The EMBO Journal vol.13 no.23 pp.5786–5792, 1994

A precursor terminal protein–trinucleotide intermediate during initiation of adenovirus DNA replication: regeneration of molecular ends *in vitro* by a jumping back mechanism

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Communicated by P.C.van der Vliet

The adenovirus type 5 origin sequence starts with 3′ GTAGTA. Initiation of replication occurs by a protein priming mechanism in which the viral precursor terminal protein (pTP) is covalently linked to the first nucleotide of the nascent chain, a dCMP residue. This suggests that a pTP–dCMP (pTP–C) complex functions as an initiation intermediate. Employing a reconstituted replication system and both synthetic oligonucleotides and the natural TP–DNA as templates, we show that pTP–CAT rather than pTP–C is an intermediate in initiation. By replicating oligonucleotide templates mutated at different positions and analyzing the product lengths, we observed that the GTA at positions 4–6, rather than 1–3, are used as a template for pTP–CAT formation. Moreover, deletions of one or two nucleotides at the molecular ends were regenerated upon *in vitro* replication. Our results support a model in which the pTP–CAT intermediate, synthesized opposite to positions 4–6, jumps back to position 1 of the template to start elongation. In order to permit elongation, some base pairing between pTP–CAT and template residues 1–3 is required. This jumping-back mechanism ensures the integrity of terminal sequences during replication of the linear genome.

Key words: adenovirus/DNA polymerase/DNA replication/terminal repeat/trinucleotide

Introduction

To initiate DNA replication all known DNA polymerases require a template and a free 3′ OH group, which acts as a primer. Such a primer is generally provided by short stretches of RNA bound to the template. However, linear DNA molecules can also make use of an OH group provided by an amino acid. In this initiation reaction the protein delivering the amino acid will become covalently linked to the DNA via a phosphodiester bond between the β-hydroxyl group of the amino acid and the α-phosphoryl group of a nucleotide. This process is called protein priming (reviewed in Salas, 1991).

Adenoviruses contain a linear double-stranded genome of 36 kbp with two origins of replication located in the inverted terminal repeats. The adenovirus (Ad) type 5 origin sequence starts with 3′ GTAGTA. The 5′ ends are covalently linked to the 55 kDa viral terminal protein (TP). The development of a soluble *in vitro* replication system (Challberg and Kelly, 1979) led to our current understanding of the mechanism of Ad5 DNA replication at the molecular level. The initiation reaction, in which the first nucleotide, a dCMP residue, is linked to serine 580 in the precursor of the terminal protein (pTP), is thoroughly characterized. It requires two viral proteins, the 80 kDa pTP and the 140 kDa Ad5 DNA polymerase (pol). In addition, two cellular transcription factors, NFI and Oct-1, have been shown to stimulate this reaction (Nagata *et al.*, 1982; Hay, 1985; Leegwater *et al.*, 1985; Pruijn *et al.*, 1986; Rosenfeld and Kelly, 1986; Rosenfeld *et al.*,1987; Verrijzer *et al.*, 1990). However, at high pTP–pol concentrations the stimulating effect of these factors is largely lost (Mul *et al.*, 1990). After the initiation reaction, the resulting complex serves as a primer for DNA synthesis via a strand displacement mechanism catalyzed by the same DNA polymerase and requiring the Ad DNA binding protein (DBP). Late in infection pTP is cleaved by a virus-encoded protease into TP and the precursor part (for reviews, see Challberg and Kelly, 1989; Hay and Russell, 1989; Stillman, 1989; van der Vliet *et al.*, 1991).

Initiation and elongation are performed by the same DNA polymerase, but differ in sensitivity to inhibitors. Both ddNTPs (Enomoto *et al.*, 1981; Nagata *et al.*, 1983) and HPMPApp (Mul *et al.*, 1989) inhibit elongation, but initiation is not affected by these nucleotide analogs. Moreover, initiation is resistant to aphidicolin, whereas elongation is partially sensitive (Nagata *et al.*, 1983; Sussenbach and van der Vliet, 1983). This suggests that structural changes occur in the polymerase when proceeding from initiation to elongation. In agreement with this notion, kinetic studies revealed that the K_m for dCTP is lower for initiation than for elongation (Mul and van der Vliet, 1993). As a consequence, at low dCTP concentrations part of the pTP does not participate in elongation, but remains trapped in an early intermediate form. Accumulation of this product indicates a barrier after initiation, despite the presence of all nucleoside triphosphates. We have suggested that this early intermediate represents pTP containing the first three nucleotides (pTP–CAT), rather than pTP–C (Mul and van der Vliet, 1993).

In the present study we characterized initiation intermediates using highly purified pTP–pol and both the natural TP–DNA and single-stranded oligonucleotides as templates which are also active in initiation (Tamanoi and Stillman, 1982; Guggenheimer *et al.*, 1984; Harris and Hay, 1988; Kenny and Hurwitz, 1988; Dobbs *et al.*, 1990). We show here that a pTP–CAT intermediate is indeed formed on both templates. This intermediate is formed by using the template residues 4–6. We propose that this intermediate jumps back to position 1 of the template in

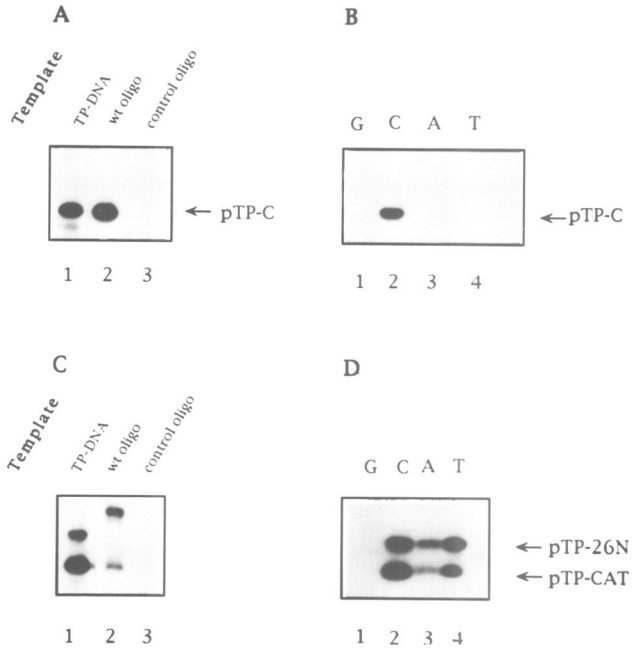

Fig. 1. Formation of a pTP–CAT intermediate during initiation. (**A**) Formation of the pTP–dCMP complex using different templates. Five hundred nanograms of TP–DNA (lane 1), 700 ng of a ss 30mer containing the Ad5 origin, designated as wt (lane 2) or 700 ng of a control ss 30mer containing an unrelated sequence (lane 3) were incubated with pTP–pol and [α-^{32}P]dCTP. Products were analyzed by SDS–PAGE (see Materials and methods for details). (**B**) Formation of the pTP–dNMP complex with each of the four [α-^{32}P]dNTPs. The wt oligonucleotide was mixed with pTP–pol in the presence of one of the [α-^{32}P]dNTPs as indicated (G, C, A or T). (**C**) Truncated replication assay. The same amounts of templates as in (A) were mixed with pTP–pol in the presence of [α-^{32}P]dCTP, dATP, dTTP and ddGTP. (**D**) Truncated replication assay using TP–DNA as template. The template was mixed with one [α-^{32}P]dNTP and the other unlabeled dNTPs; ddGTP was added instead of dGTP.

order to proceed to elongation. This model has many similarities with the sliding back models proposed for ϕ29 and PRD1 DNA replication (respectively Mendez *et al.*, 1992; Caldentey *et al.*, 1993), lending credence to the suggestion that this is a universal mechanism for protein primed DNA replication which enables correction of deletions and mutations at the molecular ends.

Results

Formation of a pTP–CAT intermediate during initiation

When the purified pTP–pol complex was mixed with the natural TP–DNA template and [α-^{32}P]dCTP was added as the only nucleotide, a labeled pTP–dCMP complex was observed on SDS–polyacrylamide gels (Figure 1A, lane 1). The same result can be obtained in the presence of a single-stranded oligonucleotide representing the template strand of Ad5 (nucleotides 1–30), but to obtain the same level of pTP–dCMP complex much higher template concentrations are required (Figure 1A, lane 2). Nevertheless, this labeling is specific, since a control oligonucleotide, added at the same concentration but lacking the origin sequence, gave a 20-fold lower signal (Figure 1A, lane 3), comparable with the level obtained without template (not shown). These results are in agreement with previous data (Harris and Hay, 1988; Dobbs *et al.*, 1990).

Formation of this complex is specific for dCTP. Upon replacement of dCTP by any of the other labeled dNTPs no complex could be observed (Figure 1B, lanes 1–4). Even the weak signal (20-fold lower) obtained without template was specific for dCTP (not shown). Thus, pTP can only accommodate dCTP as the first nucleotide during initiation. When Mn^{2+} was used instead of the standardly used Mg^{2+}, all four nucleotides could be linked to pTP equally well in the absence of a template, whereas in the presence of single-stranded template the preference for dCMP was much weaker and in particular incorporation of dAMP and dTMP was observed at up to 20% of the level obtained with dCMP (results not shown). Thus, Mn^{2+} weakens the specific nucleotide selection process of the Ad polymerase, although it can be used efficiently for initiation (Pronk *et al.*, 1994)

When all four nucleotides are added, elongation starts. To study elongation we added ddGTP, which blocks elongation at position 26, the first C residue in the template strand. Under these conditions and using the natural TP–DNA as template a pTP–26 nt partial elongation product is formed which migrates as a 90 kDa band in SDS–polyacrylamide gels (Figure 1C, lane 1). An elongation product is also formed when the single-stranded template is used, but this band has a lower mobility (Figure 1C, lane 2). Analysis of this product on a sequence gel after removal of the pTP showed a 26 nt band, as with the natural template, indicating that the lower mobility is due to renaturation of the product with the excess template strand.

In addition to the partial elongation products, a band is seen which almost coincides with the pTP–dCMP band. This is caused by the low dCTP concentration, which leads to accumulation of an intermediate, since the K_m for dCTP for initiation is lower than for elongation. If higher dCTP concentrations are used this product is rapidly converted into the pTP–26 elongation product, indicating that it is a precursor to elongation (Mul and van der Vliet, 1993). Since both dATP and dTTP are present, this product may be composed of pTP bound to the first three nucleotides, pTP–CAT rather then pTP–C. To investigate this we used all four α-^{32}P-labeled dNTPs and studied incorporation in both bands (Figure 1D lanes 1–4). Along with [α-^{32}P]dCTP, labeling of the lower band was also observed with [α-^{32}P]dATP and [α-^{32}P]dTTP, albeit somewhat less than with dCTP, showing that the intermediate product contains C, A and T. We assume that it represents pTP–CAT, since the mobility of the band closely resembles the mobility of pTP–C. The difference in molecular mass between pTP–C and pTP–CAT is only 0.6 kDa, or 0.7%, which will not be separable on this gel. Larger complexes such as pTP–CATCAT would have been detectable as a shift in mobility. Other experiments described below (Figure 3B) confirm that the product represents pTP–CAT and not pTP–CATCAT.

The pTP–CAT intermediate is formed at residues 4–6 in the template

The template sequence 3′ GTAGTAGTTA... contains three positions (G1, G4 and G7) which can be used to start formation of a pTP–dCMP complex and two positions

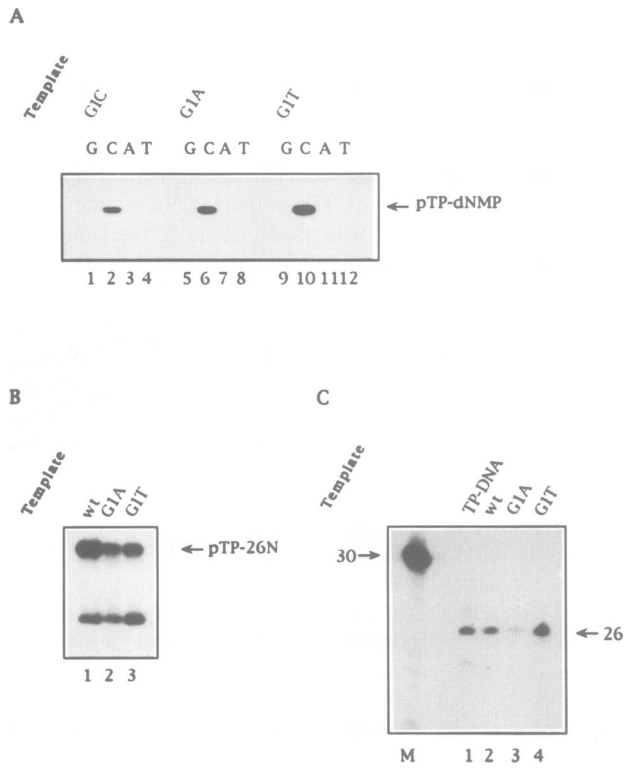

Fig. 2. Templates mutated at position 1 are fully competent for replication. (**A**) Formation of the pTP−dNMP complex with each of the four [α-^{32}P]dNTPs using templates mutated at G1. Templates were mixed with pTP−pol and one of the [α-^{32}P]dNTP as indicated. (**B**) Truncated replication assay. Various templates were mixed with pTP−pol and [α-^{32}P]dCTP, dNTPs and ddGTP. (**C**) Determination of the length of partial elongation products from TP−DNA, wt and G1 mutated oligonucleotide templates. Partial elongation products were formed and separated as in (B). The oligonucleotide products were isolated and analyzed on a 15% denaturing polyacrylamide gel. M, marker of 30 nt.

(G1 and G4) to start pTP−CAT. Indeed, early experiments employing TP-free DNA templates indicated that internal G residues could be used under certain conditions such as low template concentrations (van Bergen *et al.*, 1983), since in those cases a product of 23 rather than 26 nt was produced when ddGTP was added. To investigate the actual start position we mutated the G at position 1 to C, A or T. This did not change the amount of pTP−dCMP formation when [α-^{32}P]dCTP was added as the only nucleotide. Like with the wild-type origin, none of the other dNTPs could substitute for dCTP (Figure 2A), confirming the preference for incorporation of dCMP even when the first nucleotide is mutated. The same result was obtained when G4 or G7 were mutated. When all three G residues were mutated incorporation dropped to a level comparable with that in the absence of template (not shown). Furthermore, Dobbs *et al.* (1990) have shown that mutation of G1 and G4 greatly decreases the incorporation of dCTP. This indicates that a G residue is essential for the efficient formation of the pTP−dCMP complex, but the position of this G-residue is less important and, in particular, G1 and G4 can be used for pTP−dCMP formation.

A different pattern was observed when partial elongation was studied employing [α-^{32}P]dCTP and the other unlabeled dNTPs. The mutants G1A and G1T could be elongated almost as efficiently as the wild-type (Figure 2B, lanes 2 and 3). Since efficient initiation requires a G residue, the formation of pTP−CAT must have taken place opposite G4 or G7 in the case of the G1 mutants (G1A and G1T). If this pTP−CAT intermediate is subsequently elongated, this will lead to a partial elongation product of 23 or 20 nt respectively. However, when we analyzed the products on a sequence gel after the removal of the pTP we observed, surprisingly, that both products were the same length as TP−DNA or wt, i.e. 26 nucleotides (Figure 2C). We explain these results by assuming that a pTP−CAT intermediate is formed from bases 4–6, which subsequently jumps back to position 1 to start elongation, leading to the observed length of 26 nucleotides. We consider a start at G7 less likely, since this would lead to a pTP−CAAT intermediate which, when jumping back to GTAG at position 1–4, would lead to mismatches.

To further confirm that the start occurs opposite G4 we used a template in which the A in position 6 was replaced by a C (GTAGTC). This mutant behaved normally when only one dNTP was offered and only incorporated dCTP (Figure 3A, lanes 1–4). However, when elongation was permitted, another pattern was observed. The initiation band now contained C, A and G rather than C, A and T (Figure 3B, lanes 1–4). This indicates a pTP−CAG intermediate, which could only have been formed with nucleotides 4–6 as template. Since there was no incorporation of dTTP in the intermediate, it also indicates that the intermediate contains a trinucleotide and not a hexanucleotide (pTP−CATCAG). In this particular experiment the elongation products obtained with [^{32}P]dCTP and [^{32}P]dATP were rather weak, but they could be observed after a longer exposure of the film. The elongation products are weak anyhow, presumably because of mismatches occurring when the pTP−CAG intermediate jumps back, leading to instability. This may also explain the double band seen in lanes 1 and 4 of Figure 3B, indicating that elongation also occurs without translocation.

When, in a similar experiment, the template A3C was used, pTP−CAT was formed, but not pTP−CAG (not shown), whereas elongation was similar to that in Figure 3B. Furthermore, when the template G4A was similarly tested, we found only formation of a pTP−C complex. No labeling of the intermediate complex was found with labeled dATP or dTTP and elongation was blocked (Figure 3C, lanes 1–4). This suggests that pTP−CAT synthesis can only occur when G4 is used as the complementary base and that formation of this intermediate is required for elongation.

A deletion of one or more nucleotides at the origin can be regenerated in vitro

In the jumping back mechanism proposed here the role of the first three nucleotides in the template is just to accept the pTP−CAT intermediate through base pairing. To investigate the extent of base pairing that is required and to see if position 1 can nevertheless be used to start initiation, we deleted one, two or three nucleotides at the 3′ end and repeated the experiment by allowing partial elongation and assaying the length of the product. In the case of start at position 1 a shortened product would be produced, 25 nt for Δ1, 24 nt for Δ2 and 23 nt for Δ3.

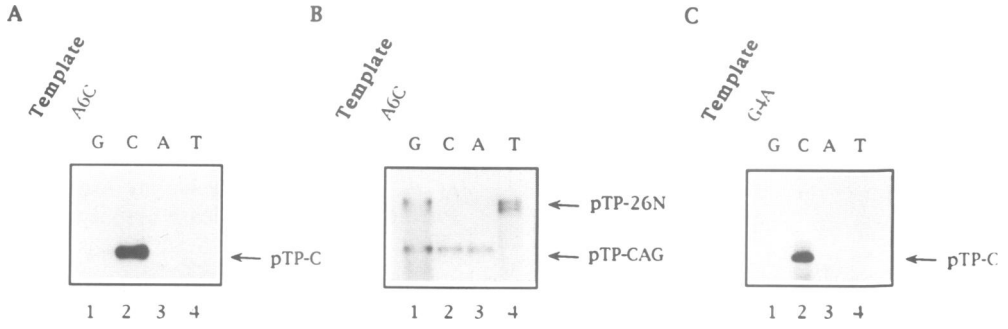

Fig. 3. The pTP–CAT intermediate is formed at template residues 4–6. (**A**) Formation of the pTP–dNMP complex using a template mutated at position 6 (A6C) and one of the [α-^{32}P]dNTPs, as indicated. (**B**) Truncated replication assay performed on the A6C template. (**C**) Similar experiment to that in (B) using a template mutated at position 4 to A (G4A).

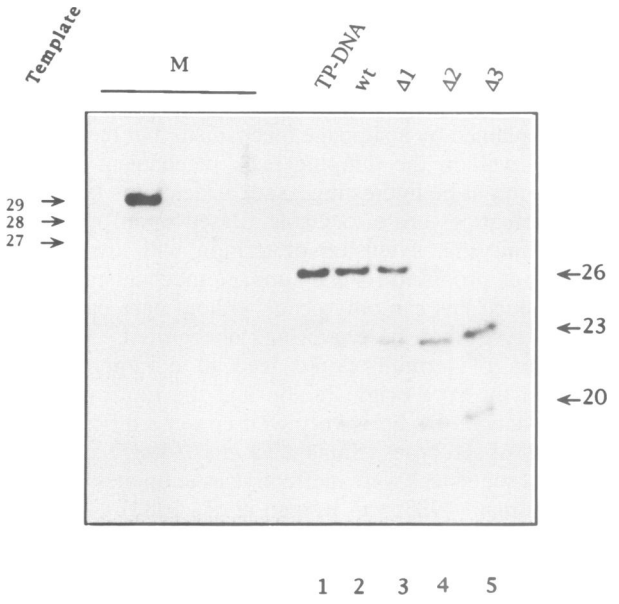

Fig. 4. Regeneration of 3′ ends. Partial elongation products were formed as described under Materials and methods from various templates, including templates with deletions at the 3′ end (Δ1, Δ2 and Δ3). The oligonucleotide products were isolated and analyzed on a 15% denaturing polyacrylamide gel. Markers are as indicated. The markers of 28 and 27 nt length are weak in this picture, but were easily seen upon longer exposure of the gel.

However, analysis of the replication products (Figure 4) showed that a 26 nt product was produced with Δ1 and to a lesser extent Δ2. The Δ3 template generated only 23 and 20 nt products (the latter resulting from start at the less favored start site G7). This suggests that the pTP–CAT intermediate formed at nucleotides 4–6 can only jump back and continue elongation when some base pairing is still possible, with two nucleotides (Δ1) or one nucleotide (Δ2). Without base pairing (Δ3) no restoration of the first nucleotides is possible and the pTP–CAT intermediate proceeds to elongation without jumping back.

Discussion

Based upon an analysis of truncated elongation products and a mutational study, we propose a model for initiation of adenovirus DNA replication as depicted in Figure 5. The G residue at position 4 in the template directs the coupling of a dCMP to pTP, followed by incorporation of A and T, also template-directed, leading to a pTP–CAT intermediate. This reaction requires a conformation of the DNA polymerase which is characterized by resistance to several inhibitors of DNA replication and by a high affinity for dCTP. Probably, after the formation of the pTP–CAT intermediate the conformation of the polymerase active site changes to elongation mode, characterized by a lower affinity for dCTP and an increase in sensitivity to ddNTPs, HPMPApp and aphidicolin. This step may include dissociation of pTP from pol, but this remains to be tested. In a subsequent step, the pTP–CAT intermediate translocates via a jump to permit base pairing with the first three nucleotides of the template. This has already been suggested by Mendez *et al.* (1992) and Caldentey *et al.* (1993), based upon our previous experiments (Mul *et al.*, 1993).

Sliding versus jumping

A sliding back model was first proposed by Mendez *et al.* (1992) for bacteriophage ϕ29, which also initiates by a protein priming mechanism, and was later also suggested for bacteriophage PRD1 (Caldentey *et al.*, 1993). For ϕ29, the second nucleotide directs synthesis of TP–dAMP and for PRD1, the fourth consecutive C residue in the template is responsible for synthesis of TP–dGMP. In both cases the main evidence for such an internal start came from studies in which mutation of these template residues led to a different dNMP being coupled to TP in a template-directed fashion. Our attempts to use a similar approach for adenovirus were unsuccessful, since the Ad polymerase could only link dCMP to pTP, at least in the presence of Mg^{2+} ions. Therefore mutation of G4 still yields pTP–dCMP, presumably directed by one of the other G residues, since mutating all G residues was detrimental to initiation, in agreement with previous results (Dobbs *et al.*, 1990). This indicates that some flexibility must exist in the position of the bound template when single-stranded DNA is used. However, when TP–DNA was used, the pTP–CAT intermediate was formed in agreement with a start at position 4. Presumably the requirement for G4 is more stringent in this case, since TP increases the affinity of pTP–pol for the origin (Pronk and van der Vliet, 1993) and may help to position the polymerase, similarly to NFI (Bosher *et al.*, 1990; Chen *et al.*, 1990; Mul and van der Vliet, 1992) and the Oct-1 POU domain (Coenjaerts *et al.*, 1994). However, this could not be tested directly, since

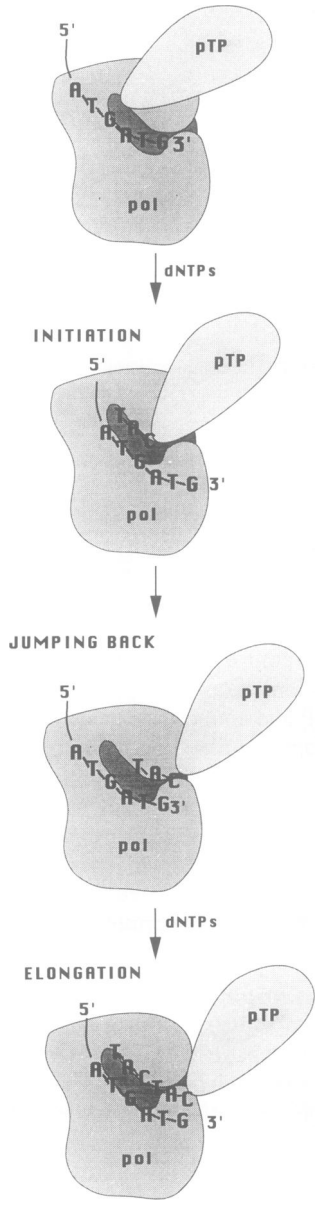

Fig. 5. Jumping back model for transition from initiation to elongation. Protein primed initiation occurs opposite to the fourth template residue. From this position a pTP−CAT intermediate is formed. After the formation of this product a conformational change presumably occurs in the complex and the pTP−CAT jumps back to be positioned opposite the first three nucleotides at the 3′ end, after which elongation can start. Ad polymerase is modeled based upon the three-dimensional structure for the Klenow fragment (Beese *et al.*, 1993). The structure of pTP is arbitrary.

mutated TP-containing templates cannot be prepared easily.

The sliding back model for ϕ29 and PRD1 was suggested based upon a repeat of two T residues (ϕ29) or four C residues (PRD1) which enable continuous base pairing of the TP−dNMP complex during translocation. Our results differ in that the intermediate contains a trinucleotide rather than just one dNMP. This makes it necessary to propose jumping rather than sliding, to prevent mismatches occurring during a sliding back process. Despite this slight difference, the similarities between the three systems are remarkable and indicate a universal advantage for such a mechanism, which may be instrumental in correcting errors during initiation (Mendez *et al.*, 1992). In this respect it is interesting that the protein primed initiation reaction in ϕ29 is quite inaccurate compared with elongation. The 3′→5′ exonuclease activity of ϕ29 DNA polymerase cannot act on the initiation complex (Esteban *et al.*, 1993). Presently we are testing if this is also true for the 3′→5′ exonuclease activity present in the Ad pol (Field *et al.*, 1984).

Regeneration of terminal deletions

We show here that deletion of one or two nucleotides can be repaired, while longer deletions cannot, although the information to regenerate a three nucleotide deletion is present in the pTP−CAT intermediate. Apparently, base pairing with at least one nucleotide is required to stabilize pTP−CAT after jumping back to the terminus. Interestingly, regeneration of two missing bases has been observed *in vivo* as well. A plasmid lacking two base pairs at one end and 11 base pairs at the other end produces intact termini after transfection (Graham *et al.*, 1989). This has been explained by a slippage mechanism, but these results fit very well in the jumping back mechanism proposed here. It might be interesting to see if deletions larger than two nucleotides are also viable. Based upon our results, we assume that extension of termini with trinucleotide repeats, as proposed for the slippage mechanism, are less likely, since this can only occur without base pairing and we observe that base pairing is required. Moreover, extension of termini would lead to a larger distance between the core origin region and the molecular ends. This distance of 8 bp is very well conserved between the various Ad serotypes (Shinagawa *et al.*, 1987) and longer terminal sequences were ineffective as templates (Tamanoi and Stillman, 1983; van Bergen *et al.*, 1983).

With one exception, all Ad serotypes contain terminal repetitions of two (CT), three (CAT) or four (CTAT) nucleotides (Shinagawa *et al.*, 1987). Thus, if the other serotypes also employ a jumping back mechanism, differences must exist in the starting position and intermediates of pTP−CT or pTP−CTAT should be formed. This will probably depend on subtle differences in polymerases and precursor terminal proteins and/or the use of different mechanism to enhance replication through auxiliary sequences. This may lead to a different positioning of polymerase and points to an inherent flexibility in the initiation system, in which the presence of a repeat is apparently more important than the actual length or base sequence.

In conclusion, adenoviruses have developed several mechanisms to preserve the integrity of their linear genome. In addition to terminal proteins protecting the 5′-termini against nucleases and inhibitory end-binding proteins, such as the Ku protein (De Vries *et al.*, 1989), the inverted terminal repeats safeguard mutations in one of the termini through panhandle formation. Initiation at position 4 and jumping back of the pTP−CAT intermediate provide a further level of control, enabling restoration of small terminal deletions and mutations.

Materials and methods

Proteins, nucleotides and DNA templates

The complex of the precursor terminal protein and the adenovirus DNA polymerase (pTP−pol) was purified from recombinant vaccinia virus-

infected cells. HeLa cells (6 l) were co-infected with three recombinant vaccinia viruses encoding pTP, Adpol and T7 RNA polymerase (Nakano *et al.*, 1991). Extracts were made and the pTP–pol complex was purified essentially as described by Mul *et al.* (1989). The extracts containing 300 mM KCl were adjusted to 100 mM KCl with buffer A (25 mM 2[*N*]-morpholinoethane sulfonic acid, pH 6.2, 1 mM dithiothreitol, 1 mM EDTA, 0.02% Nonidet P-40, 0.1 mM phenylmethylsulfonylfluoride, 20% glycerol) and were applied to a 50 ml DEAE column. After washing with 100 ml buffer A, 100 mM KCl, the column was developed with a linear gradient ranging from 100 to 500 mM KCl in buffer A. The column fractions were screened for pTP–pol activity using an *in vitro* replication assay (Mul *et al.*, 1989). The pTP–pol-containing fractions were pooled and applied to a 8 ml Mono S column run at 0.5 ml/min and developed with a linear salt gradient ranging from 100 to 600 mM KCl in buffer A. Subsequently the pTP–pol-containing fractions were loaded on a 16 ml ss-DNA column which was developed with a linear gradient ranging from 150 to 600 mM of KCl in buffer A. Finally, the active fractions were loaded on a 1 ml Mono S column run at 0.5 ml/min and developed with a linear salt gradient ranging from 100 to 600 mM KCl in buffer A. Protein concentrations were determined using the Bio-Rad dye reagent with BSA as a standard. The total yield was ~4 mg purified protein, with a specific activity of 1760 U/mg (for unit definition see Mul *et al.*, 1990). As determined by SDS–PAGE followed by silver-staining, the estimated purity was 90%. Furthermore, all protein preparations were tested for the presence of nucleases by monitoring the breakdown of a 5′-labeled oligonucleotide, with negative results.

Adenovirus type 5 TP-DNA was isolated as described previously (Coenjaerts and van der Vliet, 1994). Unlabeled deoxynucleotides and dideoxynucleotides were purchased from Pharmacia. The four [α-^{32}P]dNTPs were from Amersham International. Single-stranded oligonucleotides were synthesized in a Pharmacia LKB Gene Assembler Plus. The sequence of the wild-type 30mer containing the template strand of the Ad5 origin is 3′-GTAGTAGTTATTATATGGAATAAAACCTAA-5′. The sequence of the control template with an 'unrelated' sequence is 3′-CGGCGCGACTACGGTACCGCG-5′.

Initiation complex formation.

The standard incubation mixture (25 µl) contained 1 µg pTP–pol and 700 ng ss-oligonucleotide or 500 ng Ad5 TP–DNA as template in a buffer containing 20 mM HEPES, pH 7.5, 1 mM dithiotreitol (DTT), 1 mM $MgCl_2$, 1 µg BSA and KCl to a final concentration of 55 mM. Where indicated, 1 mM $MnCl_2$ was added instead of 1 mM $MgCl_2$. The initiation reaction was allowed to proceed for 1 h at 30°C in the presence of 0.75 µM of one of the [α-^{32}P]dNTP (5 µCi). The reaction was stopped by addition of 10 µl 0.25 M sodium pyrophosphate and 2 µl 0.2 M EDTA. Reaction products were precipitated with 5 µl trichloroacetic acid (TCA; 20% final concentration) for 20–30 min at 0°C. The precipitate was spun down by centrifugation for 15 min at 12 000 r.p.m. 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% bromophenol blue). Samples were heated for 5 min at 100°C and separated by electrophoresis in 7.5% polyacrylamide–SDS gels and detected by autoradiography.

Analysis of truncated replication products

Initiation coupled to partial elongation was performed under similar conditions as initiation, except for the added dNTPs. One of the four dNTPs was labeled with α-^{32}P and added at a concentration of 0.75 µM. The other dNTPs were unlabeled and present at 40 µM, except dCTP, which was always present at 0.75 µM, and dGTP, which was substituted by 30 µM ddGTP. This results in an elongation block after position 26, the first cytidine residue in the template. After incubation at 30°C, reactions were stopped and products were separated by SDS–PAGE as described for the initiation assay. When indicated, the elongation products were cut out of the wet gel and recovered by electroelution in a buffer containing 25 mM Tris, 0.2 M glycine and 0.1% SDS in the presence of 100 µg/ml BSA. The DNA–protein complex was precipitated with 20% TCA, pellets were washed with a 15% TCA solution and dissolved in 0.2 N NaOH, followed by an incubation for 2 h at 37°C to hydrolyze the serine–dCMP phosphodiester linkage. After neutralization the oligonucleotides were extracted with a mixture of phenol:chloroform:isoamylalcohol (25:24:1). To the remaining phenol layer was added 50 µl 0.2 N NaOH to completely extract the oligonucleotide. The DNA was precipitated by adding 0.1 volumes of 3 M sodium acetate, pH 7.0, 0.6 volumes of isopropanol and 5 µg glycogen. The precipitates were dissolved in a sample buffer containing 50% formamide and were analyzed on a 15% polyacrylamide gel containing 7 M urea.

Acknowledgements

We are grateful to W.T.Teertstra for purified pTP–pol and to Dr R.Padmanabhan for the gift of the two recombinant vaccinia viruses encoding pTP (vvRN-pTP) and pol (vvRN-pol). We thank Dr B.Moss for providing v-TF7-3. Furthermore, we thank H.Th.M.Timmers and J.Dekker for critical reading of the manuscript. This work was supported in part by the Netherlands Foundation for Chemical Research (SON), with financial support from the Netherlands Organization for Scientific Research (NWO).

References

Beese,L.S., Derbyshire,V. and Steitz,T.A. (1993) *Science*, **260**, 352–355.
Bosher,J., Robinson,E.C. and Hay,R.T. (1990) *New Biologist*, **2**, 1083–1090.
Coenjaerts,F.E.J. and van der Vliet,P.C. (1994) *Methods Enzymol.*, in press.
Coenjaerts,F.E.J., van Oosterhout,J.A.W.M. and van der Vliet,P.C. (1994) *EMBO J.*, **13**, 5401–5409.
Caldentey,J., Blanco,L., Bamford,D.H. and Salas,M. (1993) *Nucleic Acids Res.*, **21**, 3725–3730.
Challberg,M.D. and Kelly,T.J.,Jr (1979) *Proc. Natl Acad. Sci. USA*, **76**, 655–659.
Challberg,M.D. and Kelly,T.J.,Jr (1989) *Annu. Rev. Biochem.*, **58**, 671–717.
Chen,M., Mermod,N. and Horwitz,M.S. (1990) *J. Biol. Chem.*, **265**, 18634–18642.
De Vries,E., van Driel,W., Bergsma,W.G., Arnberg,A.C. and van der Vliet,P.C. (1989) *J. Mol. Biol.*, **208**, 56–78.
Dobbs,L., Zhao,L.-J., Sripad,G. and Padmanabhan,R. (1990) *Virology*, **178**, 43–51.
Enomoto,T., Lichy,J.H., Ikeda,J.-E. and Hurwitz,J. (1981) *Proc. Natl Acad. Sci. USA.*, **78**, 6779–6783.
Esteban,J.A., Salas,M. and Blanco,L. (1993) *J. Biol. Chem.*, **268**, 2719–2726.
Field,J., Gronostajski,R.M. and Hurwitz,J. (1984) *J. Biol. Chem.*, **259**, 9487–9495.
Graham,F.L., Rudy,J. and Brinkley,P. (1989) *EMBO J.*, **8**, 2077–2085.
Guggenheimer,R.A., Stillman,B.W., Nagata,K., Tamanoi,F. and Hurwitz,J. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 3069–3073.
Harris,M.P.G. and Hay,R.T. (1988) *J. Mol. Biol.*, **201**, 57–67.
Hay,R.T. (1985) *J. Mol. Biol.*, **186**, 129–136.
Hay,R.T. and Russell,W.C. (1989) *Biochem. J.*, **258**, 3–16.
Kenny,M.K. and Hurwitz,J. (1988) *J. Biol. Chem.*, **263**, 9809–9817.
Leegwater,P.A.J., van Driel,W. and van der Vliet,P.C. (1985) *EMBO J.*, **4**, 1515–1521.
Mendez,J., Blanco,L., Estaban,J.A., Bernad,B. and Salas,M., (1992) *Proc. Natl Acad. Sci. USA*, **89**, 9579–9583.
Mul,Y.M. and van der Vliet,P.C. (1992) *EMBO J.*, **11**, 751–760.
Mul,Y.M and van der Vliet,P.C. (1993) *Nucleic Acids Res.*, **21**, 641–647.
Mul,Y.M., van Miltenburg,R.T., De Clercq,E. and van der Vliet,P.C. (1989) *Nucleic Acids Res.*, **17**, 8917–8929.
Mul,Y.M., Verrijzer,P.C. and van der Vliet,P.C. (1990) *J. Virol.*, **64**, 5510–5518.
Nagata,K., Guggenheimer,R.A., Enomoto,T., Lichy,J.H. and Hurwitz,J. (1982) *Proc. Natl Acad. Sci. USA*, **79**, 6438–6442.
Nagata,K., Guggenheimer,R.A. and Hurwitz,J. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 4266–4270.
Nakano,R., Zhao,L.-J. and Padmanabhan,R. (1991) *Gene*, **105**, 173–178.
Pronk,R. and van der Vliet,P.C. (1993) *Nucleic Acids Res.*, **21**, 2293–2300.
Pronk,R., van Driel,W. and van der Vliet,P.C. (1994) *FEBS Lett.*, **337**, 33–38.
Pruijn,G.J.M., van Driel,W. and van der Vliet,P.C. (1986) *Nature*, **322**, 656–659.
Rosenfeld,P.J. and Kelly,T.J. (1986) *J . Biol. Chem.*, **261**, 1398–1408.
Rosenfeld,P.J., O'Neill,E.A.,Wides,R.J. and Kelly,T.J. (1987) *Mol. Cell. Biol.*, **7**, 875–886.
Salas,M. (1991) *Annu. Rev. Biochem.*, **60**, 39–71
Shinagawa,M., Iida,Y., Matsuda,A.,Tsukiyama,T. and Sato,G. (1987) *Gene*, **55**, 85–93.
Stillman,B.W. (1989) *Annu. Rev. Cell Biol.*, **5**, 197–245.
Sussenbach,J.S. and van der Vliet,P.C. (1983) *Curr. Topics Microbiol. Immunol.*, **109**, 53–73.
Tamanoi,F. and Stillman,B.W. (1982) *Cold Spring Harbor Symp. Quant. Biol.*, **47**, 741–750.

Tamanoi,F. and Stillman,B.W. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 6446–6450.
van Bergen,B.G.M., van der Ley,P.A., van Driel,W., van Mansfeld,A.D.M. and van der Vliet,P.C. (1983) *Nucleic Acids Res.*, **7**, 1975–1989.
van der Vliet,P.C. (1991) *Semin. Virol.*, **2**, 271–280.
Verrijzer,C.P., Kal,A.J. and van der Vliet,P.C. (1990) *EMBO J.*, **9**, 1883–1888.

Received on July 21, 1994; revised on September 6, 1994