Initiation of adenovirus DNA replication occurs by a jumping back mechanism in which the precursor terminal priming protein (pTP) forms a pTP-trinucleotide complex (pTP-CAT) catalyzed by the viral DNA polymerase (pol). This covalent complex subsequently jumps back three bases to permit the start of chain elongation. Before initiation, pTP and pol form a tight heterodimer. We investigated the fate of this pTP-pol complex during the various steps in replication. Employing in vitro initiation and elongation on both natural viral templates and synthetic oligonucleotides followed by glycerol gradient separation of the reaction products, we established that pTP and pol are separated during elongation. Whereas pTP-C and pTP-CA were still bound to the polymerase, after the formation of pTP-CAT 60% of the pTP-pol complex had dissociated. Dissociation coincides with a change in sensitivity to inhibitors and in Km for dNTPs, suggesting a conformational change in the polymerase, both in the active site and in the pTP interaction domain. In agreement with this, the polymerase becomes a more efficient enzyme after release of the pTP primer. We also investigated whether the synthesis of a pTP initiation intermediate is confined to three nucleotides. Employing synthetic oligonucleotide templates with a sequence repeat of two nucleotides (GAGAGA... instead of the natural GTAGTA...) we show that G5 rather than G3 is used to start, leading to a pTP-tetranucleotide (CTCT) intermediate that subsequently jumps back. This indicates flexibility in the use of the start site with a preference for the synthesis of three or four nucleotides during initiation rather than two.

The replication of the linear 36-kilobase pair adenovirus (Ad) DNA initiates at two origins located at either molecular end employing a protein-priming mechanism. Initiation requires at least two viral proteins, the DNA polymerase (pol) and the precursor of the terminal protein (pTP), which acts as the primer and is linked covalently to the first nucleotide, a dCMP molecule. Initiation can be stimulated by the cellular transcription factors NFI and Oct-1 which guide the pTP to the core origin employing their DNA binding domains. This leads to stabilization of the preinitiation complex and increases the number of initiation events by enhancing the Vmax of the reaction. In addition, the virus-coded single-stranded DNA-binding protein stimulates initiation by lowering the Km for dCTP (1).

The virus uses a jumping back mechanism for initiation. At position 4 from the 3′-end of the template a pTP-CAT intermediate is synthesized which jumps back to be paired to the template residues 1–3 before elongation starts (2). This jumping or sliding-back mechanism was first described for φ29 (3) and appears to be universal for protein-priming replication systems (4–6), enabling errors made during initiation and small deletions to be restored. Subsequent elongation concomitant with the displacement of the non-template strand further requires the Ad DNA-binding protein in addition to the polymerase (for review, see Refs. 7 and 8).

The multiprotein preinitiation complex assembled at the origin consists of five proteins that interact with DNA and with each other with different affinities. Several of the protein-protein interactions have been studied individually. The polymerase interacts with the NFI DNA binding domain (9–11) and possibly also with the DNA-binding protein. pTP contacts the Oct-1 POU homeodomain (12, 13). These interactions are mostly weak, and dissociation occurs already at limited (200 mM) salt concentrations. In contrast, pTP and pol form a stable heterodimer that can only be dissociated in vitro under strong denaturing conditions (1.7 M urea) (14) or by binding of specific antibodies (15, 16).

We are interested in the dynamic aspects of association and dissociation events occurring during initiation as well as during the transition to chain elongation. Previous studies employing an immobilized DNA replication system have shown that the interaction between NFI and pTP-pol is disrupted already early in initiation (17), in agreement with its role as temporally acting recruitment factor. Recently dissociation of Oct-1 from pTP-pol complex was shown to take place upon elongation (13). No information exists about dissociation of the pTP-pol complex. A priori these two proteins could stay together during replication, but this would require high flexibility of the end of the nascent strand anchored to the pTP which would have to be present in the moving fork.

We have studied the dissociation of pTP and polymerase employing separation of initiation and elongation products by glycerol gradient centrifugation and find that the heterodimer is disrupted early after the synthesis of the pTP-pol initiation intermediate. This is accompanied by changes in the catalytic properties of the polymerase. Moreover, we provide evidence for flexibility in the choice of the position in the origin at which the formation of initiation intermediates starts.

**EXPERIMENTAL PROCEDURES**

**Nucleotides and DNA Templates**—Ad serotype 5 TP-DNA was isolated as described previously (18). The [γ-32P]dNTPs were from ICN, and [γ-32P]ATP (3,000 Ci/mmol) was obtained from Amersham International. Unlabeled deoxynucleotides, deoxyribonucleotides, and oligonu-
cleotide templates were obtained from Pharmacia Biotech Inc. The sequence of the wild-type 30-mer containing the template strand of the Ad5 origin is 5′-GTAATGTTATTATGTTAGAATAACCTAAATG-3′. The template strand of the GA repeat template is 5′-GAAGAGAGATATGGAATAAAACCTAA-3′. Oligonucleotides SP1 (5′-GATCGA-CAGTGTTCATGACATGATGATG-3′) and SPIC-6 (5′-TCTGTGACTCGTAGTC-3′) were purified by electrophoresis on 8 M urea, 20% polyacrylamide gel electrophoresis. Oligonucleotide SP1 was 5′-end labeled with [γ-32P]ATP and T4 polynucleotide kinase and purified further by polyacrylamide gel electrophoresis. Partially double-stranded primer-template structures were made by hybridizing labeled SP1 to the non-labeled SPIC-6 oligonucleotide in the presence of 0.2 mM NaCl and 60 mM Tris-HCl, pH 7.5. The mixture was heated at 70 °C and was allowed to cool down slowly to room temperature.

The pTP-pol complex and the free Ad DNA polymerase were expressed in Sf9 cells employing recombinant baculoviruses that were constructed as described Ref. 19. Purification of both the pTP-pol complex and the free Ad DNA polymerase to apparent homogeneity was described previously (20). Polyclonal antibodies were raised against the pTP-pol complex in rabbits as described before (12).

Initiation and Partial Elongation—The standard incubation mixture (25 μl) for initiation contained 0.4 μg of pTP-pol and 0.7 μg of single-stranded oligonucleotide as template in a buffer containing 20 mM Hepes, pH 7.5, 1 mM dithiothreitol, 1 mM MgCl2, 1 μM of bovine serum albumin, and NaCl to a final concentration of 55 mM. The initiation reaction was allowed to proceed for 1 h unless otherwise indicated. At 30 °C in the presence of a 0.75 μM concentration of one of the [α-32P]dNTPs (5 μCi). The reaction was stopped by the addition of 10 μl of 0.25 M sodium pyrophosphate and 2 μl of 0.2 M EDTA. Reaction products were precipitated with 5 μl of trichloroacetic acid (20% final concentration) for 20–30 min at 0 °C. The precipitate was spun down by centrifugation for 15 min at 12,000 rpm in an Eppendorf centrifuge, and the pellet was dissolved in Laemmli buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 100 mM Tris, pH 9.0, 0.02% bromphenol blue). Samples were heated for 5 min at 100 °C and separated by electrophoresis in 7.5% polyacrylamide-SDS gels and detected by autoradiography.

Initiation coupled to partial elongation was performed under conditions similar to those for initiation, except for the added dNTPs. One of the four dNTPs was labeled with [α-32P]dATP (0.75 μM unless otherwise indicated). The other dNTPs were unlabeled and present at 40 μM; dGTP was replaced by 20 μM deoxyGTP. This results in an elongation block after position 26, the first cytidine residue in the template. For the kinetic studies the concentrations of the dNTPs were variable as indicated. The other dNTPs were unlabeled and present at 40 μM; dGTP was replaced by 20 μM deoxyGTP. Under these conditions partial elongation until position 26, the first cytidine residue in the template, can take place, whereas also part of the pTP-CAT product accumulates. At higher dCTP concentrations all pTP-CAT products can be extended, indicating that it is an intermediate in replication (1). By using low dCTP concentrations sufficient amounts of the intermediate product accumulate to enable the study of both products in one reaction. The reaction products were subsequently separated by glycerol gradient centrifugation. To disrupt the interaction between proteins and DNA we included heparin at 14 μg/ml in the gradient. This concentration was chosen carefully such that the pTP-pol heterodimer remained intact as much as possible during centrifugation. When higher heparin levels or high salt were used, we observed spontaneous dissociation of the pTP-pol complex, most likely induced by centrifugal forces.

Fractions were collected and analyzed by immunoblotting with a polyclonal antiserum raised against the pTP-pol complex, which preferentially recognizes epitopes on pTP and is less reactive against pol. Fig. 1A shows that most of the pTP coelutes with the polymerase near the bottom of the gradient, which corresponds to the position of the intact pTP-pol heterodimer, present in excess. The 32P-labeled pTP-26 replication product sedimented around the position of the 104-kDa marker protein (Fig. 1, B and C), corresponding with its molecular mass (97 kDa, assuming that the DNA is double-stranded because of renaturation). This shows that this replication product has dissociated from the polymerase. In contrast, the pTP-CAT intermediate was found distributed over two peaks (Fig. 1C), one cosedimenting with pTP-26 and one at the pTP-pol position, indicating that approximately 40% of the pTP-CAT is still bound to the polymerase. Employing a mutant single-stranded origin that was mutated at position 7 from G to C (C7), we also analyzed the sedimentation behavior of a pTP-7N product. This product sedimented similarly to the pTP-26 product (data not shown).

Replication products formed on the natural TP-DNA template and analyzed in the same way showed a similar dissociation pattern of the replication products, with pTP-26 dissociated and the pTP-CAT intermediate distributed over two peaks (Fig. 2, A and D). The same results were obtained at various pTP-pol or template concentrations or when NFI was included in the reaction (not shown). To determine the moment of dissociation in more detail we performed the reaction in the presence of one (dCTP) or two (dCTP and dATP) nucleotides allowing only the formation of pTP-C or pTP-CA products. In contrast to pTP-CAT, both products cosedimented almost exclusively with the pTP-pol complex (Fig. 2, B–D). This shows that dissociation only starts after the synthesis of pTP-CAT.

After pTP-CAT formation the intermediate jumps back. One possibility is that jumping back and dissociation are somehow
coupled. To test this we used an oligonucleotide template lacking the first three nucleotides and mutated at position 7 (Δ3G7C, 3'G,TACTTA...). On this template jumping back is not possible, and the presence of a C at position 7 forces the use of the first G in the mutant template for pTP \textsubscript{z}CAT formation. As expected, replication on this template in the presence of dCTP, dATP, and dTTP resulted in the synthesis of pTP \textsubscript{z}CAT only without pTP \textsubscript{z}26 formation. Subsequent analysis in a glycerol gradient (Fig. 3) showed a distribution of pTP \textsubscript{z}CAT similar to that with wild-type, despite the absence of a jump. These results suggest that dissociation occurs independently of the jumping back step.

Dissociation from pTP Enhances the Polymerase Activity—Dissociation of the pTP-pol complex implies that the free polymerase performs elongation. To compare the polymerizing efficiencies of the free and pTP-bound enzyme we prepared highly purified Ad DNA polymerase and pTP-pol complex from recombinant baculovirus-infected insect cells and measured the rate of DNA synthesis on a DNA-primed template. Equimolar amounts of the free and the complexed polymerase were preincubated with all four nucleotides and a 5'-end-labeled 15-mer primer base paired to a 21-mer template (see “Experimental Procedures”), in the absence of Mg\textsuperscript{2+}. After initiation of the reaction by the addition of 1 mM MgCl\textsubscript{2}, extension of the primer was allowed for the indicated periods, and the products were analyzed on a sequence gel. As shown in Fig. 4, almost of all primers were elongated within 30 s by uncomplexed DNA polymerase, whereas this occurred only after 32 min for the pTP-pol complex. By quantitation of the products we calculated a 10-fold higher initial rate of elongation for the free polymerase compared with the pTP-pol complex, indicating that dissociation from pTP after initiation renders the DNA polymerase more efficient for elongation. Formally we cannot exclude that the properties of free polymerase and dissociated DNA polymerase are not identical, but it is technically very difficult to study the properties of the dissociated polymerase since only a
Dissociation of the Ad pTP-Pol Complex

Properties and Inhibitor Sensitivity of the Polymerase—Previ-
ously we observed that the optimal conditions for formation of
pTP-dCMP differ considerably from those required during elon-
gation. The initiation reaction is insensitive to ddNTPs, aphidi-
colin, and (S)-g-(3-hydroxy-2-phosphonylmethoxypropyl)ad-
enine diphosphoryl, all strong inhibitors of elongation (21–25).
Moreover, the \( K_m \) for dCTP is 3.2-fold lower during initiation
(1). Similar results were obtained when we compared the \( K_m \)
values for dATP and dTTP (Fig. 5). For the formation of the
pTP-CAT intermediate, values of 3.8 and 1.0 \( \mu \)M were found,
respectively, whereas for pTP-26 this was 5.1 and 5.5 \( \mu \)M. The
various parameters are summarized in Table I. The most likely
explanation for these results is that changes occur in the poly-
merase active site upon transition from initiation to elonga-
tion, concomitant with dissociation from pTP and jumping back.

Ad5 DNA Polymerase Can Use Different Initiation Sites De-
pending on the Template—With the Ad5 template (3’-GTAG-
TAGTTA . . .) the second GTA triplet is used for the synthesis
of the pTP-CAT intermediate. To investigate whether it is
always the second repeat that is used by the Ad5 DNA poly-
erase, we employed a template with a terminal repeat consist-
ing of four times GA (3’-GAGAGAGATA . . .), which occurs
naturally in Ad4. As on the Ad5 template (2), only dCMP could
be coupled when any of the \( ^{32} \)P-labeled dNTPs was added (Fig.
6A, lanes 1–4). Elongation until position 26 was possible on the
GA repeat as with wild-type (Fig. 6A, lanes 5 and 6). To exam-
ine which of the 4 G residues was used for initiation we mu-
tated the A residues at the positions 2, 4, 6, and 8 one by one to
a C and studied incorporation of \( \alpha\)-\( ^{32} \)P-labeled nucleotides in
the intermediate using partial elongation conditions. As shown
in Fig. 6B, the initiation intermediate could only be labeled
with \( [\alpha\text{-}\text{\( ^{32} \)P}]dCTP \) and \( [\alpha\text{-}\text{\( ^{32} \)P}]dTTP \) employing the mutants
A2C and A4C (lanes 1–4 and 5–8), indicating that the synthe-
sis of the intermediate had not started from template residues
1 or 3. With A6C and A8C, however, the intermediate could
also be labeled with \( [\alpha\text{-}\text{\( ^{32} \)P}]dGTP \) (lanes 9–12 and 13–16). These
results are consistent with a start of synthesis from template
residue G5 from which a pTP-tetrancleotide is synthesized
which jumps back to enable elongation. For the A6C and A8C
mutants, these intermediates would be pTP-CGCT and pTP-CTCG,
respectively. Alternatively, the results could be explained by
the synthesis of a mixture of pTP-CG and pTP-CT originating
from the use of both G5 and G7, which would also result in labeling with C, G, and T. We consider the latter
possibility less likely since replacing dGTP by 20 \( \mu \)M ddGTP
completely blocked elongation on the A8C mutant (not shown).
If a pTP-dinucleotide (pTP-CT) would have been formed start-
ing at G5 this would have been able to jump back and continue
elongation. However, in the case of synthesis of pTP-CTCG the
presence of the dideoxy group at the 3’-end would prevent
elongation, and this is exactly what we observed.

In conclusion, for Ad5 DNA polymerase the choice of the G
residue seems not to be determined by the most proximal
repeat but rather by the position of this residue. A preference
exists for position 4 or 5 rather than 3, provided that a repeat
is still present and starts with a G residue. This indicates a
certain amount of flexibility in the choice of the starting
nucleotide.

**DISCUSSION**

Dissociation of the pTP-Pol Complex Occurs at or after
pTP-CAT Formation—By studying the sedimentation prop-
eries of four well defined replication products (pTP-C, pTP-CA,
pTP-CAT, and pTP-26) we have obtained strong evidence for
dissociation of the pTP primer and the DNA polymerase early
during the initiation process. In these experiments it was es-
cential to remove the proteins from the template while keeping
the pTP-pol interaction intact. We observed that, although the
interaction between pTP and pol is reported to be very stable
(14), the application of centrifugal forces in combination with

**FIG. 3.** Dissociation is not coupled to jumping back. An oligo-
nucleotide lacking the first 3 bases and containing a mutation at posi-
tion 7 was used as template, thereby preventing jumping back. Panel A,
the resulting pTP-CAT products synthesized in the presence of dCTP,
dATP, and dTTP were analyzed as in Fig. 1. Panel B, plot of the results.

**FIG. 4.** Dissociation increases the polymerizing efficiency. A par-
tial duplex consisting of a 5’-\( ^{32} \)P end-labeled 15-mer and a nonra-
dicactive 21-mer (SP1-SPI+6) was used as primer-template for DNA
polymerization. Kinetics of DNA synthesis was monitored by gel elec-
trophoresis of the products. Equimolar amounts of the Ad DNA poly-
erase were compared, 25 ng of the free Ad DNA polymerase and 37.5 ng
of the pTPpol complex. Control lane 1 shows the nonelongated SP1-
SPI+6. Arrows indicate the position of the 15-mer (nonelongated
 primer) and the position of the 21-mer (completely elongated primer).
The bands in lane 2 are somewhat less intense because of a small
loading difference.

small percentage (less than 0.1%) of pTP-pol participates in the
reaction.

**Dissociation Is Accompanied with Changes in the Catalytic
Properties and Inhibitor Sensitivity of the Polymerase—Previo-
ously we observed that the optimal conditions for formation of
pTP-dCMP differ considerably from those required during elon-
gation. The initiation reaction is insensitive to ddNTPs, aphidi-

high salt or high heparin concentrations disrupted the complex. We finally found conditions (low concentrations of heparin) under which the pTP\(^z\) pol complex remained intact while the contacts with DNA were disrupted.

We observed dissociation of the pTP\(^z\) pol complex after the synthesis of the pTP\(^z\) CAT intermediate. Approximately 60% of the pTP\(^z\) CAT product becomes dissociated. That dissociation is not yet complete might indicate that the events triggering dissociation take place gradually. One of the events occurring after the pTP\(^z\) CAT formation is the jumping back step. However, a link between dissociation and jumping back could not be established since the replication product formed by employing the \(\Delta3G7C\) template (on which no jumping back of pTP\(^z\) CAT is possible) showed the same distribution pattern as observed using the wild-type template. After elongation of the pTP\(^z\) CAT intermediate additional dissociation occurs and is (almost) complete when seven nucleotides have been synthesized.

We have strong indications that other events, besides jumping back, are taking place after pTP\(^z\) CAT formation. Before and after pTP\(^z\) CAT formation the catalytic properties of the DNA polymerase differ considerably as revealed by changes in the \(K_m\) for dCTP, dATP, and dTTP and by changes in inhibitor sensitivities (summarized in Table I). This indicates a change in the active site of Ad DNA polymerase after pTP\(^z\) CAT formation. Such a conformational change in the polymerase active site might also influence the pTP interaction domain of the

**FIG. 5.** The \(K_m\) values for dATP and dTTP during pTP\(^z\) CAT formation and during elongation are different. Panel A, partial elongation reactions were performed with either \([\alpha-^{32}\text{P}]\)dATP (left) or \([\alpha-^{32}\text{P}]\)dTTP (right) as described under “Experimental Procedures.” Reactions were incubated for 20 min at 30 °C. The dATP and dTTP concentrations ranged from 0.05 to 10 \(\mu\text{M}\). Panel B, Lineweaver-Burk plots. For calculation of the \(K_m\) values for pTP\(^z\) CAT and pTP\(^z\) 26 the data were corrected for the difference in the number of A and T residues. Similar values were obtained at other incubation times.
apparently determined by the template sequence rather than by the distance from the molecular end or the conserved pTP-pol binding site. Assuming that the binding of pTP-pol to the core origin is independent of the sequences near the molecular end, this suggests a certain amount of flexibility in locating a G residue of the template in the active site. Recent structural comparisons of a proofreading complex of the Kle
now fragment of Escherichia coli DNA polymerase I and the "polymerizing" complex of the Taq DNA polymerase show that DNA polymerases can make a sliding motion along the template, indicating considerable flexibility in protein-DNA contacts (30).

Using a variety of templates, the first residue coupled to pTP is always a dCMP, suggesting that this is an intrinsic property of Ad5 pol. Similar restrictions for a specific starting nucleotide are not found in other protein-priming DNA replication systems that use a similar initiation mechanism (3–5). Bacteriophages φ29 and Cp-1 seem to have a built-in property to start replication from the second and third template residues, respectively, irrespective of the nucleotide in this position (3, 5), indicating that the position rather than the nucleotide determines the start site. In the case of adenovirus, a limited amount of flexibility in the position seems to be permitted, probably dictated by the need for a G in the template. Employing the GA repeat template, the Ad5 DNA polymerase prefers to start from position G5 rather than G3. Given the fact that on the natural template the start is at G4, why would G5 be favored over G3? Most terminal sequences present in all adenovirus serotypes (31) allow the formation of a pTP-trinucleotide or pTP-tetranucleotide. The benefit of an initiation intermediate of three or four nucleotides long, compared with two, could be to enable proofreading because this requires the synthesis of at least three or four nucleotides to initiate, at least in E. coli pol I (32).

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TABLE I

Comparison of the various properties of the Ad DNA polymerase during initiation and elongation

| Ref. | Initiation | Elongation |
|------|------------|------------|
| dCTP (+ DBP) | 0.44 μM | 1.40 μM |
| dCTP (− DBP) | 3.7 μM | ND |
| dATP (− DBP) | 3.8 μM | 5.1 μM |
| dTTP (− DBP) | 1.0 μM | 5.5 μM |
| (S)-HPMPApp | Resistant (9% reduction at 50 μM) | Sensitive (50% reduction at 1.8 μM) |
| Aphidicolin | Resistant (up to 100 μM) | Sensitive (50% reduction at 8 μM) |
| ddNTPs | Resistant (up to 200 μM) | Sensitive (50% reduction at 14.2 μM) |

* DBP, DNA-binding protein; HPMPApp, (S)-g(3-hydroxy-2-phosphonylmethoxypropyladenine diphosphoryl.)

Figure 6. Formation of a pTP-tetranucleotide intermediate on a mutant template. Single-stranded template 30-mers containing the wild-type origin (GTAGTAGTT . . . ) or G to C transversions of the latter at positions 2, 4, 6, or 8 were used. Panel A, incubation of the GA repeat with one α-32P-labeled dNTP as indicated (lanes 1–4) or with α-32P-labeled dCTP, dATP, dTTP, and ddGTP (lane 6) allowing partial elongation. Lane 5 contains the wild-type origin. Panel B, partial elongation reactions on the indicated mutant templates, using one α-32P-labeled dNTP as indicated as well as the other unlabeled dNTPs at 40 μM except ddGTP, which was added at 0.7 μM.
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