The adenovirus terminal protein influences binding of replication proteins and changes the origin structure

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ABSTRACT

The adenovirus terminal protein (TP) is covalently linked to the 5' ends of the adenovirus genome and enhances DNA replication *in vitro* by increasing template activity. To study the effect of TP in more detail we isolated short origin fragments containing functional TP using anion exchange chromatography. These fragments were highly active as templates for DNA replication in a reconstituted system. Employing band-shift assays we found that the affinity of the precursor terminal protein-DNA polymerase complex for the TP-containing origin was increased 2 to 3-fold. Binding affinities of two other replication stimulating proteins, NFI and Oct-1, were not influenced by the terminal protein. Upon DNaseI footprinting we observed, unexpectedly, that the breakdown pattern had changed at various positions in the origin, notably in the area 3–6 and 41–51 by the presence of TP. Some differences in the footprint pattern of NFI and Oct-1 were also found. Our results indicate that TP induces subtle changes in the origin structure that influence the interaction of other replication proteins.

INTRODUCTION

Several DNA and RNA viruses contain a protein covalently bound to the ends of their genome. Examples include the human adenovirus, bacteriophages ϕ U29, PRD1 and HB-3, hepadnavirus, and several RNA viruses including poliovirus, foot and mouth disease virus and arabis mosaic virus (reviewed in reference 1). In general the function of these proteins is not well understood. They may protect the DNA or RNA *in vivo* from exonucleolytic digestion or from other end-binding proteins that inhibit replication. Other proposed functions include nuclear matrix attachment (2), enhanced integration (3) or enhancement of replication (1). The presence of the genome bound VPg protein in RNA viruses is most probably correlated with a role in initiation of RNA replication (for review see reference 1 and references cited therein), whereas the protein attached to hepadnavirus is generally believed to serve as a primer for minus-strand DNA reverse transcription (4).

The presence of a protein covalently attached to adenovirus DNA isolated from virions was first demonstrated by Robinson and Bellett using electron microscopy of Ad2 DNA; this protein was later designated terminal protein (TP) (5). Subsequent biochemical studies identified TP as a viral 55 kDa protein linked to the 5' dCMP residue through a phosphodiester bond with serine residue 580. Analysis of other serotypes showed that the protein is highly conserved indicating an essential function in the viral lifecycle (6,7). TP is synthesized in infected cells as a 80 kDa precursor (pTP) which binds strongly to the viral DNA polymerase (pol), thereby forming a pTP-pol heterodimer and is also important for nuclear transport of the polymerase (8). Initiation of adenovirus DNA replication occurs by protein-priming whereby pTP functions as a primer and thus becomes covalently bound to the first nucleotide of the nascent strand, a dCMP molecule. The 3'OH group in this pTP-dCMP initiation complex serves as a primer for further elongation. For optimal initiation three other proteins are required, the viral DBP and two cellular transcription factors, NFI and Oct-1 (for reviews, see references 9–12). Late in infection pTP-DNA is cleaved by a viral protease into TP-DNA (13).

Several functions have been proposed for the parental TP. *In vivo*, it is involved in attachment of the DNA to the nuclear matrix. This attachment is important for efficient transcription of adenovirus DNA and possibly also for DNA replication (2,14). Furthermore, the presence of TP renders the viral DNA inaccessible to 5' exonucleases (15) and also prevents binding of end-recognizing proteins such as the NFIV/Ku protein which inhibit DNA replication when a plasmid devoid of TP is used as a template (16). *In vitro*, the presence of TP was shown to have at least two functions. It considerably increases the template activity compared to protein-free DNA and it prevents the use of internal G residues at positions 4 and 7 as start points for initiation. pTP-containing templates are as efficient templates as TP-containing templates both *in vivo*, as shown by the use of templates derived from virions defective in the viral protease (13), and *in vitro* (17,18).

In order to study the functions of the (precursor) terminal protein a number of mutants have been constructed and tested for their effects on DNA replication and viability. These studies

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have indicated that the region around the proteolytic cleavage site is relatively resistant to insertions, but that most other mutations, both in the precursor moiety and in the TP moiety are detrimental for viability and *in vitro* DNA replication (19–21). N-terminal deletions of two to six amino acids render pTP gradually inactive in *in vitro* initiation and DNA replication assays, whereas replacement mutations of Ser⁵⁸⁰, to which DNA binds, completely abolish pTP function (22). However, further identification of specific functional domains in pTP has not yet been achieved. Using mutagenesis it will be even more difficult to analyze specific functions of the parental TP since most mutations will primarily have an effect on the function of pTP as an initiation protein, thus preventing further analysis of the role of the parental TP.

Biochemical studies of the role of TP in DNA replication have been hampered by the inability to couple pTP or TP to DNA fragments in the absence of DNA replication and by the difficulty to purify pTP-DNA after DNA replication. Moreover, technical problems are connected with the handling of covalent protein-DNA complexes since it is not possible to use common nucleic acid handling techniques such as phenol extraction and ethanol precipitation. In order to circumvent these problems we have isolated a short terminal fragment containing functional TP, which allowed us to study the effect of TP on origin structure and binding of initiation proteins to the native adenovirus origin.

MATERIALS AND METHODS

Purification of pTP-pol and other replication proteins

The precursor terminal protein and DNA polymerase were overexpressed in HeLa cells using a vaccinia virus expression system (23). The purification of the pTP-pol complex obtained from doubly infected HeLa cells was as described previously (24). The final preparation was more than 90% pure as judged by silver staining. One unit (U) is defined as the amount of pTP-pol that incorporates 20 pmol of α -³²P-dCTP (5.5 Ci/mmol) into adenovirus terminal fragments under standard replication conditions using 20 ng XhoI-digested Ad5 DNA as a template and equals approximately 1 μ g of pTP-pol (25).

The replication competent DNA binding domain of NFI (NFI-BD, amino acids 1–243) and the Oct-1 POU domain (amino acids 269–440) were purified to homogeneity from recombinant vaccinia virus infected HeLa cells as described (25). A binding unit (bu) is defined as the amount of protein required for 50% binding in a band-shift assay of a radioactive probe (0.1 ng) containing one binding site and corresponds to approximately 1.4 ng for NFI-BD and 1 ng for the Oct-1 POU domain (25).

DBP was purified from Ad5 infected HeLa cells as described previously (26).

Isolation and labeling of DNA fragments containing the terminal protein

Intact Ad5 DNA-TP complex was purified from virion particles (27,28). 60 μ g Ad5 DNA-TP was digested with SspI giving two TP-containing DNA fragments of 339 and 1303 basepairs, respectively, in addition to internal fragments of 19034, 10797, 2298, 1211, 682 and 271 basepairs. The digest was loaded onto a 1 ml mono-Q FPLC column (Pharmacia) equilibrated with 25 mM Tris-HCl [pH 7.6], 1 mM EDTA, 1 mM DTT, 0.1 mM phenyl methylsulfonyl fluoride, 1 μ g/ml tosyl-L-phenylalanine chloromethyl ketone (buffer A) containing 300 mM NaCl and

eluted with a 17 ml gradient from 300 mM to 600 mM NaCl, followed by a 65 ml gradient from 600 to 720 mM NaCl and a steep gradient from 720 to 1500 mM NaCl in buffer A over a 6 hr period at 4°C. Fractions of 1.5 ml were collected and screened for the presence of DNA by digestion with MspI followed by end-labeling with α -³²P-dCTP and DNA polymerase I (Klenow fragment) followed by gel electrophoresis.

To produce radioactively labeled TP-containing fragments of the desired length the 339 bp fragment (TP-339) was either digested with MspI followed by Klenow end-labeling to produce TP-188 and a protein free 151 bp fragment, or digested with MspI and HaeIII followed by Klenow end-labeling to produce TP-188 and a protein free 80 bp fragment, as well as a non-labeled 71 bp fragment. The latter procedure was used to prepare probes for DNase I footprinting, since the 151 bp fragment would interfere with the analysis. Alternatively TP-339 was digested with AflIII and HaeIII followed by Klenow end-labeling of the AflIII site which produced labeled TP-143 and a labeled protein free fragment of 196 bp. The enzymes remained present during further DNA replication studies. After replication the Klenow fragment was heat inactivated (30 min 60°C) and the protein free fragment of 196 bp was cleaved with MspI into a labeled fragment of 43 bp and an unlabeled fragment of 153 bp. Inactivation of the Klenow fragment was performed in order to prevent labeling of the 153 bp fragment, which would interfere with the 143 bp fragment devoid of TP.

TP-free Ad5 origin containing 143 bp fragments were isolated from XD7 plasmid DNA (29) by isolation of a 270 bp EcoRI-SspI fragment, followed by AflIII digestion and Klenow end-labeling. The TP-free Ad2 origin containing DNA of 114 bp was isolated from plasmid pHRI (30) by digestion with EcoRI and XbaI. The DNA concentration of TP-188 was determined by comparison of Klenow mediated α -³²P-dCTP incorporation between TP-188 and a known concentration of the protein free 114 bp fragment.

When used for gel retardation assays, the fragments were ultrafiltrated repeatedly in buffer A containing 100 mM NaCl using a low-protein binding PLGC membrane (Millipore) to remove free α -³²P-dCTP.

Complete degradation of the protein moiety of TP-188 was achieved by overnight digestion of the TP-188 with 5 mg matrix-bound chymotrypsin (Enzygel, Boehringer Mannheim) at 37°C followed by removal of the chymotrypsin by centrifugation and inactivation of remaining traces of chymotrypsin by heating to 60°C for 30 min.

DNA replication *in vitro*

Replication assays were performed in 15 μ l reaction mixtures containing 1 μ g DBP, 0.5 bu NFI-BD, 0.5 bu POU domain, 40 mu pTP-pol, and either Klenow end-labeled TP-143 (4 pg of DNA) or a protein free fragment of the same length and size (40 pg) in 25 mM HEPES-KOH [pH 7.5], 5 mM MgCl₂, 0.4 mM DTT, 4 mM ATP, 40 μ M of dGTP, dATP and dTTP and 2.5 μ M α -³²P-dCTP (5.5 Ci/mmol). For replication of protein-free fragments the α -³²P-dCTP concentration was increased to 7.5 μ M. Incubation was for 30 min at 37°C, and the reaction was terminated by addition of 3.5 μ l stopmix (20% sucrose, 5% sodium dodecyl sulphate (SDS), 0.05% bromophenol blue). The products were analyzed by 6% polyacrylamide gel electrophoresis (39:1 acryl:bisacryl). The gels were dried and autoradiographed and the results were quantified by scanning of the autoradiographs using an LKB ultrascan XL.

Gel retardation and DNase I footprint analysis

Binding reactions for gel retardation were carried out in 10 μ l binding buffer (25 mM HEPES-KOH [pH 7.5], 5 mM $MgCl_2$, 1 mM DTT, 100 μ g/ml BSA, 0.01% NP40) and contained 8 pg TP-188 (DNA), 8 pg chymotrypsin-treated TP-188 (tp-188), or an equimolar amount (5 pg) of the 114 bp fragment. As a non-specific competitor poly(dI-dC)·poly(dI-dC) was added as described. Samples were incubated for 60 min on ice and subsequently 1.5 μ l loading buffer (40% sucrose, 0.01% bromophenol blue) was added and complexes were separated on a 4% polyacrylamide gel (4:1 acryl:bisacryl) for 3 hr at 6 V/cm. Gels were dried and autoradiographed.

For DNase I footprint analysis 50 pg TP-188 or tp-188 were incubated in 25 μ l with purified NFI-BD or the Oct-1 POU domain for 30 min on ice in 25 mM HEPES-KOH [pH 7.5], 5 mM $MgCl_2$, 1 mM DTT, 100 μ g/ml BSA, 0.01% NP40 with various NaCl concentrations. DNase I digestion was performed for 120 seconds at 15°C with 1.2 U and the reaction was stopped by adding SDS and EDTA to final concentrations of 0.5% and 5 mM, respectively. After treatment with 40 μ g proteinase-K for 1 hr at 37°C, herring-sperm DNA (1.5 μ g) was added and the mixture was heated for 3 min at 100°C, phenol extracted and precipitated overnight with ethanol. The products were processed further as described (31).

RESULTS

Isolation of 339 bp Ad5 left terminal fragment containing functional TP

We reasoned that isolation of DNA fragments covalently bound to TP should be separable from protein-free DNA fragments by anion exchange chromatography based upon charge differences between the DNA containing the terminal protein and protein free DNA. To obtain small fragments of TP-DNA we digested DNA-TP obtained from virions with SspI which cleaves Ad5 DNA in 6 internal fragments varying in size between 19034 and 271 bp as well as two terminal fragments of 339 (left) and 1303 (right) bp, respectively. This mixture was loaded on an FPLC mono-Q column and eluted with a very shallow NaCl gradient from 600 to 720 mM NaCl. Care was taken to apply a low flow speed (0.15 column volumes per minute). As indicated in Fig. 1A, over 98% of the DNA eluted in fractions 19 to 28 in several distinct peaks, and a small peak was observed in fraction 16. The various fractions were digested with MspI, Klenow end-labeled and analyzed by gel electrophoresis (Fig. 1B). Fractions 20–28 contain many fragments, whereas fractions 16 and 18 consisted almost exclusively of two equally labeled fragments migrating at positions corresponding to approximately 800 bp and 150 bp, respectively. To establish that these fractions contained the TP-188 and 151 fragments derived from MspI digestion of TP-339 a small part was treated with chymotrypsin. This led to a shift of the band migrating at position 800 to a position at 190 bp whereas the 151 bp fragment did not change in position (not shown), indicating that the band at position 800 represented TP-188. Therefore this anion exchange chromatography procedure separated TP-339 from all other fragments, including TP-1303, which eluted just before or partially coinciding with the protein-free fragments (18). To test whether the TP was still functional we assayed the Klenow end-labeled TP containing origin fragment in an *in vitro* DNA replication system using purified replication proteins (Fig. 2).

Figure 1. Separation of the terminal protein containing origin fragment from all other fragments. (A) Elution pattern of the various restriction fragments obtained after cleaving TP-DNA with SspI followed by chromatography on an FPLC mono-Q column eluted with a shallow NaCl concentration gradient as indicated. (B) Klenow end-labeling of fractions eluted from the mono-Q column and digested with MspI. The terminal protein containing fragment of 339 bp (TP-339) eluting in fractions 16–18 is cut into a protein-free 151 bp fragment (lower band) and a 188 bp fragment containing the terminal protein (upper band, TP-188), which runs at the position of 800 bp due to the presence of covalently bound TP. A 3-fold shorter exposure of fractions 18–30 is shown. 'L' and 'FT' denote the load and the flow-through respectively.

For this purpose we used TP-143 rather than TP-188 or TP-339 since we wanted to compare replication with a protein-free fragment of identical sequence and size.

After incubation in the presence of non-radioactive dNTP's, a new band can be observed in addition to the parental TP-143 band (Fig. 2, lane 2). This band represents single stranded TP-143 that is formed as a result of displacement synthesis indicating that the template is active (Fig. 2, lanes 2 and 3). When α - 32 P-dCTP is included in the replication mixture a third band is observed (lane 3), representing 143 bp double-stranded DNA containing pTP as a result of protein priming. Prelabeling of the template also enables an estimate of the percentage of active template. In this experiment 40% of the input TP-143 was replicated. Under optimal conditions over 90% of the TP-143 can be replicated (18). Replication of an identical origin fragment lacking TP (Fig. 2, lanes 5–7) appeared very inefficient and could only be observed when high α - 32 P-dCTP and template concentrations were used (lane 7).

From a comparison of the intensity of the bands we estimate that TP-free DNA is at least 100-fold less efficient as a template

than TP-DNA. In previous reports a 30-fold lower efficiency for TP-free DNA compared to TP-DNA has been suggested (28,32). A reason for this discrepancy could be that TP-containing templates prepared this way seem to be more active than templates

used in previous studies, where only 5–10% of the input templates were used for DNA replication (33,34).

Binding of pTP-pol to the origin

There are several possible mechanisms to account for the stimulatory effect of the terminal protein on replication. TP could stabilize or facilitate formation of a multi-protein preinitiation complex or it could assist in unwinding or other structural changes in the origin in order to facilitate initiation of replication. TP might also increase the affinity of the origin for pTP-pol, NFI or Oct-1, thereby allowing initiation to proceed more efficiently. To test the effect of TP on binding of pTP-pol to the origin we employed a band-shift assay in which we compared TP-188 with TP-free DNA as well as with the 151 bp fragment that does not contain the adenovirus origin and functions as an internal control. Both the TP-188 fragment and the 151 bp fragment are bound by pTP-pol but the TP-188 fragment shifts more efficiently as shown by the disappearance of the free probe (Fig. 3A, lanes 1–4). The 114 bp Ad2 origin fragment behaves like the aspecific 151 bp fragment indicating that under these conditions the sequence specificity of pTP-pol binding is low. Although the effects are weak, they are well reproducible. Care was taken to ensure the same specific activity of the fragments and identical DNA concentrations. This is most easily obtained in those cases where the two fragments are derived from the same TP-339 molecule (Fig. 3A). No distinct retarded complexes are observed for the TP-188/pTP-pol and 151/pTP-pol complexes, probably because of dissociation during electrophoresis, or because the gel system used (1:4 acryl:bisacryl, containing very large pores) makes it difficult to separate high molecular weight complexes. This gel system is required to allow the large protein/TP-188 complexes to migrate into the gel. In order to quantify the amount of DNA/protein complex formed we measured the decrease in free probe. The results of densitometric scanning of the

Figure 2. Efficient replication of short origin fragments requires terminal protein. Replication in vitro was studied using as templates TP-143 (lanes 1–3), the same fragment after chymotrypsin degradation (tp-188, lane 4) and the protein-free 143 bp origin fragment isolated from plasmid DNA (lanes 5–7). Lanes 1, 4 and 5 contain input DNA, lanes 2 and 6 contain DNA after replication with unlabeled dCTP, and lanes 3 and 7 contain DNA after replication in the presence of α - 32 P-dCTP. Arrows indicate the positions of TP-143, the precursor terminal protein containing 143 bp fragment (pTP-143), the displaced single stranded TP-143 and the 143 bp Ad5 origin containing plasmid DNA.

Figure 3. pTP-pol prefers binding to a TP-containing origin fragment. (A) Increasing amounts of pTP-pol were incubated with either the labeled TP-188/151 mixture or with the 114 bp Ad2 probe and analyzed by gel retardation. Lanes 1 and 5, no pTP-pol added; lanes 2 and 6, 20 μ M pTP-pol; lanes 3 and 7, 40 μ M pTP-pol; lanes 4 and 8, 80 μ M pTP-pol. (B) Graph in which the ratio of bound versus free DNA is plotted against the amount of pTP-pol added, as determined by densitometric scanning of the autoradiograph shown in A. ($\blacklozenge$) TP-188, ($\circ$) tp-188, ($\triangle$) Ad2.

autoradiographs are shown in Fig. 3B. Binding of pTP-pol to TP-188 appears 2–3 fold more efficient than to the 151 bp fragment or the 114 bp fragment.

The presence of TP does not significantly change the binding affinity of NFI and Oct-1

Specific binding of NFI and Oct-1 to the auxiliary origin enhances DNA replication of TP-DNA 50-fold and 5-fold, respectively, under conditions of low pTP-pol concentration (25). To test if binding of these two transcription factors was influenced by the presence of TP we employed both gel retardation and DNase I footprinting.

We used the binding domains of NFI (NFI-BD) and Oct-1 (POU domain) in these assays because their stimulatory effects on DNA replication are identical to the intact proteins (35–37). For the binding of NFI-BD we compared the intact TP-188 with tp-188, in which the terminal protein was degraded completely by chymotrypsin as shown in control experiments (Fig. 4A and 4C). NFI-BD bound in band-shift assays to both TP-188 and tp-188 with almost the same affinity, whereas binding to the 151 fragment, that does not contain the NFI recognition sequence, was negative.

Like NFI-BD, the POU domain bound in band-shift assays to TP-188 and tp-188 with almost identical affinity, whereas binding

to the 151 bp control fragment, in spite of addition of 100 ng poly(dI-dC)·poly(dI-dC) was observed as well, albeit with approx. 5-fold reduced affinity (Fig. 4C and D). Binding to the aspecific 151 bp fragment is caused by the low concentration of DNA used, whereby the binding reaction is driven by the POU domain. Under high concentrations of the POU domain, the POU domain binds easily to secondary sites.

DNase I footprinting reveals changes in the origin structure

To study possible qualitative differences of binding of NFI and Oct-1 we employed DNase I footprinting on the top strand (Fig. 5A and B). First we investigated whether the presence of TP would lead to a protected region in the core origin by itself. This turned out not to be the case, indicating that the TP extends away from the end of the DNA or at least does not have strong interactions with the core origin by itself. However, close inspection of the two degradation profiles (compare Fig. 5A, lanes 1 and 7 and Fig. 5B, lanes 1 and 6) showed that the presence of TP alters the DNase I breakdown pattern considerably at various positions. We observed strong hypersensitivity in the core origin at positions 5 and 6 in the presence of the terminal protein, as well as hypersensitivity at positions 41, 42 and 48. We also observed a protection in the presence of TP at position 3, whereas position 4 was not influenced by the presence of TP. This most

Figure 4. Gel retardation assays reveal no quantitative differences of binding of NFI and Oct-1 to TP-DNA and TP-free DNA. (A) Gel-retardation assay using NFI-BD and TP-188 or tp-188. Lanes 1–5 contain the TP-188/151 mixture, lanes 6–10 contain the tp-188/151 mixture. Lanes 1 and 6, no protein added; lanes 2 and 7, 0.21 bu NFI-BD; lanes 3 and 8, 0.42 bu NFI-BD; lanes 4 and 9, 0.85 bu NFI-BD; lanes 5 and 10, 1.7 bu NFI-BD. (B) Graph in which the ratio of bound versus free DNA is plotted against the amount of NFI-BD added, as determined by densitometric scanning of the autoradiograph shown in A. (♦) TP-188, (○) tp-188. (C) Gel retardation assay using the POU domain and TP-188 or tp-188. Lanes 1–5 contain the TP-188/151 mixture, lanes 6–10 contain the tp-188/151 mixture. 100 ng poly(dI-dC)·poly(dI-dC) is included in every assay. Lanes 1 and 6, no protein added; lanes 2 and 7, 0.16 bu POU; lanes 3 and 8, 0.31 bu POU; lanes 4 and 9, 0.62 bu POU; lanes 5 and 10, 1.25 bu POU. (D) Graph in which the ratio of bound versus free DNA is plotted against the amount of POU added, as determined by densitometric scanning of the autoradiograph shown in A. (♦) TP-188, (○) tp-188.

Figure 5. DNase I footprinting of NFI and the POU domain is qualitatively influenced by the presence of the terminal protein. (A) DNase I footprints of NFI-BD using TP-188 and tp-188. Lanes 1–6 and 7–12 contain 0, 1.4, 2.8, 5.7, 8.6 and 11.4 bu NFI-BD, respectively. (B) DNase I footprints of POU using TP-188 and tp-188. Lanes 1–6 and 7–12 contain 0, 2, 4, 8 and 16 bu POU, respectively.

likely indicates that TP induces structural changes in the origin, although other explanations may exist (see Discussion).

When we added NFI-BD, the size of the footprint on TP-188 at first glance seemed shorter than that on tp-188 (Fig. 5A). On tp-188, NFI-BD clearly protects nucleotides 19 to 42 of the top strand, which is identical to previous results employing protein-free origin fragments (38). However, with TP-188 the hypersensitivity of nucleotides 41 and 42 made it difficult to measure protection. Scanning of the gels showed that also these nucleotides became partially protected, similar as with tp-188 where nucleotides 41 and 42 are hardly visible. Thus, the footprint extends to the same borders in both cases. NFI-BD induces hypersensitivity by itself around positions 48, 50 and 51 and this hypersensitivity was more pronounced using TP-DNA as a probe.

DNase I footprinting with the POU domain (Fig. 5B) showed that also with this protein the size of the footprint is not influenced by the terminal protein. Both for TP-188 and tp-188 protection of the top strand was observed between nucleotides 35 and 54, as shown before (39). However, there are some notable differences between TP-188 and tp-188. Bases 50 and 51 were well protected against DNase I degradation upon binding of the POU domain to TP-188, whereas these bases were less protected against DNase I attack upon binding of the POU domain to tp-188. The various changes in the footprint pattern are summarized in Fig. 6.

Figure 6. Summary of the differences in the DNase I footprint pattern of TP-188 compared to tp-188 in the absence and presence of NFI-BD or Oct-1.

DISCUSSION

Using restriction endonuclease digestion and ion exchange chromatography, we have succeeded in separating a short, terminal protein containing adenovirus DNA fragment from internal DNA fragments. This fragment including the origin of replication was fully active in *in vitro* replication assays. With this fragment we were able to study the structure of the adenovirus origin in the context of a native, functional terminal protein. The fragment could be used both in bandshift assays if a large pore containing gel system was used and for footprinting of the top strand. Footprinting of the bottom strand was not possible since

specific labeling of the bottom strand requires dephosphorylation of the TP-containing fragment followed by phosphorylation by T4 kinase. It appeared impossible to remove the phosphatase without inactivation of TP.

DNase I digestion of the adenovirus origin with the covalently linked terminal protein showed no protected region, indicating that the terminal protein does not shield phosphodiester bonds from DNase I digestion and thus may extend away from the DNA. However, strong DNase I hypersensitivity at positions 5 and 6 was observed as well as at positions 41, 42 and 48, although less pronounced. DNase I digestion of the same DNA after degradation of the terminal protein showed no hypersensitivity at any of these positions, similar as protein-free plasmid derived DNA. One explanation of the increased DNase I sensitivity at positions 5 and 6, close to TP, might be that TP binds weakly to DNase I thus enabling the enzyme to digest DNA close to the origin stronger. However, we feel that this explanation is less likely since breakdown at another position close by, position 3, was completely blocked. Moreover, such a sticking model could not explain hypersensitivity at positions further away from the molecular end such as 41, 42 and 48. We consider it more likely that TP induces subtle structural changes in the DNA at the origin of replication, extending over some distance. The hypersensitive sites could be caused by a change in twist angle, base-pair propeller-twist or roll and tilt angles of nucleotides in the DNA, factors that influence the cleavage specificity of DNase I (40). Such changes in the configuration of the origin may be the result of twisting of the DNA as a consequence of the covalently bound terminal protein.

In order to test whether the terminal protein affected binding of proteins involved in initiation of DNA replication we investigated the binding of pTP-pol, NFI and Oct-1 to both an origin containing a terminal protein and an origin devoid of terminal protein employing band-shift assays and DNase I footprint analyses. We found that the pTP-pol complex binds rather aspecifically to the DNA, as had been shown before (41,42). However, binding to a TP-containing adenovirus origin was two to three-fold increased compared to a TP-free origin. It is not likely that this effect alone is responsible for the large difference in template activity but the role of TP may not be confined to the binding affinity alone. TP might also contribute to an improved positioning of the pTP-pol complex to enable higher initiation rates. At present we do not know whether this increased binding is a consequence of the altered DNA structure in TP-DNA or the result of a direct interaction between the origin-linked terminal protein and the pTP-pol complex. Protein cross-linking using glutaraldehyde showed no interaction between pTP-pol and the terminal protein (not shown), but possibly the right conditions for detecting such an interaction have not yet been achieved. DNase I footprint analysis failed to show any areas in the origin that were specifically protected by the pTP-pol complex (not shown).

The affinities of NFI-BD and the Oct-1 POU domain were equal for TP-DNA and protein-free DNA, whereas the footprint patterns displayed differences (see Fig. 6). The reason for these differences could also reside in an altered DNA structure of TP-DNA. A similar situation in which a protein changes DNA structure and thereby changes the binding characteristics of transcription factors to DNA has been reported previously for the adenovirus DNA binding protein (43) and the E. Coli HU protein (44).

How does TP enhance initiation?

The binding affinity and the level of saturation of the auxiliary origin determine the effect of NFI and Oct-1 on DNA replication (12). Since the changes induced by TP in NFI and Oct-1 binding apparently do not change the binding affinity of NFI and Oct-1, it is unlikely that TP enhances replication through changed binding of NFI or Oct-1. Moreover, the stimulatory effect of TP occurs also in the absence of NFI and Oct-1 and these proteins stimulate initiation both on TP-containing and TP-free DNA. Since the increased template activity can only partially be explained by the increased pTP-pol binding, we hypothesize that the main stimulatory effect of TP could lie in the induction or facilitation of changes in the origin required for initiation, such as unwinding occurring after formation of a preinitiation complex. We cannot exclude that NFI and Oct-1 cooperate somehow with TP in this respect, and a direct experimental approach to study origin unwinding will be required to establish this. Alternatively, the difference in pTP-pol binding between TP-containing and TP-free templates may be much larger in the presence of other replication proteins which is difficult to test. A final possibility is that TP may prevent abortive initiations which eventually leads to an overall lower efficiency of TP-free DNA templates.

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