

# Characterisation of the adenovirus preterminal protein and its interaction with the POU homeodomain of NFIII (Oct-1)

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## ABSTRACT

**Formation of the preinitiation complex for adenovirus DNA replication involves the incoming preterminal protein–adenovirus DNA polymerase heterodimer being positioned at the origin of replication by protein–DNA and protein–protein interactions. Preterminal protein directly binds to the cellular transcription factor nuclear factor III (Oct-1), via the POU homeodomain. Co-precipitation of POU with individual domains of preterminal protein expressed by *in vitro* translation indicated that POU contacts multiple sites on preterminal protein. Partial proteolysis of preterminal protein in the presence or absence of POU homeodomain demonstrated that many sites accessible to proteases in free preterminal protein were resistant to cleavage in the presence of POU homeodomain. The accessibility of sites in free preterminal protein to cleavage by trypsin was strongly dependent on the ionic strength, suggesting that preterminal protein may undergo a sodium chloride-induced conformational change. It is therefore likely that the POU homeodomain contacts a number of sites on preterminal protein to induce a conformational change which may influence the initiation of adenovirus DNA replication.**

## INTRODUCTION

The adenovirus genome is a linear double-stranded DNA molecule of 36 000 bp with inverted terminal repeats (ITRs) of ~100 bp. Located within the ITRs are the *cis*-acting DNA sequences which define *ori*, the origin of DNA replication (1). Covalently attached to each 5'-end is a terminal protein (TP) which is likely to be an additional *cis*-acting component of *ori* (2). Within the terminal 51 bp of the adenovirus 2 (Ad 2) genome, four regions have been defined that are involved in initiation of replication. The terminal 18 bp are regarded as the minimal replication origin (3) and limited initiation can occur at this site with just the three viral proteins involved in replication: preterminal protein (pTP), DNA polymerase (pol) and DNA-binding protein (DBP). However, in Ad 2, two cellular transcription factors, nuclear factor I (NFI) and nuclear

factor III (NFIII or Oct-1) are required for efficient levels of replication (4,5). In order to initiate replication it is thought that the origin of replication is first coated with DBP. This protein acts cooperatively with NFI, which binds to a recognition site within the origin of replication, separated from the 1–18 bp core by a precisely defined spacer region (6). NFIII also binds at a specific recognition site between nt 39 and 48. Protein–protein interactions, between NFI and pol (7–9) and pTP and the POU homeodomain (POUhd) of NFIII (10), help recruit the pTP–pol heterodimer into the preinitiation complex. Interaction between the heterodimer and specific base pairs 9–18 in the DNA sequence ensures correct positioning (11) and the complex is further stabilised by interactions between the incoming pTP–pol and the genome-bound TP (12). DNA replication is then initiated by a protein priming mechanism in which a covalent linkage is formed between the  $\alpha$ -phosphoryl group of the terminal residue, dCMP, and the  $\beta$ -hydroxyl group of a serine residue in pTP, a reaction catalysed by pol (13). The 3'-hydroxyl group of the pTP–dCMP complex is then used as a primer, by pol, for synthesis of the nascent strand. Base pairing with the second GTA triplet of the template strand guides the synthesis of a pTP–trinucleotide, which then jumps back three bases, to base pair with the first triplet (also GTA) and synthesis then proceeds by displacement of the non-template strand (14). Dissociation of pTP from pol begins as the pTP–trinucleotide is formed and is almost complete by the time 7 nt have been synthesised (15). NFIII dissociates as the replication fork passes through the NFIII binding site (10). Here we investigate the interaction between NFIII and pTP showing that widely separated portions of the linear sequence of pTP interact with the POUhd of NFIII.

## MATERIALS AND METHODS

### Purification of pTP, NFIII, POU, POUhd and GST–POUs

Ad 2 pTP was expressed in *Spodoptera frugiperfa* sf9 cells using recombinant baculoviruses and purified as described previously (11,16). NFIII was expressed as a fusion with glutathione *S*-transferase (GST) (plasmid obtained from P. O'Hare, Marie Curie Research Institute, Oxted, UK) and purified by affinity chromatography on glutathione–agarose. Digestion with thrombin gave free NFIII from which the GST was removed by reapplication on glutathione–agarose. Plasmids containing POU, POUhd and POU specific domain

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(POUs) as GST fusions were obtained from P. C. van der Vliet (Utrecht University, Utrecht, The Netherlands). The plasmids were transformed into *Escherichia coli* strain B834. Cells induced by IPTG were resuspended in 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol (DTT), 5 mM Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 0.5 mM phenylmethylsulphonyl fluoride (PMSF) (buffer A), lysed with lysozyme and Triton X-100 added to 1%. After sonication and centrifugation the supernatant was passed through a DEAE-cellulose column equilibrated with buffer A. This flow-through was applied to a glutathione-agarose column equilibrated with 50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 5 mM Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 15% glycerol (buffer B). The column was washed with buffer B and eluted with 25 mM potassium acetate pH 6.8, 50 mM NaCl, 1 mM DTT, 0.1% Triton X-100, 10 mM glutathione, 10% glycerol. For the GST-POU fusion protein the material thus obtained was digested with thrombin to give free POU, which was further purified by FPLC on a Mono-S column (Pharmacia). POU was eluted by applying a linear gradient of NaCl from 50 mM NaCl to include a greater proportion of buffer C (25 mM potassium acetate pH 6.8, 1 M NaCl, 1 mM DTT, 10% glycerol). POU elutes at ~230 mM NaCl. GST-POUhd was also digested by thrombin and POUhd further purified on a Mono-S column eluted with a linear gradient of NaCl starting with 25 mM potassium acetate pH 8.0, 100 mM NaCl, 1 mM DTT, 10% glycerol, 0.1% Triton X-100 and increasing the NaCl concentration up to 500 mM. GST-POUs was used only as the fusion protein.

### SDS-PAGE and immunoblotting

Protein samples were denatured and reduced by boiling in the presence of 2% SDS and 0.72 M 2-mercaptoethanol and analysed by SDS-polyacrylamide/DATD gel electrophoresis (PAGE). Proteins were electrophoretically transferred (mini Trans-Blot cell; Bio-Rad) onto polyvinylidene difluoride (PVDF) membrane. Membranes were incubated in blocking buffer [phosphate-buffered saline (PBS) containing 5% non-fat milk], which was used in all subsequent incubations, and then with primary antibody at the appropriate dilution. Membranes were washed with PBS containing 0.1% Tween 20 and antibody-antigen complexes detected by incubation of the membrane with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies and an enhanced chemiluminescence (ECL) system.

### Primary antibodies

3D11, 5E3, 7H1 and 44E1 are mouse monoclonal antibodies raised against Ad 2 pTP (16) and were used as a cocktail unless otherwise stated. Antibody MAD3 10B is a mouse monoclonal antibody raised against IκBα (17). The SV5 Pk tag mAb (18) was used to detect Pk-tagged pTP fragments. Rabbit polyclonal serum was raised against a peptide corresponding to the N-terminal 15 amino acids of pTP (19).

### GST co-precipitations

GST-POU, GST-POUhd, GST-POUs or GST (400 ng) was bound to glutathione-agarose (5 μl) and, after washing and blocking with 1% BSA in 25 mM potassium acetate pH 7.5, pTP (150 ng) was allowed to associate with the GST proteins by incubation on a tumbling wheel for 1 h. After further

washing with 1% BSA in 25 mM potassium acetate pH 7.5, and then 25 mM potassium acetate pH 7.5, samples were analysed by SDS-PAGE and immunoblotting.

### *In vitro* transcription/translation of pTP fragments

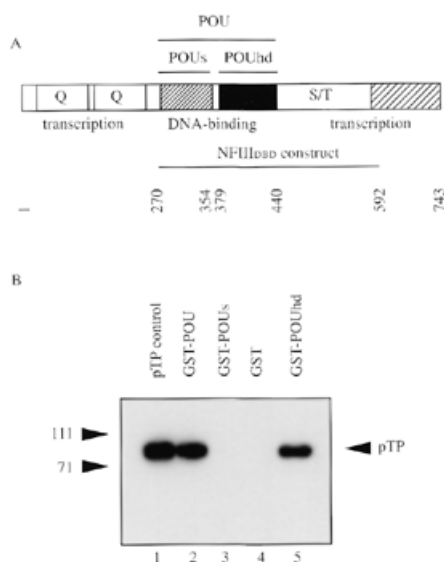
DNA fragments corresponding to from the N-terminus of pTP to the first iTP cleavage site (amino acids 1–175) (NTs), from the N-terminus to the TP cleavage site (amino acids 1–349) (NTb) and from the TP cleavage site to the C-terminus (amino acids 350–671) (TP) were excised from pGEX plasmids containing these fragments and inserted into a pcDNA3 vector (Invitrogen) into which an oligonucleotide coding for the 14 amino acid Pk tag sequence (18) had been inserted upstream of, and in-frame with, the *Bam*HI site (pcDNA-N-tag, a gift of Lesley Stark). The fragment corresponding to from the first iTP cleavage site to the TP cleavage site (amino acids 176–349) (M) was amplified by PCR from the NTb construct using primers introducing N-terminal *Bam*HI and C-terminal *Eco*RI sites. The resulting fragment was cloned into pcDNA-N-tag. These constructs were used as templates in the TNT Coupled Wheat Germ Extract System (Promega) according to the manufacturer's instructions. Products from the *in vitro* transcription/translation reactions were incubated with GST or GST-POU glutathione-agarose beads and after extensive washing samples were analysed by SDS-PAGE. The gel was fixed and dried and [<sup>35</sup>S]methionine-labeled protein detected by exposure to a phosphorimager screen and scanning by a Fujix BAS 1000 phosphorimager and analysed with MacBAS software using a linear signal intensity scale.

### Trypsin assay

Trypsin activity was measured at various NaCl concentrations using a modification of the method of Erlanger *et al.* (20). An aliquot of 435 μg of *N*-benzoyl-DL-arginine-D-nitroanilide, dissolved in 10 μl DMSO, was added to 1 ml 25 mM potassium acetate, pH 7.5, containing the appropriate NaCl concentration. Trypsin (2.5 μg) was added and the mixture incubated at room temperature for 30 min before quenching with 50 μl glacial acetic acid. The optical density was measured at 410 nm and plotted against NaCl concentration.

### Proteolytic digestions of pTP

pTP was preincubated with a 50-fold excess, unless otherwise stated, of NFIII, POU, POUhd or protease-free BSA (as a control), for 30 min at room temperature, in 25 mM potassium acetate, pH 7.5, unless otherwise stated. Trypsin or chymotrypsin were added at a protein:protease ratio of 15:1 and the samples incubated at room temperature. Aliquots were removed at various time points and quenched by the addition of PMSF. Similarly, digestions were performed at 37°C with Ad 2 protease [provided by Goncalo Cabrita and preactivated by a 15 min incubation with the activating peptide (GVQS-LKRRRCF) at 37°C; 19] quenching the aliquots with dithio-dipyridine (DTDP). Endoproteinase Asp-N digestions were also performed at 37°C, with a protein:protease ratio of 7.5:1 and quenched with EDTA. Control digestions were performed by digesting IκBα with chymotrypsin in the presence of the appropriate NFIII fragment or BSA as described in Jaffray *et al.* (17).



**Figure 1.** Co-precipitation of pTP with GST-POU fragments. (A) Schematic diagram of NFIII (24) indicating the positions of the POU, POUhd and POU<sub>s</sub> and of the NFIIDBD construct used. Regions marked Q are glutamine-rich and that marked S/T serine/threonine-rich. Regions involved in transcription and DNA binding are indicated. (B) GST fusions of the POU domain, the POU<sub>s</sub> and the POUhd (400 ng) were immobilised on glutathione-agarose beads (5  $\mu$ l). Following washing and blocking of the beads, pTP (150 ng) was allowed to associate with the fusion proteins bound to the beads and, after extensive washing, pTP binding was assessed by western blotting probed with a cocktail of anti-pTP mAbs. The control track contains 30 ng pTP.

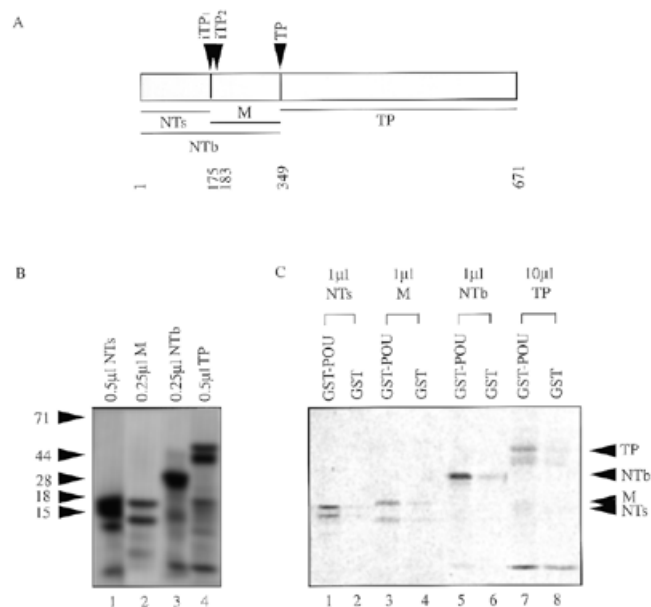
### N-terminal peptide sequencing

Peptide products of partial tryptic digestion of pTP were separated by SDS-PAGE as described above except that freshly made acrylamide:piperazine diacrylamide solution (37.5:1) was used and sodium thioglycolate (0.1 mM final concentration) was added to the upper electrophoresis buffer. Fragments were electrophoretically transferred to PVDF membranes (Amersham) and stained with Coomassie Brilliant Blue for a few seconds. The membrane was washed extensively in distilled water and the bands excised. The sequence was determined by Paul Talbot and Graham Kemp using a Procise microsequencer (Applied Biosystems) with on-line phenylthiohydantoin analysis.

## RESULTS

### pTP interacts with the POUhd of nuclear factor III

It has previously been demonstrated that the POU domain of NFIII, the POUhd, but not the POU<sub>s</sub> domain (Fig. 1A), are required for interactions of the pTP-pol heterodimer (21) and are sufficient to enhance the binding of pTP-pol to the viral origin of DNA replication (10). To characterise the interactions between purified pTP and POU, GST fusions of the POU, POUhd and POU<sub>s</sub> domains were immobilised on glutathione-agarose and their ability to interact with baculovirus-expressed

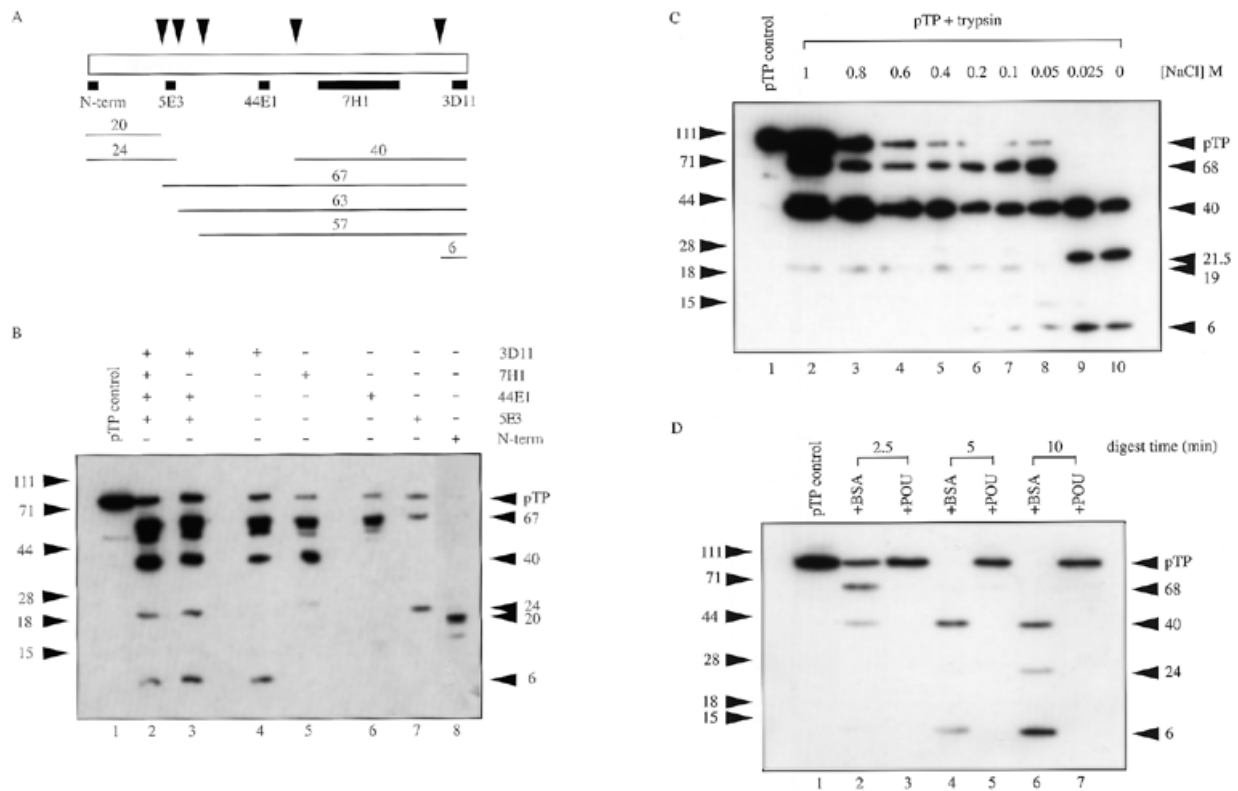


**Figure 2.** Co-precipitation of pTP fragments with GST-POU. (A) Schematic diagram of pTP indicating the fragments expressed by *in vitro* transcription/translation and their relationship to the cleavage sites of the Ad 2 protease. NT<sub>s</sub> is a fragment of amino acids 1–175, NT<sub>b</sub> of amino acids 1–349, M of amino acids 176–349 and TP of amino acids 350–671. (B) Samples of the *in vitro* transcribed and translated pTP fragments were separated by SDS-PAGE. The fixed and dried gels were exposed to a phosphorimager screen. (C) GST-POU, or GST as a negative control, was immobilised on glutathione-agarose beads. The pTP fragment was allowed to associate with the GST proteins bound to the beads and, after extensive washing, the immobilised material was separated by SDS-PAGE. The fixed and dried gels were exposed to a phosphorimager screen and the bands quantitated.

pTP assessed. GST-POU efficiently binds pTP as does GST-POUhd, while neither GST-POU<sub>s</sub> nor the control GST are capable of binding pTP (Fig. 1B).

### Mapping regions of pTP interacting with the POU domain

Fragments of pTP corresponding to those produced by digestion with Ad 2 protease (Fig. 2A) were cloned and expressed in a pcDNA3 vector, modified to produce proteins N-terminally tagged with the Pk tag from SV5 (18). *In vitro* transcription and translation in the presence of [<sup>35</sup>S]methionine gave the N-terminally tagged pTP fragment of correct molecular weight (confirmed by western blotting with the anti-Pk tag antibody and monoclonal antibodies to the C-termini of the fragments where available) as the major labelled product. Other bands did not react with the anti-Pk tag antibody and are due to initiation at secondary sites and proteolytic degradation from the lysate. Binding of the *in vitro* transcribed and translated products to GST-POU immobilised on glutathione-agarose compared to GST immobilised on glutathione-agarose was assessed under the same binding and washing conditions as used for the studies with full-length pTP. All four pTP fragments were shown to interact with GST-POU (Fig. 2C), albeit with different affinities, indicating that there must be multiple sites within the pTP molecule which interact with



**Figure 3.** Digestion of pTP with trypsin. **(A)** Schematic diagram indicating the position and apparent molecular weight of tryptic digest fragments of pTP identified with mAbs which recognise epitopes located as shown. **(B)** pTP (210 ng) was digested with trypsin (14 ng) for 5 min at 37°C before quenching with PMSF to 5 mM, the partially digested sample was divided into seven aliquots and the digestion products separated by SDS-PAGE and blotted onto PVDF membrane. Each track was probed individually with either rabbit polyclonal serum raised against the N-terminal 15 amino acids, an anti-pTP monoclonal antibody normalised to give equivalent intensity signals for a defined amount of pTP or a mixture of antibodies, followed by the appropriate horseradish peroxidase conjugate, and the bands visualised by ECL. **(C)** pTP (34 ng) was preincubated with BSA (200 ng) in 25 mM potassium acetate, pH 7.5, containing the appropriate NaCl concentration. Trypsin (2 ng) was added and the samples incubated at room temperature for 5 min before quenching with PMSF to 5 mM. The samples were fractionated on an SDS-polyacrylamide gel and blotted onto PVDF membrane. The blots were probed with a cocktail of anti-pTP mAbs and anti-mouse horseradish peroxidase and detected by ECL. **(D)** pTP was digested by trypsin (1:15 protease:protein ratio) in the presence of a 50 molar excess of NFIIIDBD, POU or POUhd or BSA as a control protein, aliquots containing 30 ng pTP and 140 ng NFIIIDBD (or the molar equivalents of POU or POUhd) or 200 ng BSA were removed at various time points, quenched with PMSF to 5 mM, run on an SDS-polyacrylamide gel and blotted onto PVDF membrane. The blots were probed with a cocktail of anti-pTP mAbs and anti-mouse horseradish peroxidase and detected by ECL.

POU. Each experiment was performed several times and showed a consistently five times greater binding to GST-POU than to GST for the NTs and NTb fragments, three times greater binding for the M fragment and two and a half times greater for TP fragment. The NTb fragment bound most efficiently, followed by the M fragment, the NTs fragment and, finally, the TP fragment, which bound only poorly. This suggests that the iTP-TP (M) region contains an important site(s) for interaction, with interactions in the N-terminal section being next strongest and the TP portion playing only a minor role. The structure of the POUhd, at only 7500 Da, has been shown, by NMR and X-ray crystallographic techniques, to be a compact globular structure of three  $\alpha$ -helices with an N-terminal arm of just nine amino acids (22,23). Therefore distant sites within the linear sequence of the 87 kDa pTP must be spatially close, if portions of each fragment are all to contact the POUhd. This may be the case in the free pTP structure or may be induced upon POU binding.

### Probing the pTP-POU complex by partial proteolysis

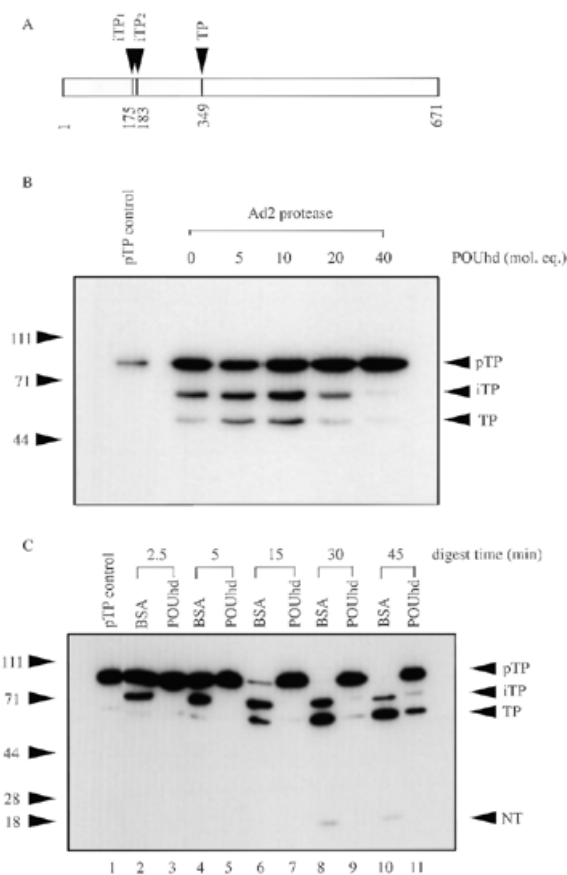
Limited proteolysis of pTP with a variety of proteases gave distinctive cleavage patterns which were elucidated using monoclonal antibodies (mAbs) raised against pTP, which have known linear epitopes (Fig. 3A), and by N-terminal protein sequencing of the digestion fragments. There are 77 predicted trypsin cleavage sites in pTP. Whilst only a small number of these are actually cleaved the susceptible sites are reasonably well spread along the length of the molecule and were therefore analysed in some detail (Fig. 3B). The majority of the fragments produced, as seen on a Coomassie stained polyacrylamide gel, were detected by the panel of monoclonal antibodies against pTP used (data not shown). The most rapid cleavage detected takes place 20 kDa from the N-terminus of the protein to give a 67 kDa fragment, recognised by the mAb 5E3, for which N-terminal sequencing gave the sequence HLRPN, indicating cleavage at an arginine two amino acids C-terminal of the second iTP cleavage site (19) at amino acid

185. (The second iTP site is at amino acid 183.) There are also two other cleavages just C-terminal to this, giving fragments of 63 and 57 kDa, respectively, which are recognised by antibodies binding at the more C-terminal epitopes (44E1, 7H1 and 3D11). The N-terminal fragment corresponding to cleavage at the 63 kDa site can be visualised by both 5E3 and polyclonal antibodies raised against the N-terminal 15 amino acids. Therefore cleavage can occur independently at this site and not only by clipping of the larger 67 kDa fragment. Similarly, cleavage to give a 40 kDa fragment (16) can occur without prior cleavage at 67 kDa, as shown by immunoprecipitation of pTP by mAb 5E3 onto protein A-agarose, which blocked the 67 kDa cleavage site from trypsin proteolysis but still allowed proteolysis at the 40 kDa site to occur (data not shown). There is also a slower cleavage 6 kDa from the C-terminus. At higher salt concentrations the digestion profile changes such that the 6 kDa C-terminal cleavage product is not produced (Fig. 3C). Furthermore there is a reduction in digestion rate with increasing NaCl concentration which occurs in two steps between 25 and 50 mM NaCl and between 0.6 and 0.8 M NaCl. This suggests that a two step conformational change may be taking place, induced by changes in ionic strength, between a more open pTP structure at low concentrations of NaCl and a more compact structure in high concentrations of NaCl. It was shown that alterations in the digestion pattern of pTP could not be accounted for by NaCl-induced changes in trypsin activity as the rate of digestion of a chromogenic substrate by trypsin varied only 2-fold over the range 0–1 M NaCl.

pTP which had been preincubated with an excess of NFII-IDBD, POU, POUhd or BSA as a control was subjected to limited proteolysis with trypsin. Aliquots were removed and quenched at certain time points. After 5 min incubation of pTP in the presence of BSA with trypsin no full-length pTP remained but in the presence of POU the pTP was still intact even after 10 min (Fig. 3D). At long incubation times (30 min) a small amount of cleavage occurred at the 67 kDa site but the other sites remained protected. This may indicate that this site is less protected than the others or may just be because it is always the first site to be cleaved.

pTP was afforded very good protection from proteolysis by interaction with POU as compared with a BSA control (Fig. 3D). Identical results were obtained with NFIIIDBD and POUhd (data not shown). As identical proteolysis patterns were obtained with each construct this suggests that the protection seen is due to pTP-POUhd interaction at or close to these sites and not just steric hindrance from non-interacting portions of the protein.

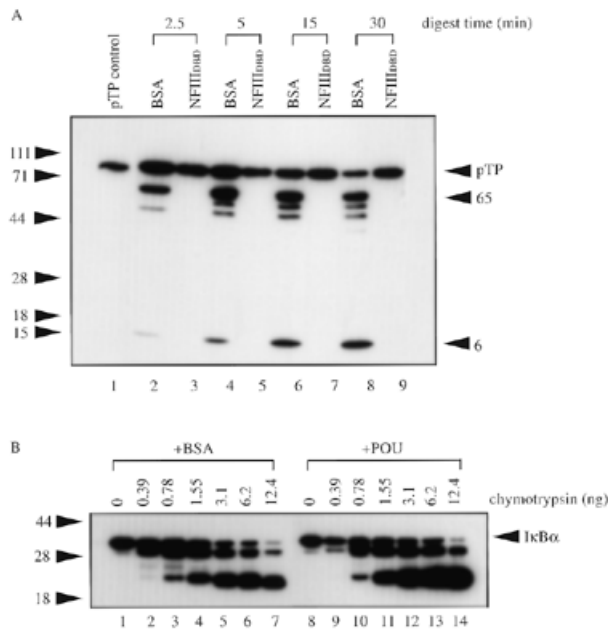
Ad 2 protease cleaves pTP at completely defined iTP and TP sites (19; Fig. 4A). Protection from digestion by POUhd was seen at both of these sites. Digestion of pTP by Ad 2 protease in the presence of increasing equivalents of POUhd for a fixed time showed that 40 molar equivalents of POUhd relative to pTP were required to achieve complete protection (Fig. 4B). After prolonged digestions of pTP in the presence of POUhd, limited cleavage of pTP was detected, but at the TP site and not the iTP site (Fig. 4C), which is the initial cleavage site in the unprotected molecule. Taken in conjunction with the observation that in the case of the trypsin digest it is the 67 kDa site which is most susceptible to cleavage in the presence of POU, this may suggest either a site of interaction centred close to the iTP site but giving some protection to the 67 kDa trypsin



**Figure 4.** Digestion of pTP with Ad 2 protease. (A) Schematic diagram indicating the cleavage sites of the Ad 2 protease on pTP. (B) pTP (30 ng) was preincubated with 0, 5, 10, 20 and 40 molar equivalents of POUhd and a corresponding amount of BSA to give a constant amount of protein in each experiment. The samples were then digested with Ad 2 protease (50 ng), activated by 15 min preincubation with activating peptide (19) for 15 min at 37°C and quenched with DTDP to 0.2 mM. The digestion products were separated by SDS-PAGE, blotted onto PVDF membrane and probed with a cocktail of anti-pTP mAbs, followed by anti-mouse horseradish peroxidase, and the bands visualised by ECL. (C) pTP was digested by Ad 2 protease in the presence of a 50 molar excess of POUhd or BSA as a control protein, aliquots containing 30 ng pTP and 140 ng POUhd or 200 ng BSA were removed at various time points, quenched with DTDP to 0.2 mM, fractionated on an SDS-polyacrylamide gel and blotted onto PVDF membrane. The blots were probed with a cocktail of anti-pTP mAbs and anti-mouse horseradish peroxidase and detected by ECL.

cleavage site 10 amino acids away or that a conformational change in pTP is brought about by the binding of POU rendering the TP site relatively more susceptible to cleavage than the iTP site.

Chymotrypsin initially digests pTP to give a 65 kDa fragment and then subsequent cleavage generates fragments of 50, 57 and 6 kDa. NFIII afforded pTP protection from digestion by chymotrypsin at all these sites, even after extended periods of digestion (Fig. 5A). Control digestions of I $\kappa$ B $\alpha$  by chymotrypsin in the presence of POU compared to BSA confirmed that the protection effect was specific to pTP and not as a result of any contaminating inhibitory effect on chymotrypsin (Fig. 5B).

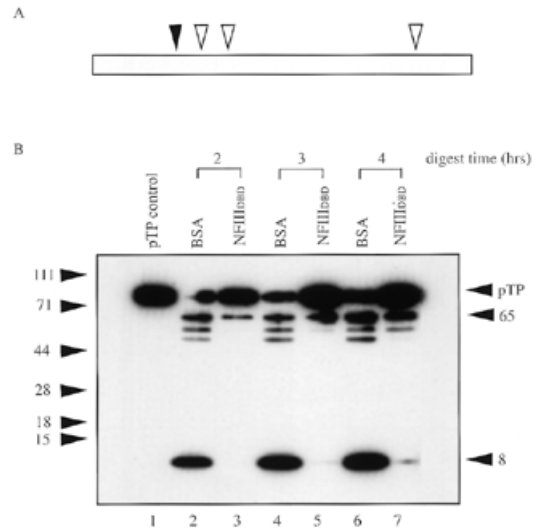


**Figure 5.** Digestion of pTP with chymotrypsin. **(A)** pTP (120 ng) was digested with chymotrypsin (2 ng) in the presence of a 50 molar excess of NFIII or BSA as a control protein, aliquots containing 30 ng pTP and 140 ng NFIII or 200 ng BSA were removed at various time points, quenched with PMSF to 5 mM, fractionated on an SDS–polyacrylamide gel and blotted onto PVDF membrane. The blots were probed with a cocktail of anti-pTP mAbs and anti-mouse horseradish peroxidase and detected by ECL. **(B)** IκBα (75 ng) was preincubated with POU (720 ng) or BSA (2400 ng) and then digested with various concentrations of chymotrypsin from 12.4 ng and doubling dilutions thereof, at room temperature for 5 min. The digestions were quenched with PMSF to 5 mM, run on an SDS–polyacrylamide gel and blotted onto PVDF membrane. The blots were probed with an anti-IκBα mAb (10B) and anti-mouse horseradish peroxidase and detected by ECL.

Endoproteinase Asp-N cleaves pTP to give similar size cleavage products to chymotrypsin, i.e. products of 65, 57 and 50 kDa and an 8 kDa C-terminal fragment (Fig. 6). Incubations had to be set up over a longer time period with this protease than the others, giving time points of 2, 3 and 4 h. Even with such long incubations a reasonable degree of protection at most sites was afforded by bound NFIII. However the cleavage site which generates the 65 kDa species appears to be equally accessible in free and NFIII-bound pTP.

## DISCUSSION

Multiple DNA–protein and protein–protein interactions stabilise the preinitiation complex formed at the adenovirus origin of DNA replication. It was originally reported that the POUhd of NFIII interacts with the pTP–pol heterodimer (21) and later established that the enhanced binding of pTP–pol to the origin of DNA replication detected in the presence of NFIII was mediated by a direct contact between pTP and POU (10). Here we have investigated the regions of pTP which participate in this interaction.



**Figure 6.** Digestion of pTP by endoproteinase-Asp-N. **(A)** Schematic diagram indicating the endoproteinase Asp-N cleavage sites on pTP. The open arrows indicate the protected sites and the solid arrow the less protected site. **(B)** pTP (15 ng) was digested with endoproteinase Asp-N (2 ng) in the presence of a 50 molar excess of NFIII or BSA as a control protein, aliquots containing 30 ng pTP and 140 ng NFIII or 200 ng BSA were removed at various time points, quenched with EDTA to 50 mM, fractionated on an SDS–polyacrylamide gel and blotted onto PVDF membrane. The blots were probed with a cocktail of anti-pTP mAbs and anti-mouse horseradish peroxidase and detected by ECL.

Partial proteolysis protection studies showed that POU gave good protection from proteolysis at a number of sites across the pTP molecule. Under these limited proteolysis conditions, only cleavage sites within exposed regions on the surface of the protein, which, for example, form disordered loop regions between areas of well-defined secondary structure, are attacked. These exposed regions are also, by definition, likely to be available for and involved in contact with other proteins. It is noteworthy that all four proteases particularly target a region between 67 and 50 kDa from the C-terminus producing a range of cleavage products. This, therefore, suggests that the region between the iTP and TP cleavage sites is relatively more exposed than the rest of the molecule.

A number of the cleavage sites were studied in some detail to determine whether delayed proteolysis at a site was a direct consequence of protection at that site or caused by protection at another site delaying a prerequisite cleavage that reveals the second site for proteolysis. It was found that several sites throughout the molecule were independent cleavage sites and thus their protection could be seen to imply multiple contact points for the POUhd on pTP. This was confirmed by cloning and expressing fragments corresponding to those produced by Ad 2 protease cleavage. Expression in the TNT coupled wheat-germ extract system should allow the fragments to fold properly into independent domains. All of these pTP domains interacted with POU, confirming that multiple contacts are made. The relative strength of the interactions indicated that the iTP–TP section already identified as the most surface exposed region of the protein makes the most important

contribution to binding, followed by the N-terminal to iTP section, and that the TP portion was least involved in the interaction. As the POUhd has a compact globular structure and is only 7.5 kDa, portions of pTP widely separated in the linear sequence must be brought together, possibly by a conformational change, to form the components of a POUhd binding site. Some evidence has been obtained for a possible conformational change in pTP which is induced by changes in NaCl concentration, suggesting that it may at least be possible to induce a change in the conformation of pTP. A conformational change in pTP would allow for a mechanism of dissociation of pTP from NFIII once NFIII has accomplished the task of helping to direct the pTP-pol heterodimer to the origin and positioning it correctly for interaction with base pairs 1–18. pTP and POU do not rapidly dissociate when complexed unless dissociation is specifically triggered, when it is then facile. It is possible that the action of covalently linking a dCMP residue onto Ser580 in the TP domain of pTP may act as such a trigger. This would explain why NFIII interacts with the TP fragment but apparently not with TP covalently attached to DNA (12). However van Leeuwen *et al.* (10) have demonstrated that NFIII is bound to the DNA until the replication fork passes through. This mechanism would involve NFIII remaining bound at the origin but not interacting with pTP whilst some 40 residues are added to the growing nucleotide chain. Thus NFIII would not be performing an active stabilising role during this time. However the NFIII interaction with DNA may be too strong to allow its efficient removal from the recognition site by any other mechanism than rendering the recognition site single stranded, as occurs when the replication fork passes through.

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#### REFERENCES

- Hay, R.T. and Russell, W.C. (1989) *Biochem. J.*, **258**, 3–16.
- Robinson, A.J., Younghusband, H.B. and Bellet, A.J.D. (1973) *Virology*, **56**, 54–59.
- Challberg, M.D. and Rawlins, D.R. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 100–104.
- Nagata, K., Guggenheimer, R.A. and Hurwitz, J. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 4266–4270.
- Pruijn, G.J.M., Van Priel, W. and van der Vliet, P.C. (1986) *Nature*, **322**, 656–659.
- Cleat, P.H. and Hay, R.T. (1989) *EMBO J.*, **8**, 1841–1848.
- Bosher, J., Robinson, E.C. and Hay, R.T. (1990) *New Biol.*, **2**, 1083–1090.
- Chen, M., Mermod, N. and Horwitz, M.S. (1990) *J. Biol. Chem.*, **265**, 18634–18642.
- Mul, Y.M., Verrijzer, C.P. and van der Vliet, P.C. (1990) *J. Virol.*, **64**, 5510–5518.
- Van Leeuwen, H.C., Rensen, M. and van der Vliet, P.C. (1997) *J. Biol. Chem.*, **272**, 3398–3405.
- Temperley, S.M. and Hay, R.T. (1992) *EMBO J.*, **11**, 761–768.
- Pronk, R. and van der Vliet, P.C. (1993) *Nucleic Acids Res.*, **21**, 2293–2300.
- Challberg, M.D., Desiderio, S.V. and Kelly, T.J. (1980) *Proc. Natl Acad. Sci. USA*, **77**, 5105–5109.
- King, A.J. and van der Vliet, P.C. (1994) *EMBO J.*, **13**, 5786–5792.
- King, A.J., Teertstra, W.R., Blanco, L., Salas, M. and van der Vliet, P.C. (1997) *Nucleic Acids Res.*, **25**, 1745–1752.
- Webster, A., Leith, I.R. and Hay, R.T., (1997) *J. Virol.*, **71**, 539–547.
- Jaffray, E., Wood, K.M. and Hay, R.T. (1995) *Mol. Cell. Biol.*, **15**, 2166–2172.
- Southern, J.A., Young, D.F., Heaney, F.C., Baumgartner, W. and Randall, R.E. (1991) *J. Gen. Virol.*, **72**, 1551–1557.
- Webster, A., Leith, I.R. and Hay, R.T. (1994) *J. Virol.*, **68**, 7292–7300.
- Erlanger, B.F., Kokowsky, N. and Cohen, W. (1961) *Arch. Biochem. Biophys.*, **95**, 271–278.
- Coenjaerts, F.E.J., van Oosterhout, J.A.W.M. and van der Vliet, P.C. (1994) *EMBO J.*, **13**, 5401–5409.
- Klemm, J.D., Rould, M.A., Aurora, R., Herr, W. and Pabo, C.O. (1994) *Cell*, **77**, 21–32.
- Cox, M., van Tilborg, P.J.A., de Laat, W., Boelens, R., van Leeuwen, H.C., van der Vliet, P.C. and Kaptein, R. (1995) *J. Biomol. NMR*, **5**, 23–32.
- Sturm, R.A., Das, G. and Herr, W. (1988) *Genes Dev.*, **2**, 1582–1599.