP-pol on the origin, we anticipated that, like the POU domain, POUhd would also stimulate initiation. Previous results indicated, however, that POUhd inhibited replication (20). The system used to assay this effect employed Ad5 TP-DNA predigested with XhoI. We repeated the experiment with a more extensive concentration range, with the same result. In contrast to the POU domain, which stimulated 3–4-fold under the conditions chosen (a relatively high pTP-pol concentration), addition of POUhd resulted in a 2-fold reduction (Fig. 6, A and B).

The inability of POUhd to stimulate replication could be caused by its higher dissociation rate, resulting in a less stable complex, or by incorrect targeting of the pTP-pol complex. Deletion of the POU subdomain results in loss of specificity (21), and the large number of other POUhd binding sites on the
36-kbp viral genome might lead to incorrect targeting of the pTP-pol complex. To test this, we used a small (110 bp) origin-containing fragment as template rather than the whole viral genome. Under the same replication conditions, we observed that POUhDis indeed able to stimulate replication of this small fragment (Fig. 6C). The level of stimulation is not as high as that obtained with the intact POU domain (Fig. 6D), possibly due to the higher off-rate of the POUhD (22). This experiment shows that the observed interaction of POUhD with pTP is functional, although a covalent linkage to the POU domain is required for accurate targeting and stability on the intact viral DNA. A drop in replication is observed with increasing amounts of POU protein on the 110-bp origin fragment (Fig. 6D). We assume that the pTP-pol complex is squelched by the POU domain at high concentrations. The terminal protein, which stabilizes the replication complex, is absent on this plasmid-derived fragment, making it more sensitive to squelching by the POU domain.

**DISCUSSION**

We show that the Oct-1 POU domain stabilizes the preinitiation complex via a direct interaction between pTP and POUhD. Enhanced pTP-pol binding in the presence of Oct-1 correlates well with the levels of stimulation, with the restriction that DNA binding assays and replication assays are performed under different conditions. Although binding of pTP is essential, it is not necessarily the only function of the POU domain. The POU domain is also capable of bending its recognition sequence slightly (23, 24), and such a bend in the origin might facilitate the pTP-pol-POU interaction on DNA.

Detailed mapping of the interaction domains on POUhD or pTP has not yet been achieved. POU proteins from all six classes are able to stimulate replication (25), suggesting that the interaction domain is a conserved region. Mutation of sev-

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**Fig. 5. The Oct-1 POU domain dissociates when the replication fork passes.** A, experimental set-up: GST-POU bound to a radiolabeled origin fragment was immobilized on glutathione agarose beads. Replication was initiated by the addition of pTP-pol, DBP, and nucleotides. Arrows indicate the expected product length when a limited number of nucleotides were added. B, dissociation of the POU domain from the origin fragment was monitored by the release of the radiolabeled fragment in the supernatant after elongation with the consecutive nucleotides. After replication of the immobilized complexes, the beads were spun down and the products were analyzed on a polyacrylamide gel. The bound origin fragment is shown in the upper panel, released fragments in the lower panel. C, replication products formed using an unlabeled origin fragment and [32P]dCTP (indicated with C*) were analyzed on a polyacrylamide gel. The products formed were as indicated.

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2 A. van der Flier, personal communication.
eral surface exposed residues, however, did not reduce the stimulatory action of the POU domain (data not shown). Previously (16) we observed that mutation of Oct-1 POUhd residues Q24 and E29 gave rise to enhanced stimulation, but the mechanism of this is not clear, and this effect might also have been due to a slight contamination of the mutant proteins with NFI.

A Model for Assembly of the Adenovirus Initiation Complex—We envisage the following model for initiation and early elongation (Fig. 7). The four proteins (pTP-pol, NFI and Oct-1) can form a complex even in the absence of DNA (Fig. 2B). Whether such a DNA free complex is formed in vivo and is sufficiently stable is presently unknown. The multiprotein complex (altogether, approximately 480 kDa) binds the origin, thereby positioning and stabilizing pTP-pol correctly in a preinitiation complex. Stabilization both by Oct-1 (Fig. 4) and NFI (13) is achieved by lowering the off-rate of the pTP-pol-DNA interaction. Because Oct-1 and NFI interact with different subunits of the pTP-pol complex they supplement each other, thereby explaining the synergism in assembly and activation observed before (7). Although the four proteins form a complex, they do not all interact with each other. No interaction between NFI and pTP or between Oct-1 and the polymerase was detected (Fig. 2A). We also do not know whether both NFI subunits interact with the polymerase. It is likely that only one subunit of NFI is involved, but this is difficult to establish because monomeric NFI cannot be isolated (11, 26). Although a weak interaction between NFI and Oct-1 was observed (Fig. 2B), we do not think that this is functional because maximal stimulation levels of Oct-1 and NFI do not seem to be influenced by each other (7). Only slight cooperative DNA binding was observed between NFI and Oct-1 on the Ad2 origin (7). Such cooperativity was shown to occur in another sequence context on the human papillomavirus enhancer (27).

Not indicated in Fig. 7 is the role of DBP in initiation. We do not have evidence that DBP forms a complex with the other four proteins in the absence of DNA nor can it be found stably in the preinitiation complex. Nevertheless DBP has a pronounced effect on initiation because it decreases the $K_m$ of the polymerase for the initiator dCTP (28) and enhances the bindi
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FIG. 7. Model of the events occurring during and shortly after initiation of adenovirus DNA replication. The covalently bound TP and DBP have been left out of the preinitiation complex for reasons of clarity. For further details see text.

passing polymerase unwinds the octamer recognition site (Fig. 5). This suggests that the POU domain may also stabilize the complex after the first dCTP coupling, during formation of early elongation products. The pTP-pol heterodimer dissociates early in elongation.3 During elongation, DBP binds cooperatively to the displaced strand (34), thereby assisting the polymerase in DNA unwinding.4

POU Domain Proteins as Regulators of Viral Replication—The use of a POU domain protein for efficient multiplication is not restricted to adenoviruses. Direct protein-protein interaction was reported between Oct-6 and the JC papovavirus T antigen (35) and between Oct-1 and the Herpes simplex transactivator protein (36), both resulting in enhanced viral gene activation. Other viruses that use a POU transcription factor include SV40, Epstein-Barr virus, murine mammary tumor virus, human papillomavirus, and hepatitis B virus (37–41). Presumably, their high level of conservation, in particular of the POU domain, and wide expression have made them attractive targets for invading viruses during evolution. Adenovirus, however, is the only virus known to use Oct-1 for efficient DNA replication. There is some indirect evidence that Oct-1 is involved in eukaryotic replication. Octamer sequences have been found in several chromosomal origins of replication (42, 43), and enhanced replication of a transfected plasmid depended on an intact octamer site (42). Whether this is a direct effect on replication remains to be elucidated.

Acknowledgments—We are indebted to R. Hay for pTP and pol expressing baculoviruses, W. Teertstra for purifying pTP and pol, J. Dekker for purified NFI, and Frits Fallaux for critical reading of the manuscript.

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