Stoichiometry and Mechanism of Assembly of SV40 T Antigen Complexes with the Viral Origin of DNA Replication and DNA Polymerase α-Primase†

Shu-Gui Huang,‡ Klaus Weisshart,§ Ilka Gilbert,|| and Ellen Fanning*,||

Institute of Physical Biochemistry, University of Munich, Schillerstrasse 44, 80336 Munich, Germany, Institute of Molecular Biotechnology, Beutenbergstrasse 11, 07745 Jena, Germany, Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235, and Vanderbilt Cancer Center, Nashville, Tennessee 37232-6838

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ABSTRACT: The interactions of simian virus 40 (SV40) large T antigen with DNA carrying the viral origin of DNA replication, as well as its interactions with cellular replication proteins, have been investigated by using fluorescent ATP analogues as specific probes. The enhanced fluorescence of 3′(2′)-*O*-(2,4,6-trinitrophenyl)adenosine diphosphate (TNP-ADP) induced by T antigen binding to the nucleotide was decreased upon binding of T antigen to origin DNA. Similarly, the enhanced fluorescence induced by T antigen binding to TNP-ADP or TNP-ATP was decreased upon binding to human DNA polymerase α-primase (pol α), but not to replication protein A (RPA). Fluorescence titrations revealed noncompetitive inhibition of TNP-ADP binding by origin DNA, and noncompetitive inhibition of TNP-ADP and TNP-ATP binding by pol α, suggesting that T antigen complexed with either origin DNA or pol α was not able to bind the TNP nucleotide. From these titrations, we have measured a binding stoichiometry of 11.5 ± 0.8 T antigen monomers per viral origin DNA, in agreement with the double hexamer assembly of T antigen on the origin as reported earlier. The stoichiometry of pol α binding to T antigen was measured to be 5.5 ± 0.6 mol of T antigen per mole of pol α. While monomeric T antigen−nucleotide complex was a preferred ligand over free T antigen in the double hexamer assembly reaction, preformed T antigen hexamers were incapable of forming double hexamers on the DNA. The results support a model in which double hexamer assembly on the viral origin occurs by successive binding of 12 free T antigen or monomeric T−nucleotide complexes to the DNA. In contrast with this stepwise assembly of T antigen monomers on DNA, hexameric T antigen was able to bind directly to pol α with concomitant release of the bound TNP nucleotide. The possible implications of these results for the mechanism of initiation of SV40 DNA replication are discussed.

Replication of the simian virus 40 (SV40)[1] genome in infected cells in culture and in vitro requires a multifunctional viral protein, the large tumor (T) antigen, as well as 10 cellular DNA replication proteins (*1*, *2*; reviewed in *3*). T antigen orchestrates a series of protein−DNA and protein−protein interactions that allow it to control replication of the viral genome by these cellular proteins. Prior to initiation of replication, T antigen binds specifically to sequences in the viral origin of DNA replication, assembling into a double hexamer on the origin in the presence of ATP and locally unwinding and distorting the DNA duplex (reviewed in *4*). T antigen interacts physically and functionally with DNA polymerase α-primase (pol α) and the single-stranded DNA binding protein replication protein A (RPA) during both initiation of replication and lagging strand DNA synthesis (*5*−*10*). T antigen also plays a catalytic role during initiation and elongation as the only DNA helicase known to be required for viral DNA replication (*11*; reviewed in *12*).

A sequence of events during initiation of replication has been postulated based on partial reactions reconstituted with purified proteins in vitro. Following assembly of the double hexamer and local origin DNA unwinding and distortion, RPA interacts with the unwound DNA and with T antigen, thus stabilizing the T antigen−DNA complex (reviewed in *4*; *9*, *13*−*15*). Further unwinding requires ATP hydrolysis by T antigen, and either topoisomerase I or topoisomerase II activity. The complex consisting of the T antigen double hexamer, the partially unwound DNA, and RPA is thought to represent a preinitiation complex. The subsequent synthesis of primers to initiate replication requires participation of pol α, which is proposed to join the complex (*16*−*18*). The protein−protein and protein−DNA interactions in this initiation complex presumably orient the T antigen double hexamer suitably for subsequent bidirectional DNA unwinding and primer synthesis (*15*, *19*−*22*).

Despite considerable effort, these protein−protein and protein−DNA interactions are still not well understood. For instance, the interactions of T antigen with pol α and RPA have not yet been characterized in terms of association

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* Correspondence should be addressed to this author at the Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235. Fax: 615-343-6707. Email: fannine@ctrvax.vanderbilt.edu.

‡ University of Munich. Address after October 1, 1998: Tularik Inc., 270 E. Grand Ave., South San Francisco, CA 94080.

§ Institute of Molecular Biotechnology.

|| Vanderbilt University.

[1] Abbreviations: TNP nucleotide, 3′(2′)-*O*-(2,4,6-trinitrophenyl)-adenosine nucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SV40, simian virus 40; RPA, replication protein A; pol α, polymerase α-primase.

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constants and binding stoichiometry, and the sequence of interactions leading to assembly of T antigen with viral origin DNA, nucleotides, and initiation proteins remains poorly defined. In the accompanying paper, we have characterized the binding of fluorescent 3′(2′)-*O*-(2,4,6-trinitrophenyl) (TNP) adenine nucleotides to T antigen (*23*). In this paper, we employ the TNP nucleotides as a probe to investigate protein−protein and protein−DNA interactions of T antigen. The fluorescence signal has allowed us to confirm the DNA binding stoichiometry of T antigen, to characterize the mechanism of assembly of T antigen−nucleotide complexes on the viral origin, and to determine the stoichiometry of the human pol α−T antigen complex in solution.

MATERIALS AND METHODS

Nucleotides were obtained from Boehringer Mannheim. The fluorescent nucleotide analogues, 3′(2′)-*O*-(2,4,6-trinitrophenyl)-ATP and 3′(2′)-*O*-(2,4,6-trinitrophenyl)-ADP, were purchased from Molecular Probes (Eugene, OR).

Proteins and DNA. T antigen and human DNA polymerase α-primase (pol α) were purified from recombinant baculovirus-infected insect cells by immunoaffinity chromatography (*20*, *24*, *25*). The p180 subunit, the p68 subunit, and the p180−p68 complex were purified as described (*24*, *26*). Replication protein A (RPA) was purified from calf thymus (*27*) and from human HeLa cells as described (*6*). Protein concentrations were determined by UV absorption at 280 nm using calculated extinction coefficients (ϵ) of T antigen and the pol α complex of 66 000 and 405 000 M^{-1} cm^{-1}, respectively. The plasmid pUCmori containing the minimal SV40 origin of replication (*28*) was purified on Qiagen columns according to the manufacturer's instructions. The DNA concentration was determined by UV measurement at 260 nm assuming 1 absorption unit equals 50 μg/mL.

Hydrolysis of TNP-ATP by T Antigen ATPase. The assay for hydrolysis of TNP-ATP by T antigen ATPase was performed as described in the accompanying paper (*23*), but with the inclusion of either pUCmori DNA, pol α, or RPA, as indicated in the text.

Fluorescence Measurements. Fluorescence measurements were carried out on a Perkin-Elmer MPF-44A fluorescence spectrophotometer as described (*23*). pUCmori DNA or cellular proteins were incubated with T antigen for 15 min at room temperature prior to fluorescence titration with TNP nucleotides. The buffer consisted of 20 mM Hepes, 5 mM $MgCl_2$, pH 8.0. All fluorescence measurements were repeated at least twice, and the standard error is given in the figure legends and Tables.

ELISA. Enzyme-linked immunosorbent assays were performed essentially as described previously (*6*, *22*, *28*), except that T antigen was preincubated for 30 min at room temperature in PBS containing 4 mM nucleotide as indicated, 7 mM $MgCl_2$, and 0.5 mM dithiothreitol, and then 1 μg was placed in each well of the ELISA plates to form the solid phase. After blocking with bovine serum albumin, the soluble purified protein was added as indicated in the figures. Detection was carried out with monoclonal antibody 2CT25 (*5*) against pol α and peroxidase-conjugated rabbit anti-mouse antibody (1:100), or polyclonal rabbit antibody against RPA (1:2000) and goat anti-rabbit antibody (1:1000), followed by a chromogenic substrate. The absorbance at 405 nm was determined after 15 min at room temperature.

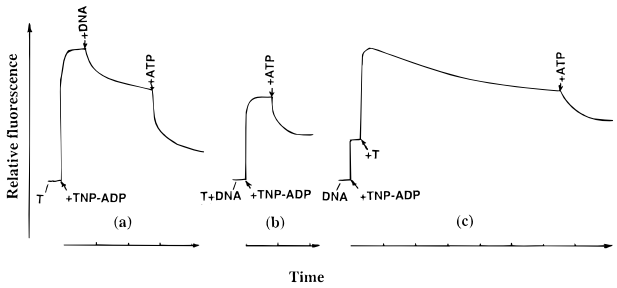


FIGURE 1: Fluorescence response of TNP-ADP bound to T antigen in the presence of pUCmori DNA. Fluorescence was monitored at $\lambda_{exc} = 415$ nm and $\lambda_{em} = 547$ nm in 20 mM Hepes, 5 mM $MgCl_2$, pH 8.0. Preincubation of T antigen alone (a), T antigen and DNA (b), or DNA alone (c) was for 15 min. The additions were made as indicated: T antigen, 1.5 μM (monomer concentration); TNP-ADP, 3.7 μM; DNA, 0.037 μM; ATP, 2.6 mM. The unit length on the time scale corresponds to 2 min.

RESULTS

Fluorescence Spectroscopic Analysis of the Assembly of T Antigen Complexes on Viral Origin DNA in Solution. As shown in the accompanying paper (*23*), TNP nucleotides bind with enhanced fluorescence to the ATP binding site of T antigen with a 1:1 molar stoichiometry and thus can be employed as specific fluorescent probes for T antigen. TNP-ATP was not hydrolyzed by the T antigen ATPase in the absence of DNA (*23*). In the presence of SV40 origin DNA, however, hydrolysis of TNP-ATP was more pronounced (data not shown), presumably due to stimulation of the ATPase activity of T antigen by DNA (*29*). For this reason, the nucleotide TNP-ADP was used as a probe for T antigen in the following DNA binding studies.

When TNP-ADP was added to T antigen, the fluorescence of the nucleotide ($\lambda_{exc} = 415$ nm, $\lambda_{em} = 547$ nm) increased as it bound to T antigen (Figure 1a). When SV40 origin DNA was then added to the mixture of T antigen and TNP-ADP, the fluorescence intensity decreased slowly (Figure 1a), suggesting that DNA binding to T antigen displaced the bound TNP-ADP from the ATP binding site. A control experiment showed that the fluorescence properties of the TNP nucleotide remained unchanged in the presence of DNA (data not shown). Since addition of a large excess of ATP displaced all prebound TNP nucleotide from the binding site (*23*), excess ATP was added to the DNA binding reaction to displace any TNP-ADP which remained bound to T antigen after addition of the DNA. The addition of ATP caused the fluorescence to decrease to a plateau; this fluorescence decrease (ΔF) allowed us to measure the amount of TNP-ADP that remained bound in the presence of DNA (Figure 1a).

When T antigen was first preincubated with the origin DNA for 15 min, and then the TNP-ADP was added, the fluorescence again increased rapidly (Figure 1b), but only to a lower level than observed when origin DNA was absent during the preincubation (compare to Figure 1a). This result suggested that part of the T antigen had bound to the DNA in the absence of nucleotide and was consequently not able to bind to TNP-ADP. If free T antigen were unable to bind to origin DNA, but required the TNP nucleotide to form hexamers prior to DNA binding (*23*), we should have observed a rapid fluorescence increase to the maximal level,

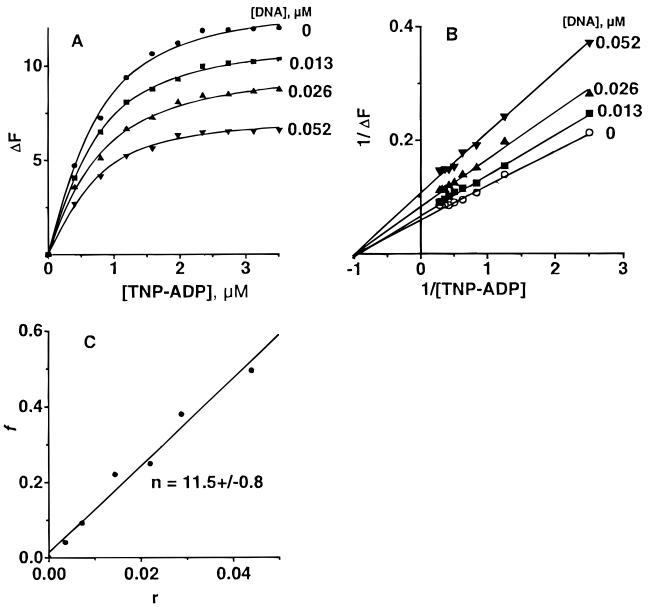


FIGURE 2: Fluorescence titration of T antigen in the presence of pUCmori DNA. The DNA at the indicated concentrations was preincubated with 1.5 μM T antigen for 15 min at room temperature prior to titration with TNP-ADP. (A) Titration curves; the solid lines are nonlinear regression fittings of the titration data (*42*) with $\Delta F = (\Delta F_{max}/2[T]_o)([T]_o + K_D + X - \sqrt{([T]_o+K_D+X)^2-4X[T]_o})$, where ΔF and ΔF_{max} are the specific fluorescence enhancement and its maximum, respectively, $[T]_o$ and K_D are the T antigen concentration and dissociation constant, respectively, and X is the concentration of added TNP-ADP. To extract the ΔF_{max} values from the fitting procedure, the K_D was set at 1.0 μM. (B) Double reciprocal plots of the titration curves. (C) Plot of the decrease (f) in specific fluorescence maximum versus the molar ratio of DNA to T antigen. The solid line is a least-squares fit according to eq 1 yielding a stoichiometry of 11.5 0.8.

followed by a decrease as the T antigen bound progressively to the origin DNA. The absence of such an initial high level of TNP-ADP binding supports the interpretation that the free monomeric T antigen had interacted with the origin DNA, to form a tight complex before addition of the TNP-ADP.

To test this interpretation further, T antigen was added to a mixture of TNP-ADP and DNA. As shown in Figure 1c, the fluorescence initially increased rapidly to the same level observed with T antigen in the absence of DNA (compare Figure 1a), but then decreased slowly to approximately the same level observed when T antigen was preincubated with origin DNA (compare Figure 1b). The simplest explanation for this observation is that T antigen first bound rapidly to TNP-ADP, while the binding of T antigen to origin DNA was slower and accompanied by displacement of the bound TNP-ADP.

To further evaluate the origin DNA interaction with T antigen, T antigen was preincubated in the presence of three different concentrations of SV40 origin DNA for 15 min at room temperature, and then titrated with increasing concentrations of TNP-ADP. The results are depicted in Figure 2. As the concentration of origin DNA in the reaction was increased, the maximal fluorescence enhancement was reduced, although T antigen binding to TNP-ADP was still detectable (Figure 2A). Double reciprocal plots of the data clearly revealed a noncompetitive pattern of inhibition of nucleotide binding by the DNA (Figure 2B). The dissociation constant of the TNP-ADP−T antigen complex (K_D = 1.0 μM) was not altered by the origin DNA; however, DNA decreased the maximal fluorescence enhancement (ΔF_{max}).

This noncompetitive inhibition pattern argues that T antigen bound initially to the origin DNA, and, once complexed with DNA, it no longer bound to the TNP nucleotide. Thus, the TNP-ADP binding reflected binding only to the free T antigen, and we can estimate the binding stoichiometry of T antigen to DNA by assuming the "inactivation" of the TNP nucleotide binding sites of that T antigen bound to DNA. As shown in the accompanying paper (*23*), the specific fluorescence enhancement (ΔF) can be used as a measure for the nucleotide binding. On this basis, we can calculate the binding stoichiometry (n) from the ΔF_{max} values according to

$$n = f/r \tag{1}$$

where f is the fraction of ΔF_{max} decrease in the presence of DNA relative to that (ΔF_{maxo}) in the absence of DNA and was calculated according to $f = (\Delta F_{maxo} - \Delta F_{max})/\Delta F_{maxo}$, and r is the molar ratio of the added origin DNA to the T antigen (r = moles of DNA/moles of T antigen monomer). From theoretical fitting of the binding curves (Figure 2A), we estimated the ΔF_{max} values. As shown in Figure 2C, a plot of the fluorescence decrease f versus the molar ratio r gave a linear dependence. A least-squares fit of these data revealed a stoichiometry of T antigen binding to origin DNA of 11.5 ± 0.8. This value suggests that 12 T antigen monomers bound to the origin DNA duplex.

In the titrations presented above, we maintained the molar ratio of [DNA]/[T antigen] at less than 0.05. At higher concentration ratios, lower stoichiometry values were obtained (data not shown), due to formation of intermediate complexes with fewer T antigen monomers per origin, which were also observed in bandshift assays (data not shown).

To confirm whether SV40 origin DNA failed to bind to the T antigen hexamer preformed by TNP-ADP binding, TNP-ADP bound to T antigen was titrated with increasing concentrations of DNA. T antigen was first preincubated for 30 min with excess TNP-ADP in seven separate reaction mixtures. Then different amounts of DNA were added to each reaction. After incubation for another 30 min, the specific fluorescence (ΔF) was measured by adding 2.6 mM ATP to each reaction. As shown in Figure 3, the ΔF followed an initial steep decrease and then reached a plateau.

If origin DNA were able to bind irreversibly to hexameric as well as to free monomeric T antigen, and released the prebound TNP-ADP from the hexamer, the free T antigen concentration should be reduced stoichiometrically as illustrated in the following two formulas, which have been simplified by omitting the hexameric T antigen term:

$$n\mathrm{T} + \mathrm{DNA} \rightarrow \mathrm{T}_n{\cdot}\mathrm{DNA}$$

$$\begin{array}{ccccc} \mathrm{T} & + & \text{TNP-ADP} & \rightleftharpoons & \\ [\mathrm{T}]_o - \Delta F/\phi - n[\mathrm{DNA}]_o & & [\text{TNP-ADP}]_o - \Delta F/\phi & & \\ & & & & \mathrm{T}{\cdot}\text{TNP-ADP} \\ & & & & \Delta F/\phi \end{array}$$

where $[T]_o$ and $[TNP\text{-}ADP]_o$ are the T antigen (micromolar) and TNP-ADP (micromolar) concentrations; $[DNA]_o$ (micromolar) and n are the added origin DNA concentration and the binding stoichiometry, respectively; ΔF and ϕ are

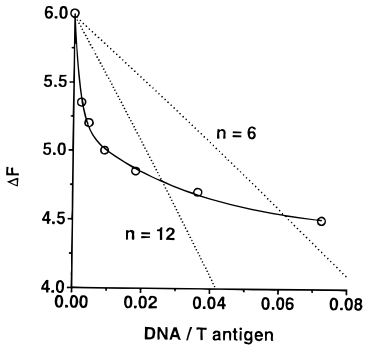


FIGURE 3: Decrease in the specific fluorescence of bound TNP-ADP as a function of pUCmori DNA concentration. 1.42 μM T antigen was first incubated with 3.51 μM TNP-ADP for 30 min prior to addition of increasing concentrations of DNA. After incubation for 30 min, the specific fluorescence ΔF was measured by adding 2.6 mM ATP. The dashed lines represent theoretical calculations assuming stoichiometric ($n = 6$ and 12) irreversible binding of DNA to T antigen (eq 2). The ϕ and K_D used were 5.9 μM^{-1} and 1.0 μM, which were determined under the same conditions.

the measured specific fluorescence decrease on addition of 2.6 mM ATP and the fluorescence yield (*23*), respectively; and $\Delta F/\phi$ represents the bound nucleotide concentration. Thus, under equilibrium conditions, the dissociation constant for T·TNP-ADP is

$$K_D = ([T]_o - \Delta F/\phi - n[DNA])([TNP\text{-}ADP]_o - \Delta F/\phi)/(\Delta F/\phi)$$

Solving for ΔF, we have

$$\Delta F = \phi \frac{b - \sqrt{b^2 - 4([T]_o - n[DNA]_o)[TNP\text{-}ADP]_o}}{2}$$

where

$$b = [T]_o - n[DNA] + [TNP\text{-}ADP]_o + K_D \qquad (2)$$

We have calculated the theoretical binding using the ϕ and K_D values determined under these conditions. The calculated lines (dashed) predict a quasi-linear decrease in ΔF as the DNA concentration increases; however, the measured ΔF data did not fit to the theoretical lines (Figure 3).

The most plausible explanation for the discrepancy between the observed and calculated curves in Figure 3 is that origin DNA bound only to the free monomeric T antigen, which induced dissociation of the monomeric T antigen−TNP-ADP complex as indicated by the initial fluorescence decrease, and that origin DNA did not bind to the hexameric T antigen−TNP-ADP complex. We further assume that the hexamer to monomer transition is very slow at room temperature. Thus, the plateau value in Figure 3 (approximately 75% of the total fluorescence) represents the remaining unquenchable fluorescence of the preformed hexameric TNP-ADP−T antigen complex. These data indicate that under the present experimental conditions, about 75% of the T antigen formed hexamer with TNP-ADP.

Binding of Pol α and RPA to T Antigen. To address the question of which forms of T antigen bound to pol α and RPA, we employed a solid-phase binding assay to compare the binding of purified pol α and RPA to T antigen in the presence and absence of ATP or the poorly hydrolyzable

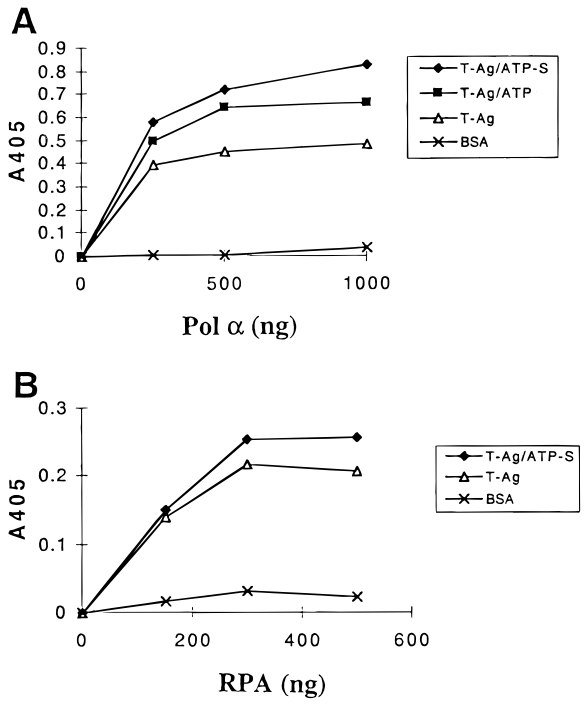


FIGURE 4: Interaction of SV40 T antigen hexamers with cellular replication proteins. Purified T antigen was preincubated with or without the indicated nucleotide (4 mM) as described under Materials and Methods for 30 min at room temperature. One microgram of T antigen was then fixed for 1 h in each well of an ELISA plate, washed with PBS, blocked with 3% bovine serum albumin (BSA) in PBS for 2 h, and washed again. Purified pol α (A) or RPA (B) was added in the indicated amounts for 2 h. After washing, the bound protein was detected by incubation with specific antibodies, peroxidase-linked second antibodies, and a chromogenic substrate as described under Materials and Methods.

ATP analogue ATPγS. Purified T antigen was preincubated with nucleotide to assemble hexamers, or without nucleotide as a control, and used as the solid phase of the ELISA (Figure 4). After washing and blocking with bovine serum albumin, purified pol α (Figure 4A) or human RPA (Figure 4B) was added in increasing concentrations. Binding of the soluble protein to T antigen was detected by incubating the complexes with a specific antibody against pol α or RPA, followed by a horseradish peroxidase-linked second antibody and a chromogenic substrate. Figure 4A shows that binding of pol α to T antigen increased in a concentration-dependent manner, while no binding to the control solid phase (bovine serum albumin) was observed, indicating that the interaction between the proteins was specific, in agreement with previous reports (*5*−*7*). Binding of pol α was markedly stimulated by preincubating the T antigen with either ATP or ATPγS (Figure 4A). RPA also bound specifically to T antigen, as reported previously (*6*, *9*, *10*), but binding was not stimulated by preincubation of T antigen with nucleotide (Figure 4B). These results suggested that pol α bound preferentially to hexameric T antigen, while RPA binding was independent of the oligomeric form of T antigen.

Fluorescence Spectroscopic Analysis of T Antigen Binding to Pol α. To apply fluorescence spectroscopy to the question of which form of T antigen bound to pol α in solution, we first investigated whether TNP nucleotides bound to pol α, since ATP is a substrate for the primase activity of the pol α complex. When TNP-ATP or TNP-ADP was added to pol α, we observed little change in either the TNP fluores-

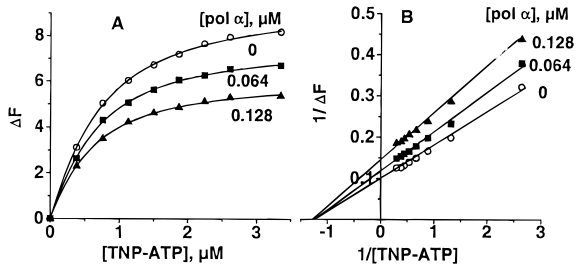


FIGURE 5: Binding of TNP-ATP in the presence of pol α. Fluorescence titration of 1.32 μM T antigen with TNP-ATP. All the measurements were conducted at pH 8.0 and room temperature. (A) Titration curves. The specific fluorescence enhancement ΔF was calculated as the difference between the fluorescence intensities in the absence and presence of 2.6 mM ATP. (B) Double reciprocal plots of the titration curves.

Table 1: Summary of the Effects of Pol α on TNP Nucleotide Binding to T Antigen[a]

T antigen	fluorescent probe	molar ratio (R) pol α/T	ΔF_{max} decrease (f)	calcd stoichiometry (n)
wild-type	TNP-ATP	0.030	0.16	5.3
	TNP-ADP	0.061	0.29	4.8
		0.061	0.50	7.4
		0.028	0.15	5.4
		0.058	0.26	4.5
T124A	TNP-ATP	0.061	0.33	5.4
				5.5 ± 0.6

[a] Fluorescence titrations of 1.1 μM T antigen were at pH 8.0, room temperature. The T antigen was preincubated with pol α for 15 min prior to fluorescence titrations. The molar ratio (R) is the ratio of pol α to monomeric T antigen. The fraction (f) of ΔF_{max} decrease is calculated from titrations in the absence (ΔF_{maxo}) and presence of pol α (ΔF_{max}), according to $f = (\Delta F_{maxo} - \Delta F_{max})/\Delta F_{maxo}$. The stoichiometry ($n$ = moles of monomeric T antigen per mole of pol α) of the T−pol α complex is calculated from $n = f/R$. Results of three different preparations of T antigen.

cence or the protein tryptophan fluorescence. Subsequent addition of excess ATP also did not cause any fluorescence change (data not shown). Using a filtration assay (30 000 NMWL Millipore filter unit; Bedford, MA), we also observed no decrease in the fluorescence intensity in the filtrate from a TNP-ATP solution in the absence and presence of excess pol α (data not shown). Together these data indicated that the TNP nucleotides did not bind pol α and could therefore be utilized as probes for T antigen binding to pol α.

To analyze the interaction of T antigen with pol α, we titrated T antigen with TNP-ATP in the presence of pol α (Figure 5). To allow complete interaction, T antigen was preincubated with the pol α for 15 min before the titration with nucleotide. The fluorescence enhancement (ΔF) observed upon TNP-ATP binding to T antigen was decreased in the presence of pol α (Figure 5A). Double reciprocal plots of the fluorescence data revealed that binding of the TNP nucleotide to T antigen was inhibited noncompetitively by pol α (Figure 5B). This finding suggests that T antigen complexed with pol α has reduced affinity for TNP nucleotide, or, in other words, the decreased fluorescence results from reduced concentrations of titratable free T antigen. From six independent titrations of three T antigen preparations, we determined a binding stoichiometry of 5.5 ± 0.6 mol of T antigen per mole of pol α (Table 1). When TNP-ADP, rather than TNP-ATP, was used as the probe, we measured essentially the same binding stoichiometry (Table 1). We

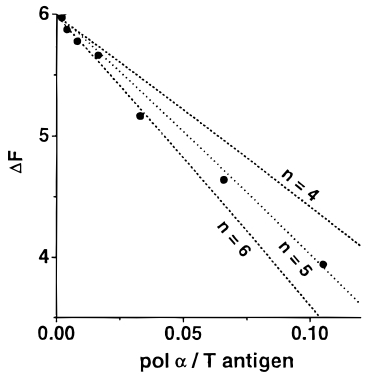


FIGURE 6: Decrease in the specific fluorescence of bound TNP-ADP as a function of the pol α concentration. 1.42 μM T antigen was first incubated with 3.51 μM TNP-ADP for 30 min prior to addition of increasing concentrations of pol α. After incubation for 30 min, the specific fluorescence ΔF was measured by adding 2.6 mM ATP. The dashed lines represent theoretical calculations assuming stoichiometric (n = 4, 5, and 6) irreversible binding of pol α to T antigen (eq 2). The ϕ and K_D used were 5.9 μM^{-1} and 1.0 μM, which were determined under the same conditions.

noted no difference between the wild-type and the replication-defective mutant T124A (*20*) T antigens in their interaction with pol α (Table 1).

We also investigated whether pol α binds to preformed T antigen hexamers. T antigen was preincubated with TNP-ATP as in Figure 3 to allow hexamers to assemble and then incubated with various concentrations of pol α. Fluorescence data were obtained as in Figure 3, and the results are presented in Figure 6. The binding of pol α to T antigen was best simulated by theoretical curves with n = 5−6 mol of monomeric T antigen per mole of pol α. This value is close to that determined from direct fluorescence titrations, suggesting that pol α bound to the preformed T antigen hexamers with concomitant release of the bound TNP nucleotide.

Previous investigations of SV40 and polyoma T antigen binding to pol α in solid-phase assays suggested that at least three of the four subunits of pol α interact with T antigen (*5*, *6*, *7*, *26*, *30*). To extend these studies to proteins in solution, we investigated the effects of pol α complexes with different subunit compositions on TNP-ADP binding to T antigen. The purified p180, p68, p180−p68 heterodimer, and the intact heterotetrameric complexes of pol α, each present at 0.12 μM, were preincubated with 0.12 μM T antigen and then titrated with TNP-ADP. As shown in Figure 7, T antigen preincubated with 0.12 μM p68 resulted in decreased TNP-ADP fluorescence as compared to the control without pol α. p180 had a similar but somewhat stronger effect. A further decrease in fluorescence was observed with the p68−p180 complex. The most dramatic reduction in fluorescence was observed with the intact heterotetrameric pol α complex. These results confirm that the two large subunits interact with T antigen independently and as a complex, and suggest that each of these subunits, and perhaps one or both of the two small subunits, contributes to the overall binding affinity.

Characterization of RPA Interactions with T Antigen in Solution. Preliminary results showed that the presence of RPA did not affect the specific fluorescence enhancement (ΔF) of TNP nucleotides upon binding to T antigen. A control experiment showed that RPA alone did not alter the

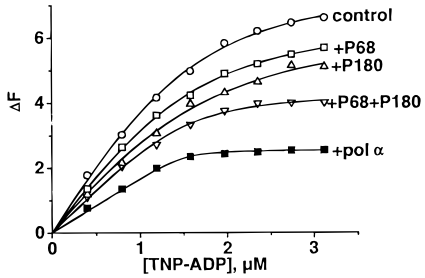


FIGURE 7: Decrease in the specific fluorescence of TNP-ADP bound to T antigen by pol α as a function of its subunit composition. p68, p180, and pol α, each at 0.12 μM, were incubated at pH 8.0 and room temperature with 0.12 μM T antigen for 30 min prior to titration with TNP-ADP.

Table 2: Summary of the Effects of RPA on TNP Nucleotide Binding to T Antigen[a]

RPA/T molar ratio	K_D (μM)	ΔF_{max}
0	0.55 ± 0.06	6.1 ± 0.1
0.17	0.64 ± 0.08	6.0 ± 0.2
0.34	0.63 ± 0.04	5.9 ± 0.1

[a] Fluorescence titration of 1.1 μM wild-type T antigen with TNP-ATP at pH 8.0 in the presence of RPA. The T antigen was preincubated with RPA for 15 min prior to fluorescence titrations. The ΔF_{max} values are the maximal fluorescence enhancements of the bound TNP-ATP or TNP-ADP in arbitrary units.

fluorescence of the TNP nucleotide. Results of fluorescence titrations of TNP nucleotides at different ratios of RPA to T antigen are summarized in Table 2. Even at rather high RPA concentrations, we observed no effect of RPA on either the nucleotide dissociation constant (K_D) or the maximal fluorescence enhancement (ΔF_{max}). We further investigated whether RPA interacted with T antigen in the presence of origin DNA or pol α by titrating the T antigen in the presence of RPA and either DNA or pol α. The results again showed that RPA did not significantly affect TNP-ADP binding to T antigen (data not shown).

DISCUSSION

The assembly of T antigen with DNA and cellular replication proteins into the initiation complex is a vital process in SV40 DNA replication (*31*). In this study, the strong fluorescence signal changes of the nucleotide analogues have allowed us to probe the interactions of T antigen with the cellular proteins and the DNA.

Comparison of the TNP Nucleotide with the Parent Nucleotide. The TNP substitution enhanced the affinity of the parent adenine nucleotides for T antigen (*23*). The introduction of this relatively bulky group had two major effects on the substrate properties. First, in contrast to ATP, TNP-ATP was not hydrolyzed in the absence of DNA by T antigen ATPase, although it bound to the ATPase site. As a result, it was a potent ATPase inhibitor (*23*). In the presence of DNA, however, TNP-ATP was hydrolyzed. Apparently the T antigen ATPase activity was enhanced by DNA such that it could hydrolyze the analogue (*29*). Second, the bulky group apparently reduced the nucleotide binding affinity for T antigen bound to DNA or pol α. While T antigen in complex with DNA or pol α must still be able to bind ATP or ADP to function as a helicase, the binding of the bulkier TNP nucleotide is not tolerated. Thus, binding of the DNA or pol α is accompanied by a concomitant release of the prebound TNP nucleotide from its binding site, giving rise to a decrease in the fluorescence. This effect is probably due to the larger size of the bulky TNP group relative to ATP or ADP.

A more severe size effect was observed in Dna B protein, which was initially thought to accommodate only three TNP nucleotides per Dna B hexamer (*32*), in contrast to six ATP or ADP molecules; only at rather high TNP nucleotide concentrations was the Dna B protein able to bind 6 mol of TNP nucleotide (*33*).

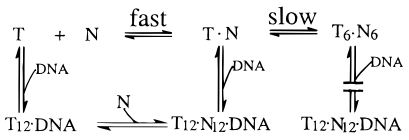
Parameters of T Antigen Binding to SV40 Origin DNA and Pol α. Our data show that 1 mol of DNA binds approximately 12 mol of T antigen. This number agrees with the stoichiometry of double hexamer formation on the DNA substrate observed in band shift experiments (*14*, *15*, *19*, *34*, *35*) and by scanning transmission electron microscopy (STEM) (*35*). The stoichiometry of 5.5 ± 0.6 mol of T antigen per mole of pol α suggests that one hexameric T antigen binds one pol α molecule. This stoichiometry agrees well with the working model for initiation, where each of the two replication forks is provided with one polymerase. These stoichiometries were determined from the decrease in the ΔF_{max} of TNP nucleotide binding, and the molar ratio of the DNA or pol α to T antigen. Possible errors may arise from the concentration determinations, but are probably small since the stoichiometry of T antigen binding to DNA agreed quite well with that determined by STEM.

Although we were able to measure the binding stoichiometry on the basis of noncompetitive inhibition, the affinity of these interactions was not determined in our studies. Since we measured only about 4% quenching of the protein tryptophan fluorescence by the DNA, the DNA−T antigen interaction could not be measured with the fluorescence quenching method, as was done for DNA binding to RPA and other proteins (*36*−*39*). We assume a very tight pol α−T, as well as DNA−T, complex, such that binding of the TNP nucleotides with a K_D of 0.3−4 μM did not reverse the formation of the complexes. Consistent with this prediction, the affinity of T antigen for origin DNA and for pol α was recently measured by surface plasmon resonance to be 10^9 M^{-1} (F. Grosse, personal communication).

Sequence of T Antigen Interactions on Origin DNA. We propose a two-step nucleotide binding reaction for T antigen, i.e., an initial formation of monomeric T−TNP nucleotide complex followed by assembly into a hexameric complex on the DNA. Our fluorescence measurements indicate that the DNA was able to interact with free monomeric T antigen (Figure 1b). The fluorescence decrease shown in Figure 1a and Figure 3 can be explained by DNA binding to the free T antigen, which induces further dissociation of the fluorescent monomeric T antigen−TNP nucleotide complex T·N in equilibrium with free T (see Scheme 1). Alternatively, the decrease could be explained by a direct binding of DNA to the T·N complex with concomitant release of the bound TNP nucleotide. Although the fluorescence data do not resolve these possibilities, the assembly reaction was faster with the monomeric complex T·N.

That the preformed hexamer $T_6{\cdot}N_6$ did not bind the DNA is supported by two lines of evidence, i.e., the results of back-titration (Figure 3) and by band shift assays using labeled origin DNA (*14*; data not shown). To draw this conclusion,

Scheme 1



we assume a slow hexamer to monomer conversion. At 37 °C, we observed a faster assembly reaction (unpublished data), which we interpret as primarily due to a faster hexamer to monomer conversion, in agreement with the observations of Hurwitz's laboratory (*14*). These findings, together with the minimal single hexamer assembly observed on double-stranded ori-containing DNA (*13, 14, 40*), can be summarized in Scheme 1. Briefly, free T antigen (monomer) quickly binds a nucleotide (N), as shown by a rapid fluorescence increase (Figure 1), to form a monomeric complex (T·N), which assembles into the hexameric complex (T_6·N_6). The monomeric and hexameric complexes are in a slow equilibrium at room temperature. The DNA can bind slowly, as shown by a slow fluorescence decrease (Figure 1), to both the free T antigen and the monomeric complex (T·N) and assembles with them into double hexamer complexes (T_{12}·DNA, or T_{12}·N_{12}·DNA). The preformed hexameric T antigen (T_6·N_6) does not bind the DNA. These observations and the proposed scheme are in agreement with reports that six T antigen molecules (T or T·N) bind in a highly cooperative way around the DNA, followed by a cooperative binding of a second set of six T antigen molecules (*13, 40*). The cooperativity in the double hexamer assembly reaction on origin DNA, but not hexamer assembly in the absence of DNA, also appears to depend on the phosphorylation state of T antigen (*40*).

Assembly of T Antigen Complexes with Replication Proteins. Pol α is able to interact with the free monomeric T antigen. The fact that the stoichiometry measured by a back-titration procedure (Figure 6) agrees with the value determined from the titrations in the presence of pol α suggests two possibilities: (1) unlike the DNA, pol α binding was able to reverse the TNP nucleotide binding equilibrium all the way from the hexamer to monomer to free monomeric T antigen; or (2) unlike DNA, it binds to the monomeric, as well as hexameric, T antigen with release of the bound TNP nucleotide. Although we have no direct kinetic data to resolve these possibilities, a very slow transition of the hexamer to the monomer form is evident under the experimental conditions, as shown with DNA in Figure 3. Thus, it seems reasonable to assume the first possibility, that pol α binds directly to the hexameric T antigen with concomitant release of the prebound TNP nucleotide.

Although the sequence of assembly of T antigen and pol α on the origin has not been tested directly, several lines of evidence suggest that pol α may assemble concurrently or even facilitate assembly of T antigen. The association of pol α with T antigen was stimulated by nucleotide binding to T antigen (Figure 4A), and preassembled hexamers appeared to be able to bind to pol α (Figure 5 and 6). Furthermore, assembly of wild-type T antigen at low concentrations on the origin was reported to be accelerated by pol α (*18*).

Unlike pol α, RPA did not influence TNP nucleotide binding to T antigen. Even in the presence of the DNA and pol α, we did not observe any significant decrease in TNP-ADP binding induced by RPA. However, ample evidence demonstrates that RPA does interact physically and functionally with T antigen (*6, 9, 10*; Weisshart et al., in preparation; Figure 4B). This apparent contradiction can be reconciled by assuming that RPA interacts with the T antigen, but this site is distant from the ATP binding site. Indeed, we have found that RPA associates with a region in the DNA binding domain of T antigen (*43*), which lies well outside the ATP and pol α binding domains (*31, 41*).

In conclusion, fluorescence spectroscopy has been utilized to confirm the stoichiometry of T antigen binding to the SV40 origin of replication in solution, to provide evidence for stepwise assembly of nucleotide-bound T antigen with origin DNA in solution, and to determine for the first time the stoichiometry of T antigen binding to pol α.

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