Domain Organization of the Adenovirus Preterminal Protein

AILSA WEBSTER,[†] IAN R. LEITH,[‡] AND RONALD T. HAY*

School of Biological and Medical Science, University of St. Andrews, Fife KY16 9AL, Scotland

Received 8 July 1996/Accepted 9 October 1996

In adenovirus-infected cells, the virus-encoded preterminal protein and DNA polymerase form a heterodimer that is directly involved in initiation of DNA replication. Monoclonal antibodies were raised against preterminal protein, and epitopes recognized by the antibodies were identified by using synthetic peptides. Partial proteolysis of preterminal protein reveals that it has a tripartite structure, with the three domains being separated by two protease-sensitive areas, located at sites processed by adenovirus protease. These areas of protease sensitivity are probably surface-exposed loops, as they are the sites, along with the C-terminal region of preterminal protein, recognized by the monoclonal antibodies. Preterminal protein is protected from proteolytic cleavage when bound to adenovirus DNA polymerase, suggesting either multiple contact points between the proteins or a DNA polymerase-induced conformational change in preterminal protein. Two of the preterminal protein-specific antibodies induced dissociation of the preterminal protein-adenovirus DNA polymerase heterodimer and inhibited initiation of adenovirus DNA replication *in vitro*. Antibodies binding close to the primary processing sites of adenovirus protease inhibited DNA binding, consistent with UV cross-linking results which reveal that an N-terminal, protease-resistant domain of preterminal protein contacts DNA. Monoclonal antibodies recognizing epitopes within the C-terminal 60 amino acids of preterminal protein stimulate DNA binding, an effect mediated through a decrease in the dissociation rate constant. These results suggest that preterminal protein contains a large, noncontiguous surface required for interaction with DNA polymerase, an N-terminal DNA binding domain, and a C-terminal regulatory domain.

Adenovirus DNA replication operates via a protein-priming mechanism, with the origins of replication being located at either end of the linear, 36-kbp virus genome and a virus-coded protein, the adenovirus preterminal protein (pTP), acting as the protein primer (reviewed in reference 35). Initiation of adenovirus DNA replication involves template-dependent transfer of dCMP to a specific hydroxyl group of pTP. This is followed by elongation of the nascent DNA strand by adenovirus DNA polymerase (Adpol), with fork movement being accompanied by DNA strand displacement (7, 9, 14, 35). In addition to pTP and Adpol, virus-coded single-stranded DNA binding protein (DBP) and two cellular factors, nuclear factor I (NFI or CTF1) and nuclear factor III (NFIII or Oct1) are required for virus DNA replication (24, 29, 43). Our understanding of the mechanisms involved in DNA replication has been considerably enhanced through the overexpression of these proteins and the development of *in vitro* assay systems using purified components (reviewed in reference 7).

Assembly of the preinitiation complex is a cooperative event involving interactions of the five aforementioned proteins, both with each other and with specific DNA sequences, located within inverted terminal repeats. pTP and Adpol form a stable heterodimer which binds to bp 9 to 18 of the genome (25, 32, 39, 41). The heterodimer is guided into position at the core origin of replication through specific associations with NFI (1–3, 24) and NFIII (6), which bind to auxiliary sequences in the inverted terminal repeats at bp 19 to 39 and 40 to 51, respectively. Binding of NFI to its recognition sequence is

enhanced by DBP through an alteration in the structure of DNA (4, 40). DBP has also been shown to stimulate initiation in an NFI-independent manner, but the mechanics of this remain to be elucidated (26). One possibility is that DBP is a component of the preinitiation complex, and some evidence exists that DBP interacts with Adpol (20). After assembly of the complex, Adpol catalyzes the transfer of dCMP to Ser₅₈₀ of pTP in the initiation event. Recently it has been shown that pTP-CAT accumulates as an intermediate in replication, and initiation has been shown to proceed via a jump-back mechanism (17), analogous to the sliding-back model described for bacteriophages ϕ 29 and PRD1 (22). This contrivance enables restoration of small terminal deletions or mutations and is thought to serve to preserve the integrity of the linear, viral genome. Surprisingly, NFI dissociates from the complex at an early stage, as a direct result of a conformational change in Adpol induced by binding of deoxynucleoside triphosphates (5). Other events in the disassembly of initiation complex and the transition between initiation and elongation remain poorly understood, although DBP, which is essential for elongation, has been shown to have helix-destabilizing properties and to increase processivity of Adpol (20, 23, 49). Virus DNA replication is followed by transcription and translation of late proteins and assembly of virus particles. After packaging of pTP-DNA into virus cores, genome-linked pTP is processed, via a 62-kDa intermediate (iTP), to mature terminal protein (TP) by adenovirus-coded protease (38, 46). Roles that have been assigned to the TP moiety, attached to the 5' end of genome, include matrix attachment, activation of transcription, distortion of origin structure, and stabilization of preinitiation complex (28, 36).

Further insight into molecular events of adenovirus DNA replication, which involve a complex series of associations, dissociations, and conformational changes, can be gained by a deeper understanding of the structures and functions of the individual proteins. Structures of the DNA binding domains of DBP and NFIII have already been solved (8, 42), but relatively

* Corresponding author. Mailing address: School of Biological and Medical Science, Irvine Building, University of St. Andrews, Fife KY16 9AL, Scotland. Phone: 44 1334 463396. Fax: 44 1334 463400. E-mail: rth@st-and.ac.uk.

[†] Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.

[‡] Present address: Biomedical Research Centre, Ninewells Hospital Medical School, University of Dundee, Dundee, Scotland.

little is known about NFI, Adpol, and the subject of this study, pTP. Previous attempts to identify functionally important domains in pTP have been confined to insertion/deletion mutagenesis (10–12, 27, 33, 34), with the general conclusions from such studies being that regions spanning the protein are required for DNA replication and virus viability. Here we describe the domain structure of pTP determined by a combination of approaches, including antibody accessibility experiments, partial proteolysis, and photochemical cross-linking. This information was used to identify regions of pTP participating in interactions with DNA and Adpol.

MATERIALS AND METHODS

Purification of pTP, Adpol, DBP, NFI, and NFII. Adenovirus type 2 (Ad2) pTP was expressed in *Spodoptera frugiperda* sf9 cells by using recombinant baculoviruses as described previously (41). Cells were resuspended in ice-cold 50 mM HEPES-NaOH (pH 8)–5 mM KCl–0.5 mM MgCl₂ supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg of pepstatin per ml, 1 µg of E-64 per ml, and 1 µg of pepstatin per ml), incubated on ice for 10 min, then disrupted by 15 strokes in a Dounce homogenizer using a type B pestle. Nuclei were collected by centrifugation (6,000 × g, 3 min, 4°C), suspended in 25 mM HEPES-NaOH (pH 8)–1 mM dithiothreitol (DTT)–1 M NaCl with protease inhibitors (see above), and incubated on ice for 15 min. Extracts were clarified by centrifugation (100,000 × g, 20 min, 4°C), diluted 1:4 in 50 mM Tris-HCl (pH 8)–1 mM EDTA–10% glycerol–10 mM 2-mercaptoethanol–1.5 M KCl (buffer A), and loaded onto a 6-ml phenyl-agarose column (Sigma P8901) equilibrated with 10 volumes of buffer A. Column was then washed with 2 volumes of the same buffer, and pTP was eluted with 50 mM Tris-HCl (pH 8)–1 mM EDTA–20% glycerol–0.6 M NaCl–10 mM 2-mercaptoethanol. Fractions containing pTP were pooled and dialyzed against 25 mM HEPES-NaOH (pH 8)–1 mM EDTA–1 mM DTT–20% glycerol–0.4 M NaCl–1 mM phenylmethylsulfonyl fluoride and added to an equal volume of 25 mM HEPES-NaOH (pH 8)–50 mM NaCl–500 mM sucrose–1 mM EDTA–0.1% octylglucopyranoside. Prior to loading onto a 4-ml single-stranded DNA–cellulose column pre-equilibrated with 25 mM HEPES-NaOH (pH 8)–1 mM EDTA–0.2 M NaCl–1 mM DTT–10% glycerol (buffer B), the sample was diluted 1:2 in buffer B and clarified by centrifugation (15,000 × g, 30 min, 4°C). pTP was eluted from the column with 25 mM HEPES-NaOH (pH 8)–1 mM EDTA–1 mM DTT–0.6 M NaCl–10% glycerol and stored in aliquots at –70°C.

Adpol, DBP, and NFI were purified from sf9 cells infected with recombinant baculoviruses as described previously (2, 23, 41). NFII was expressed as a fusion with glutathione *S*-transferase (plasmid obtained from P. O'Hare, Marie Curie Research Institute, Oxford, England) and purified by affinity chromatography on glutathione-agarose and DNA-Sepharose.

Proteolytic digestions of pTP. Ad2 pTP was digested to iTP and TP, using activated, recombinant Ad2 protease as previously described (46). pTP was incubated in the presence of endoproteinase LysC (endo-LysC), endo-AspN, chymotrypsin, and trypsin at 37°C for times and protein/protease ratios indicated. For protection experiments, pTP was preincubated with a 10-fold excess of purified Adpol or protease-free bovine serum albumin (BSA) (as a control) for 30 min at 37°C and then digested with endo-AspN or endo-LysC for 16 h at 37°C (protein/protease ratio was 50:1). The equivalent of 20 ng of pTP was loaded per lane, and digestion was monitored by Western blotting and probing with the appropriate antibody.

SDS-PAGE and immunoblotting. Protein samples were denatured and reduced by boiling in the presence of 1.25% sodium dodecyl sulfate (SDS) and 0.35 M 2-mercaptoethanol and then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting as described previously (46). Tissue culture supernatants containing anti-pTP antibodies were diluted 1:40, with the exception of 46E6 supernatant, which was undiluted, and used as primary antisera in immunoblotting. The preparation and use of polyclonal antisera specific for the N-terminal 15 amino acids (aa) of pTP has been described elsewhere (46).

Low-molecular-weight peptides were analyzed in 16.5% discontinuous Tricine gels (37).

Peptide synthesis and enzyme-linked immunosorbent assays (ELISAs). Peptides HNRDMTGGVFQRLRC, GRHLRPNSSAAVAIDAR, AAAVAIDAR, and VERLMQDYKDLRRRC were synthesized as described previously (44). Ninety-six-well plates were coated with 1% glutaraldehyde at 4°C for 8 h, washed with phosphate-buffered saline (PBS), and then incubated with peptide (0.05 mg/ml in PBS) at 4°C overnight. After removal of free peptide, plates were dried at 37°C. Plates were blocked with 10% nonfat milk in PBS and then incubated with dilution series of hybridoma-containing tissue culture supernatants for 1 h at room temperature. Wells were washed with three times with 200 µl of PBS–1% nonfat milk and incubated with horseradish peroxidase-linked anti-mouse immunoglobulin (Ig) (Amersham) diluted 1:2,000 in PBS for 1 h at room temperature. Wells were washed as before and then washed with twice with 200 µl of PBS prior to development with 3,3',5,5'-tetramethyl benzidine (TMB) color development reagent (Dynatech).

FIG. 1. Bar diagram of pTP indicating positions of sites cleaved by adenovirus protease (A) and endo-LysC (LysC). The C-terminal lysine was cleaved by endo-LysC only in the presence of 0.1% SDS. The sizes and positions of fragments generated by adenovirus protease and endo-LysC are indicated above and below the bar, respectively.

Preparation and mapping of monoclonal antibodies against pTP. Inbred BALB/c mice were immunized intraperitoneally with purified pTP (50 µg of protein per immunization) emulsified with complete Freund's adjuvant. The mice were reimmunized intraperitoneally with pTP emulsified with incomplete Freund's adjuvant at 24 days and with purified pTP alone at 55 days. Sixty days after the initial immunization, mice were sacrificed and their spleens were removed. Splenocytes were fused with SP2/0-Af-14 myeloma cells (13, 31). Hybridoma cells secreting antibodies specific for Ad2 pTP were selected by coating nitrocellulose with Ad2 pTP, incubating the sheets with media from the hybridoma cells, and identifying bound antibody with horseradish peroxidase-conjugated anti-mouse Ig and enhanced chemiluminescence (30). Sixteen hybridoma cell lines that produced antibodies reacting with native pTP in a dot blot assay were identified and isolated. Antisera from the resulting tissue culture supernatants were isotyped by using a hemagglutination kit (Seralab), and 11 of these, termed 3D11, 5E3, 6E2, 7A2, 7H1, 10B1, 11F11, 16H1, 44E1, 46E1 and 49E4, were found to be IgG1s. Western blot analysis revealed that eight of the antibodies recognized primary sequences within pTP, the exceptions being 6E2, 10B1, and 49E4, which recognised conformational epitopes and did not bind to denatured pTP.

Hybridomas were grown in serum-free medium (Gibco-BRL), and supernatants were used as the source of specific antibodies. Antibodies were purified by binding to protein A-Sepharose and eluting with 100 mM citric acid (pH 3). Following dialysis against PBS, antibodies were diluted to 1 mg/ml and stored in aliquots at –20°C (13). A monoclonal antibody raised against IkBα (16) was purified from serum-free supernatants and used as a control.

Monoclonal antibodies reacting with linear epitopes were mapped by assessing their reactivities with six specific proteolytic fragments of pTP in Western blot analysis. Fragments tested were 55-kDa TP-, two N-terminal fragments, termed NT1 (18 kDa) and NT2 (16 kDa), generated by incubation of pTP with activated adenovirus protease (46), and three polypeptides generated by endo-LysC (protein/protease ratio of 50:1, 16 h at 37°C in the presence of 0.1% SDS) with apparent molecular masses of 6, 22, and 60 kDa. The 6- and 22-kDa polypeptides were shown to contain the C and N termini, respectively, of pTP. Origins of these adenovirus protease and endo-LysC digestion products of pTP are shown schematically in Fig. 1.

Where appropriate, antibodies were tested by ELISA for binding to synthetic peptides GRHLRPNSSAAVAIDAR and VERLMQDYKDLRRRC (peptides from the region just after iTP cleavage sites; aa 184 to 200 and 213 to 227) and HNRDMTGGVFQRLRC (peptide spanning the TP cleavage site; aa 342 to 354).

Immunoprecipitations. A 50-µl bed volume of protein G-Sepharose beads (Sigma) was incubated with 1 ml of serum-free tissue culture supernatant from the appropriate hybridoma cell line for 2 h at room temperature with rotation. Beads were washed three times with 1 ml of 50 mM Tris-HCl (pH 8)–1 mM EDTA–0.4 M NaCl–0.05% Nonidet P-40 (IP buffer), and a 10-µl bed volume of antibody-coated beads was incubated with 20 µl of baculovirus cell extract containing 2 µg of pTP and 20 µg of Adpol (or protease-free BSA) for 1 h at room temperature with rotation. Prior to incubation with coated beads, extracts were diluted 1:5 in IP buffer and clarified by centrifugation (100,000 × g, 4°C, 20 min). Beads were collected by centrifugation (6,000 × g, room temperature, 10 s) and washed four times with 1 ml of IP buffer prior to boiling in the presence of SDS and 2-mercaptoethanol and analysis of the immunoprecipitated material by SDS-PAGE (12.5% polyacrylamide gel).

In vitro DNA replication assays. In vitro initiation reactions were carried out with purified recombinant proteins and purified Ad2 complex as the template. Ad2 complex was prepared by guanidinium-HCl denaturation of virus particles and gel filtration chromatography as described elsewhere (18). Assays were in a final volume of 10 µl comprising 25 mM bicine-NaOH (pH 8), 0.2 mg of protease-free BSA per ml, 2 mM DTT, 1 mM MnCl₂, 0.15 mM ATP, 500 ng of DBP, 20 ng of NFI, 20 ng of NFII, 50 ng of pTP-Adpol, 50 ng of purified monoclonal antibody, and 60 ng of Ad2 complex; 2.5 µCi of [α -³²P]dCTP (3,000 Ci/mmol; Amersham) was added, and reactions were carried out for 1 h at 30°C. Reaction mixtures were then made 10 mM with respect to CaCl₂ and 1 U of micrococcal nuclease was added to each prior to incubation for a further 30

TABLE 1. Locations of epitopes for monoclonal antibodies that recognize the Ad2 pTP

MAb ^a	DB ^b	WB ^c	IP ^d	Location of epitope
5E3	+	+	+	184–200
16H1	+	+	+	184–200
46E6	+	±	±	213–227
44E1	+	+	+	342–354
7A2	+	+	+	342–354
7H1	+	+	–	388–607
3D11	+	+	+	608–671
11F11	+	+	+	608–671
6E2	+	–	+	Conformational
10B1	+	–	+	Conformational
49E4	+	–	–	Conformational

^a All monoclonal antibodies (MAb) characterized were of the IgG1 isotype.

^b Ability to recognize purified Ad2 pTP immobilized on nitrocellulose in a dot blot assay.

^c Ability to detect Ad2 pTP by Western blotting.

^d Ability to immunoprecipitate Ad2 pTP.

min at 37°C. Samples were analyzed by SDS-PAGE (12.5% polyacrylamide) and autoradiography.

Gel electrophoresis DNA binding assays. Two complementary oligonucleotides containing positions 1 to 18 of the Ad2 origin of DNA replication, 5'-GA TCCATCATCAATAATATACCG-3' (top strand) and 5'-GATCCGGTATAT TATTGATGATG-3' (bottom strand), were annealed and labeled with the Klenow fragment of DNA polymerase I in the presence of [α -³²P]dATP and dCTP, and the labeled fragment was purified on an 8% polyacrylamide gel. DNA was eluted from the gel by passive diffusion (overnight at 20°C) into 10 mM Tris-HCl (pH 7.5)–100 mM NaCl–1 mM EDTA and stored at 4°C. Purified pTP was diluted to 2 to 100 ng/ μ l in 100 mM potassium phosphate (pH 6)–2 mM DTT–0.4 M KCl–10% glycerol–1 mg of protease-free BSA per ml (dilution buffer), and 2 to 100 ng of pTP was incubated with 1 ng of ³²P-end-labeled, double-stranded oligonucleotide for 15 min on ice in a total volume of 20 μ l containing 25 mM bicine-NaOH (pH 8), 2 mM DTT, 4 mM MgCl₂, 20 mM KCl, and 1 mg of BSA per ml. At the end of the incubation period, glycerol was added to 5% and samples were loaded onto a 6% polyacrylamide gel containing 50 mM Tris-borate and 1 mM EDTA (pH 8.3), which was electrophoresed at 150 V for 2 h. In certain assays, purified monoclonal antibodies (50 ng) were included and incubated with pTP for 15 min at room temperature prior to the addition of oligonucleotide probe. Gels were fixed in 20% methanol–10% acetic acid for 30 minutes, dried, and analyzed by autoradiography. Results were quantitated by phosphor imaging.

Association of pTP with oligonucleotide probes was determined as a function of time by incubating 200 ng of pTP with 500 ng of purified monoclonal antibody for 30 min on ice in a total volume of 210 μ l containing 25 mM bicine-NaOH (pH 8), 2 mM DTT, 1 mM EDTA, 20 mM KCl, 1 mg of BSA per ml, and 5% glycerol (DNA binding buffer). At time zero, 20 ng of ³²P-end-labeled, double-stranded oligonucleotide was added, the reaction components were mixed, and 1/10 of the reaction mixture was applied to a 6% polyacrylamide gel (already running at 200 V) at 15 s, 30 s, and 1, 2, 5, 10 and 30 min after addition of probe. Electrophoresis was continued for 90 min at 4°C, and gels were fixed, dried, and analyzed as described above. Determination of the rate of dissociation of pTP from oligonucleotide probe, and the effects of monoclonal antibodies thereon, were investigated by incubating 200 ng of pTP and 500 ng of purified monoclonal antibody with 20 ng of ³²P-end-labeled, double-stranded oligonucleotide for 30 min on ice in a total volume of 200 μ l containing DNA binding buffer (see above). At time zero, 1 μ g of unlabeled, double-stranded competitor oligonucleotide was added, and 1/10 of the reaction volume was applied to a 6% polyacrylamide gel, already running at 200 V (see above), at 0, 15, and 30 s and 1, 2, 5, 10, 20, and 30 min after addition of unlabeled competitor DNA (21).

UV cross-linking of pTP to DNA. Purified pTP (or iTP or TP) was diluted to 100 ng/ μ l in dilution buffer (see above), and 100 ng of pTP was incubated with 10 ng of the ³²P-labeled, double-stranded oligonucleotide containing the Ad2 origin of DNA replication (described above) for 15 min on ice in a total volume of 20 μ l containing 25 mM bicine-NaOH (pH 8), 2 mM DTT, 1 mM EDTA, 20 mM KCl, and 1 mg of protease-free BSA per ml. Purified monoclonal antibodies (100 ng) were included in certain assays. The reaction mixtures were transferred onto the surface of a siliconized glass plate, which was placed on ice 2 cm below a UV transilluminator (UVP Inc. model TM-36 with maximal spectral output at 300 nm) and irradiated for 45 min. After irradiation, samples were either reduced and denatured in preparation for analysis by SDS-PAGE or incubated for 6 h at 37°C in the presence of either 50 ng of activated, recombinant Ad2 protease (45), 0.1 μ g of endo-LysC, or 0.1 μ g of endo-AspN. Digestions with endo-AspN were in the presence of 10 mM MgSO₄. Samples were then reduced,

denatured, and separated on an SDS–15% polyacrylamide gel, which was dried and analyzed by autoradiography.

RESULTS

Domain organization of pTP. Monoclonal antibodies and proteases are useful tools in the determination of the domain organization of proteins, as they can be used as site-specific probes of surface accessibility. To use this approach for the analysis of pTP structure, we prepared a panel of monoclonal antibodies and determined their sites of interaction with pTP by assessing their reactivities with both specific proteolytic fragments of pTP and synthetic peptides (see Materials and Methods). Properties of the monoclonal antibodies, together with the regions of pTP to which they bind, are listed in Table 1. Epitopes recognized by the antibodies were found to cluster in three main regions of the protein; just after the iTP cleavage sites (5E3, 16H1, and 46E6), spanning the TP cleavage site (7A2 and 44E1), and close to the C terminus of pTP (3D11 and 11F11), suggesting that these regions are surface exposed (Fig. 2B).

Previous studies have identified the sites in pTP cleaved by adenovirus protease to generate iTP and mature terminal protein (38, 46). Here we probed pTP with trypsin, chymotrypsin, endo-LysC, and endo-AspN, at a range of concentrations, with a view to identifying protease-resistant domains and protease-susceptible sites. For each protease, digestion products were identified by N-terminal sequencing and by their reactivities with the N-terminal antipeptide serum or with antibody 3D11, which binds to an epitope at the C terminus of pTP (Fig. 2B). Incubation of pTP with low levels of trypsin results in an initial

FIG. 2. Domain organization of pTP. (A) Assay performed with untreated pTP (–) or 10 μ g of purified pTP treated with 10, 20, 40, and 100 ng of trypsin (Trp.) or chymotrypsin (Chy.). Digestion products were resolved in a 15% polyacrylamide gel containing SDS and detected by staining with Coomassie brilliant blue. Positions of molecular weight markers are indicated on kilodaltons on the left, while pTP and molecular masses (in kilodaltons) of its major digestion products are indicated on the right. (B) The locations of protease cleavage sites in pTP were determined by a combination of N-terminal sequencing of proteolytic fragments, after electrophoretic transfer onto Problot, and Western blot analysis with monoclonal antibodies. Sites of cleavage by proteases (A, adenovirus protease; T, trypsin; C, chymotrypsin) are indicated by arrows, while the location of epitopes for monoclonal antibodies and the nuclear localization signal (NLS) are indicated by black bars. The mature TP (processed form of pTP) is indicated by the shaded region.

cleavage close to the iTP site, while higher levels of protease result in the generation of two trypsin-resistant fragments of 40 and 22 kDa (Fig. 2A). The 22-kDa polypeptide corresponds to the N terminus of pTP, while sequence analysis of the 40-kDa fragment revealed that it had the N-terminal sequence RRVPPPP, located just after the nuclear localization sequence in TP (48). This fragment extended to the C-terminal region of the protein, as it was recognized by antibody 3D11. Chymotrypsin and endo-LysC cleavage sites just C-terminal of the iTP sites were also identified, yielding 22-kDa N-terminal and 65-kDa C-terminal fragments in each case (Fig. 2A and data not shown). Identification of the polypeptides generated by endo-LysC was simplified by the observation that pTP contains only four lysines; three are clustered in the protease-sensitive region proximal to the iTP sites (aa 222, 246, and 258), and the fourth is at aa 608 near the C terminus of the protein (Fig. 1). The latter site is cleaved only by endo-LysC in the presence of 0.1% SDS, indicating that under native conditions, the lysine at the C terminus of pTP is not surface exposed. Incubation of pTP with endo-AspN at a protein/protease ratio of 50:1 resulted in the production of a single, stable fragment of 20 kDa, corresponding to the N terminus of pTP, revealing yet another protease cleavage site proximal to iTP sites (data not shown).

Proteolysis experiments have identified two major protease-sensitive regions within pTP, the first located around the iTP sites (aa 180 to 258) and the second encompassing the TP cleavage site and the nuclear localization site (aa 342 to 388). There is a remarkable correlation between these protease-sensitive sites and the regions of pTP containing epitopes recognized by the monoclonal antibodies (Fig. 2B). It is likely, therefore, that these regions are exposed on the surface of the protein as unstructured loops, a conclusion supported by the presence of the NLS in one of these regions; it would have to be exposed to be recognized by the proteins involved in nuclear transport. Proteolysis of pTP indicates that while there are protease-sensitive sites proximal to the iTP and TP sites, the N-terminal 20-kDa domain of the protein is resistant to attack by a range of proteases, including trypsin and chymotrypsin (Fig. 2A). Furthermore, a polyclonal antiserum raised against purified pTP failed to recognize the protease-resistant N-terminal fragment, while a peptide antiserum raised against the N-terminal 15 aa of pTP, although reactive in Western blots, did not immunoprecipitate the native protein (46). These observations, taken together with proteolysis results, suggest that the N-terminal domain of pTP is maintained as a highly ordered compact structure in which the N terminus is buried.

Mapping of regions of pTP interacting with Adpol. To define the regions of pTP interacting with Adpol, a form of protein-protein footprinting was used, employing specific proteases and monoclonal antibodies raised against pTP. pTP-Adpol and pTP (BSA control) were incubated with endo-LysC or endo-AspN at a protein/protease ratio of 50:1 for 16 h at 37°C. Samples were analyzed on 16.5% discontinuous Tricine gels, and the identities of the digestion products were determined by Western blot analysis, based on their apparent molecular weights and reactivities with the monoclonal antibodies. pTP contains four endo-LysC sites (Fig. 1) and multiple endo-AspN sites which are located throughout the C-terminal two-thirds of the protein. In the absence of Adpol, pTP is extremely susceptible to endo-AspN digestion, with only the N-terminal 20-kDa of the protein being resistant to the protease. Patterns obtained were identical for 3D11 and 11F11, 44E1 and 7A2, and 16H1 and 5E3. The blots for one representative of each class are shown in Fig. 3A along with that of 7H1.

The epitope for 3D11 is located at the C terminus of pTP,

FIG. 3. Partial proteolysis of pTP in the presence of Adpol. (A) Purified pTP was digested with endo-LysC (Lys C) or endo-AspN (Asp N) in the presence of Adpol or an equivalent amount of BSA. Digestion products were resolved in a 16.5% polyacrylamide gel containing Tricine and SDS, electrophoretically transferred to a polyvinylidene difluoride membrane, and analyzed by Western blotting with the indicated monoclonal antibodies. Sizes are indicated in kilodaltons. (B) Monoclonal antibody recognition sites on pTP (black bars), adenovirus protease cleavage sites (open arrowheads), and endo-LysC cleavage sites (filled arrowheads).

allowing the location of protease cleavage sites to be determined relative to the C terminus of the protein. Endo-AspN digestion of pTP, in the presence of BSA, produces a major species of 8 kDa that reacts with 3D11 (Fig. 3A), indicating that it is a C-terminal fragment of pTP. In the presence of Adpol (Fig. 3A), however, only trace amounts of this fragment were present with most of the reactive species being in the high-molecular-mass region (60-kDa band). This result indicates that binding of Adpol to pTP gives extensive protection in the C-terminal region of pTP, and in particular that an endo-AspN site, very close to Ser₅₈₀, is masked by binding of Adpol. Examination of the distribution of the high-molecular-weight species that are reactive with 3D11 and 7H1 (Fig. 3A) indicates that processing at the cluster of lysines to the C terminus of the iTP site, by endo-LysC, is partially protected in the presence of Adpol, as indicated by the greater intensity of the 60-kDa band in the presence of BSA compared to Adpol. It is noted that in these protection experiments, no SDS was included in endo-LysC digestions and that under such conditions, cleavage at the C-terminal lysine of pTP did not take place. With antibody 44E1 used in the Western blot analysis, the most striking difference between pTP cleaved in the presence of BSA and Adpol is observed in the endo-AspN digestions. In the presence of Adpol, we observed protection of a 60-kDa fragment encompassing the TP site; in the absence of Adpol, no 44E1-reacting species were detected (Fig. 3A). Thus, endo-AspN cleavage sites in the vicinity of TP cleavage site are strongly protected by Adpol, indicating that Adpol either contacts pTP at sites proximal to TP site or causes a conformational change in this region of the protein. In assays using antibody 5E3 (Fig. 3A), the only significant difference detected is in the level of processing by endo-LysC in the presence of Adpol, again demonstrating a degree of protection in the cluster of lysines, just C terminal of the iTP sites. Since none of the antisera were directed against the N terminus of pTP, protection in this region was investigated by using an affinity-purified peptide serum directed against the N-terminal

15 aa of pTP. No difference was observed between the BSA and Adpol samples for endo-AspN, trypsin, or chymotrypsin digestions (data not shown). Although binding of Adpol to the protease-resistant N terminus of pTP cannot be ruled out, our results show no protection in this region.

Taken together, these data suggest multiple contact points between Adpol and pTP, with strong protection observed at endo-AspN sites between iTP and TP sites, around the TP cleavage site, and in the C terminus of pTP in a region proximal to Ser₅₈₀. The observation that large regions of pTP interact with Adpol is consistent with the fact that the two proteins form a very stable heterodimer, 2 M urea being required to dissociate them (19). Additional evidence that the region to the C terminus of iTP site is required for binding of pTP to Adpol comes from studies in which pTP was immobilized on a CNBr-activated Sepharose column. The coupling was through lysine residues, and although pTP contains only four lysine residues, efficient coupling was observed. The C-terminal lysine is not surface exposed under nondenaturing conditions, and so coupling is presumed to be through the three lysine residues to the C terminus of the iTP site. Immobilizing pTP through these lysines completely abolished its ability to bind to Adpol (data not shown). In conclusion, much of the pTP primary sequence, stretching from iTP sites to its C terminus, is required to bind to Adpol, and the possibility that an extensive conformational change in pTP takes place upon binding of Adpol is not ruled out.

Dissociation of pTP from Adpol induced by 6E2 and 10B1.

The ability of each of the monoclonal antibodies to recognize pTP and pTP-Adpol heterodimer was investigated by immunoprecipitation. Each of the monoclonal antibodies was immobilized on protein G-Sepharose as described in Materials and Methods and incubated with either pTP or pTP-Adpol heterodimer. All antibodies except 7H1 (Fig. 4A) and 49E4 (data not shown) could immunoprecipitate pTP from the cell extract. Antibody 46E6 immunoprecipitated pTP significantly less efficiently than the other antibodies. All antibodies that could efficiently immunoprecipitate pTP (Fig. 4A) could also immunoprecipitate pTP-Adpol with the surprising exception of antibodies 6E2 and 10B1 (Fig. 4B). In the cases of these two conformational antibodies, pTP but not Adpol was immunoprecipitated. Since, under the assay conditions used, all pTP was present as a heterodimer with Adpol, it is concluded that 6E2 and 10B1 bind to pTP to induce a conformational change that dissociates the pTP-Adpol heterodimer. An alternative possibility is that the rate at which the heterodimer dissociates and reassociates is such that the 6E2 and 10B1 antibodies bind to free pTP and once bound preclude association with Adpol. Whichever of these two explanations turns out to be correct, the end result is the same: binding of the two conformational antibodies releases Adpol from the heterodimer. Clearly, in vivo, the antibodies will not be present to do this during DNA replication, but their action shows that in principle a protein-protein interaction could dissociate the heterodimer. Whether such a dissociation takes place in vivo and, if it does, the nature of the mechanism responsible are questions requiring further investigation. The effects of the monoclonal antibodies on DNA replication in vitro have been determined. In initiation reactions, both 6E2 and 10B1 inhibited the transfer of dCMP onto pTP during initiation of DNA replication (Fig. 4C). This finding is entirely consistent with the observations described above that antibodies 6E2 and 10B1 are able to bring about the dissociation of pTP from Adpol. In addition to the effects of 6E2 and 10B1, it is also noted that antibody 7A2 immunoprecipitated pTP-Adpol less efficiently than pTP (Fig. 4A and B),

FIG. 4. Dissociation of pTP from Adpol induced by 6E2 and 10B1. The indicated monoclonal antibodies (MAb) were immobilized on protein G beads and incubated with an extract from recombinant baculovirus-infected insect cells containing 2 μ g of pTP (A) or 2 μ g of pTP plus 20 μ g of Adpol (B). Proteins bound to beads were resolved on a polyacrylamide gel containing SDS and visualized by staining with Coomassie brilliant blue. The positions of Adpol, pTP, IgG heavy chain (HC), and IgG light chain (LC) are indicated. (C) Influence of monoclonal antibodies on the initiation of adenovirus DNA replication in vitro. The indicated monoclonal antibodies were added to an in vitro assay mixture containing purified proteins and protein-linked adenovirus genome. Initiation activity was measured by the transfer of [32 P]dCMP onto pTP. Reaction products were resolved in a polyacrylamide gel containing SDS, and radioactive species were visualized by autoradiography. The position of [32 P]dCMP is indicated.

indicating a reduced avidity of this antibody for heterodimer compared to free pTP.

Identification of the DNA binding domain of pTP. In contrast to the interaction between pTP and Adpol, which is required for initiation of DNA replication, the role of the DNA binding activities of pTP remain to be established. It has previously been shown that pTP binds weakly to DNA (41), and it was noted that the DNA-protein complex migrates more slowly than expected for an 87-kDa protein, as it comigrates with the pTP-Adpol-DNA complex (41). One possibility is that an unusual primary amino acid sequence in pTP or conformation of pTP-DNA is responsible for the slower than expected migration. Alternatively, the retarded species may represent a dimer or multimer of pTP binding to DNA. Gel filtration analysis (41) and chemical cross-linking studies (data not shown) indicate that pTP is monomeric in solution under the conditions examined. In this study, we used two complementary assays to study pTP-DNA binding: UV cross-linking and gel retardation assays.

To identify regions of pTP that contact DNA, a combination

FIG. 5. Identification of the DNA binding domain of pTP. (A) Purified pTP (100 ng) was incubated with ^{32}P -labeled DNA (10 ng) containing 1 to 18 bp from the adenovirus genome and irradiated with UV light. Samples were either reduced and denatured immediately (–) or incubated for 6 h at 37°C either in the absence of added protease (–) or in the presence of 50 ng of activated recombinant Ad2 protease (Ad2 prot), 100 ng of endo-LysC (Lys-C), or 100 ng of endo-AspN (Asp-N). Samples were then reduced, denatured, and separated on a 15% polyacrylamide gel, which was dried and analyzed by autoradiography. The molecular masses (in kilodaltons) of radioactive cleavage products are indicated. (B) The locations of the ^{32}P -labeled digestion products are indicated (black bars) on a schematic of the pTP protein. The open box is the region removed by processing, whereas the shaded box represents the mature TP. Cleavage sites for the adenovirus protease (open arrowheads), endo-LysC (filled arrowheads), and endo-AspN (filled triangles) are indicated.

of UV cross-linking and specific proteolysis was used. pTP was preincubated with ^{32}P -labeled double-stranded DNA containing positions 1 to 18 from the adenovirus genome prior to being irradiated with UV light, reduced and denatured, and analyzed by SDS-PAGE. The autoradiogram (Fig. 5A) indicates that such treatment results in the covalent cross-linking of pTP to the labeled DNA. Incubation of the UV-cross-linked material with Ad2 protease, endo-LysC, and endo-AspN resulted in generation of labeled species of 18, 22, and 20 kDa, respectively (Fig. 5A). On the basis of electrophoretic mobility and reactivity with N-terminal peptide antisera, it was concluded that all of the radioactive species were derived from the N terminus of pTP (Fig. 5B). Thus, UV cross-linking experiments indicate that the N terminus of pTP is in contact with DNA. Previous work revealed that digestion of pTP to iTP abolished DNA binding activity in a gel electrophoresis DNA binding assay (46). Here we digested pTP to iTP and TP, using the conditions described previously, and carried out UV cross-linking experiments, as described above, for the three species. The N-terminal fragment, N1, is cross-linked to DNA in iTP and TP samples, while gel electrophoresis DNA binding assays (results not shown) were consistent with previous results (46) revealing that only the intact pTP, not any of its digestion products (iTP, TP, NT1, or NT2), bound to DNA. The apparently contradictory nature of the UV cross-linking and gel retardation results, with respect to the binding of NT1, can be resolved when it is considered that cross-linking requires only a transient interaction between the protein and DNA, while for an interaction to be detected in a gel retardation assay, a longer association is required.

One interpretation of these results is that the N terminus of pTP contacts DNA directly but that other regions of the protein are required to stabilize the interaction.

Effects of monoclonal antibodies on DNA binding activity of pTP. The effects of monoclonal antibodies on the DNA binding activity of pTP were determined in a gel electrophoresis DNA binding assay using an end-labeled oligonucleotide probe. pTP (20 ng) was preincubated with purified monoclonal antibodies (50 ng) prior to incubation with the labeled DNA. Antibodies 7A2 and 7H1 did not influence the binding of pTP to DNA, giving binding equivalent to that observed either in the absence of antibody or in the presence of control antibody (Fig. 6A). Antibody 16H1 and the conformational antibodies, 6E2 and 10B1, inhibited binding of pTP to the labeled DNA, while 3D11, 5E3, 11F11, 44E1, and 46E6 all decreased the electrophoretic mobility of the pTP-DNA complex (Fig. 6A). Although a shift-up of the pTP-DNA complex by antibodies 5E3 and 44E1 was observed (Fig. 6A), the total amount of pTP bound to DNA was considerably less than in the control lanes, indicating partial inhibition of DNA binding of pTP by these antibodies. In addition, it was noted that preincubation with

FIG. 6. Effects of monoclonal antibodies on the DNA binding activity of pTP. (A) The indicated monoclonal antibodies (MAB) were preincubated with purified pTP prior to addition of ^{32}P -labeled double-stranded DNA containing the adenovirus origin of DNA replication. DNA-protein complexes were resolved from free DNA by electrophoresis in a native polyacrylamide gel. Radioactive species were detected by autoradiography of the dried gel and quantitated by phosphor imaging. (B) The indicated amount of pTP was indicated with either a control antibody or 16H1 prior to analysis in a gel electrophoresis DNA binding assay as described above. (C) The indicated amount of pTP was incubated with either a control antibody or 3D11 prior to analysis in a gel electrophoresis DNA binding assay as described above.

3D11 and 11F11 resulted in increased binding of pTP to DNA, as indicated by a decrease in the proportion of free probe present relative to the controls. Similar results were obtained when pTP was incubated with DNA for 15 min prior to the addition of antibodies (data not shown), indicating that the order of addition is not important.

It is noted that 16H1, which inhibits binding of pTP to DNA, binds to an epitope close to iTP cleavage sites, a region previously proposed to be important in DNA binding (46). To investigate this point further, increasing concentrations of pTP were incubated with either a control antibody or 16H1 prior to addition of labeled DNA, and the DNA-protein complexes were resolved in a native polyacrylamide gel. At the higher concentrations of pTP used, multiple DNA-protein complexes were resolved, but the formation of all species was inhibited by the 16H1 antibody (Fig. 6B). In a similar fashion, the stimulatory effects of antibodies 3D11 and 11F11 were also investigated. In the presence of the 3D11 antibody, DNA binding activity was detected with 0.6 ng of pTP, as opposed to 20 ng in the absence of specific antibody (Fig. 6C). Similar data were obtained for 11F11 (data not shown). Thus, antibodies binding to the C terminus of pTP were found to enhance the binding of pTP to DNA, under the assay conditions used, suggesting a role for this region of pTP in regulation of DNA binding.

Antibodies 3D11 and 11F11 decrease the rate at which pTP dissociates from DNA. It is clear that antibodies 3D11 and 11F11 increase the equilibrium concentration of the DNA-bound form of pTP (Fig. 6A and C). This could be achieved by increasing the association rate constant or decreasing the dissociation rate constant for the pTP-DNA interaction. An attempt was therefore made to measure the rates at which pTP and pTP bound by antibody 3D11 associate with and dissociate from DNA. pTP, preincubated with either a control antibody or 3D11, was mixed with labeled DNA. Samples were removed at various times after mixing and applied to a running polyacrylamide gel to resolve free DNA and DNA-protein complexes. In the presence or absence of 3D11, pTP was found to associate with the 24-bp labeled DNA at the earliest time point tested (15 s). It was thus impracticable to determine the association rate constant. For the times tested, no visible difference in the rates of association of pTP with the labeled DNA was observed in the presence of 3D11 or 11F11 (data not shown) compared to control antibodies. In contrast, the specific antibodies 3D11 and 11F11 had a marked effect on the rate of dissociation of pTP from DNA compared to the control antibody, $\text{I}\kappa\text{B}\alpha$ (Fig. 7). With the control antibody, the dissociation of pTP was too rapid to measure, with only 5% of the pTP, bound at zero time, remaining bound after 15 s. In the presence of 3D11, however, 40% of pTP remained bound to DNA after 30 min of incubation with a 50-fold excess of unlabeled competitor DNA (Fig. 7). These data indicate that 3D11 and 11F11 exert their effects by binding to the C terminus of pTP and decreasing the dissociation rate constant.

DISCUSSION

pTP contributes to the complex molecular architecture of the preinitiation complex formed at the adenovirus origin of DNA replication both when covalently linked to the 5' end of the genome and as a component of incoming pTP-Adpol heterodimer. The precise roles of genome-bound pTP and newly initiated pTP in DNA replication, and the identities of their respective molecular associates throughout, remain to be determined. We describe the production of monoclonal antibodies against pTP and their use in mapping functional domains of the protein. Two of the antibodies, 7A2 and 44E1, recognize

FIG. 7. Antibody 3D11 decreases the rate at which pTP dissociates from DNA. Purified pTP (200 ng) was incubated with either a control monoclonal antibody (MAb) (500 ng) or antibody 3D11 (500 ng) prior to addition of ^{32}P -labeled double-stranded DNA containing the adenovirus origin of DNA replication. Once the reaction had reached equilibrium, a 50-fold excess of unlabeled DNA was added. At the indicated times, samples containing 20 ng of pTP were removed and applied to a running native polyacrylamide gel. Radioactive species were detected by autoradiography of the dried gel and quantitated by phosphor imaging. The positions of free DNA, DNA-pTP complex, and DNA-pTP-MAb ternary complex are indicated.

epitopes spanning the TP cleavage site, indicating that this site is both antigenic and surface exposed. The fact that their epitopes are destroyed by adenovirus protease makes them useful tools for future studies of the role of processing pTP in the infectious cycle. Proteolysis studies identified several protease-resistant domains in pTP, including an N-terminal fragment of 20 kDa (Fig. 2A).

Previous work showed that pTP and iTP, but not mature TP, could bind to Adpol. The C-terminal two-thirds of pTP is relatively rich in acidic residues and in the absence of Adpol is extensively cleaved by endo-AspN (Fig. 3). Specific endo-AspN sites, midway between iTP and TP sites, at either side of the TP cleavage site and proximal to Ser₅₈₀, were strongly protected in the heterodimer, with a 60-kDa fragment, corresponding to C terminus of pTP, remaining intact when treated with endo-AspN (Fig. 3). Six of the antibodies, however, were able to immunoprecipitate both pTP and heterodimer (Fig. 4A and B), indicating that the 15 aa C terminal to iTP site, the TP site itself, and epitopes contained within C-terminal 60 aa of pTP are not masked by binding of Adpol. Our data suggest multiple contact points between pTP and Adpol and, moreover, that structural integrity of a region stretching from iTP sites to the C terminus of the protein is required for binding, with some indications that an extensive conformational change in pTP takes place. Opinions are divided as to whether pTP and Adpol remain associated through the course of DNA replication, or whether the heterodimer dissociates at some point, either in transition from initiation to elongation, after limited elongation, or at the end of the replication cycle. Effects of antibodies 6E2 and 10B1 on pTP-Adpol reveal that the heterodimer can be separated under nondenaturing conditions. The importance of the conformation of pTP in binding to Adpol is further emphasized by the observation that TP does not bind to Adpol, despite the facts that it remains associated with NT1 and NT2 (46) and that the TP site itself is not masked by Adpol (effects of 7A2 and 44E1 in Fig. 4B). Evidence already exists to the effect that Adpol undergoes a conformational change on binding of deoxynucleoside triphosphates during initiation (5), and

FIG. 8. Diagrammatic representation of the domain organization of pTP. pol, polymerase.

so it is possible that this or another induced change could cause pTP to dissociate from the heterodimer. In a similar vein, elongation of DNA results in a string of negative charges extending from Ser₅₈₀, which is situated in a relatively acidic region of the protein (Fig. 8). Since endo-AspN sites proximal to Ser₅₈₀ are strongly protected when Adpol is bound, a negative protrusion in this region could be sufficient to disrupt the interaction between pTP and Adpol and dissociate the heterodimer.

DNA binding properties of pTP are less well understood and potentially more complex than its interaction with Adpol. pTP binds to DNA in association with Adpol, but it remains to be established whether the critical DNA contacts are made by pTP, or if contacts are made from both components of the heterodimer. Clearly pTP binds to DNA in the absence of Adpol, but the role for this activity of the protein in viral DNA replication has yet to be precisely defined. Here we show that an N-terminal, protease-resistant domain of pTP contacts DNA and that other regions of the protein are required to stabilize the association. Previous work demonstrates that integrity of the iTP sites is required for a stable association of pTP with DNA (46). UV cross-linking experiments, however, reveal that NT1 does contact DNA when pTP is digested to iTP or TP, and so the conclusion drawn is that when iTP sites are cleaved, dissociation of NT1 from DNA is too rapid for the interaction to be detected in a standard gel retardation assay. Although intact pTP does bind to DNA in such assays, the rate of dissociation is still too fast to measure (Fig. 7). In contrast, binding of antibodies to epitopes in the C-terminal 60 aa of pTP was found to reduce the dissociation rate constant to such an extent that pTP appears locked on DNA; after 30 min in the presence of a 50-fold excess of unlabeled competitor, 40% of pTP remains bound to end-labeled probe (Fig. 7). In conclusion, UV cross-linking and gel retardation assays have enabled us to distinguish between regions of pTP that make direct contact with DNA and those that affect the rate of dissociation of pTP from DNA. None of the monoclonal antibodies reacts with the N terminus of the protein (NT1), and their effects on UV cross-linking of pTP to DNA were found to be minimal (data not shown). In contrast, antibodies reacting outwith NT1 exerted a range of inhibitory and stimulatory effects in gel retardation assays (Fig. 6); an antibody binding close to iTP site inhibited DNA binding, as did conformational antibodies 6E2 and 10B1, while antibodies binding to epitopes at the C terminus of pTP markedly decreased dissociation rates. The role of the subtle DNA binding properties of pTP in the replication cycle remains to be established, with several questions arising: does pTP bind to origin DNA when covalently attached to the 5' end of the genome, does pTP bind as a dimer based on its low mobility in a gel retardation assay, and does it play some other role in DNA replication, for example, as a processivity factor? We identify the extreme C terminus of pTP as a regulatory domain, perhaps analogous to that found at C

terminus of p53 (15). Binding of a cellular or viral protein to the C-terminal tail of pTP could serve to lock it onto DNA, and if pTP binds to DNA as a homodimer, then a partner already exists linked to 5' end of genome.

We have succeeded in identifying regions of pTP that contact DNA and Adpol; the N terminus contacts DNA, while multiple regions within C-terminal two-thirds of pTP interact with Adpol. Of interest in this regard, is that mutagenesis studies of the bacteriophage ϕ 29 terminal protein identified a DNA binding region at its N terminus and polymerase binding regions, both C terminal to this and close to the C terminus of the protein (47). The similarities between these results and those presented here suggest that although the two protein primers differ considerably in size and have little homology at the primary sequence level, the organization of their DNA and polymerase binding regions is conserved. Apparent throughout this study is the importance of the conformation of adenovirus pTP to its DNA and Adpol binding functions, giving rise to subtle opportunities for interactions, or modifications, to induce conformational changes in pTP and to so modulate its function. The extreme dependence on conformation of pTP for both DNA and Adpol binding also explains why previous mutagenesis studies have failed to identify specific functional domains of the protein (10–12, 27, 33, 34).

ACKNOWLEDGMENTS

We thank Paul Szalowski for help with immunizations and cell fusions, Paul Talbot and Graham Kemp for peptide synthesis and N-terminal sequencing, and Marylne Roff for performing some of the ELISAs. We are grateful to P. O'Hare (Marie Curie Research Institute, Oxford, England), who provided the glutathione *S*-transferase–NFII expression plasmid. Thanks are also due to Catherine Botting for comments on the manuscript and to Bill Blyth for photography.

This work was supported by the Medical Research Council and the Wellcome Trust.

REFERENCES

- Armentero, M.-T., M. Horwitz, and N. Mermod. 1994. Targeting of DNA polymerase to the adenovirus origin of DNA replication by interaction with nuclear factor I. *Proc. Natl. Acad. Sci. USA* **91**:11537–11541.
- Bosher, J., E. C. Robinson, and R. T. Hay. 1990. Interactions between the adenovirus type 2 DNA polymerase and DNA binding domain of nuclear factor I. *New Biol.* **2**:1083–1090.
- Chen, M., N. Mermod, and M. S. Horwitz. 1990. Protein-protein interactions between adenovirus DNA polymerase and nuclear factor I mediate formation of the DNA replication preinitiation complex. *J. Biol. Chem.* **265**:18634–18642.
- Cleat, P. H., and R. T. Hay. 1989. Cooperative interactions between NF1 and the adenovirus DNA binding protein. *EMBO J.* **8**:1841–1848.
- Coenjaerts, F. E. J., and P. C. van der Vliet. 1994. Early dissociation of nuclear factor I from the origin during initiation of adenovirus DNA replication studied by origin immobilization. *Nucleic Acids Res.* **22**:5235–5240.
- Coenjaerts, F. E. J., J. A. W. M. van Oosterhout, and P. C. van der Vliet. 1994. The Oct-1 POU domain stimulates adenovirus DNA replication by a direct interaction between the viral precursor protein-DNA polymerase complex and the POU homeodomain. *EMBO J.* **13**:5401–5409.
- Coenjaerts, F. E. J., and P. C. van der Vliet. 1995. Adenovirus DNA replication in a reconstituted system. *Methods Enzymol.* **262**:548–560.
- Dekker, N., M. Cox, R. Boelens, C. Verrijzer, P. C. van der Vliet, and R. Kaptein. 1993. Solution structure of the POU-specific DNA-binding domain of Oct-1. *Nature* **362**:852–855.
- DePamphilis, M. L. 1993. Eukaryotic DNA replication: anatomy of an origin. *Annu. Rev. Biochem.* **62**:29–63.
- Fredman, J. N., and J. A. Engler. 1993. Adenovirus precursor to terminal protein interacts with the nuclear matrix in vivo and in vitro. *J. Virol.* **67**:3384–3395.
- Fredman, J. N., S. C. Pettit, M. S. Horowitz, and J. A. Engler. 1991. Linker intersection mutations in the adenovirus preterminal protein that affect DNA replication in vivo and in vitro. *J. Virol.* **65**:4591–4597.
- Freimuth, P. L., and H. S. Ginsberg. 1986. Codon insertion mutants of the adenovirus preterminal protein. *Proc. Natl. Acad. Sci. USA* **83**:7816–7820.
- Harlow, E., and D. Lane. 1988. *Antibodies—a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

14. Hay, R. T. Adenovirus DNA replication, p. 699–719. In M. L. DePamphilis (ed.), DNA replication in eukaryotic cells. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. Hupp, T. R., D. W. Meek, C. A. Midgely, and D. A. Lane. 1992. Regulation of the specific DNA binding function of p53. *Cell* **71**:875–886.
16. Jaffray, E., K. M. Wood, and R. T. Hay. 1995. Domain organization of I κ B α and sites of interaction with NF- κ B p65. *Mol. Cell. Biol.* **15**:2166–2172.
17. King, A. J., and P. C. van der Vliet. 1994. A precursor terminal protein-trinucleotide intermediate during initiation of adenovirus DNA replication: regeneration of molecular ends in vitro by a jumping back mechanism. *EMBO J.* **13**:5786–5792.
18. Leith, I. R., R. T. Hay, and W. C. Russell. 1989. Adenovirus subviral particles and cores can support limited DNA replication. *J. Gen. Virol.* **70**:3235–3248.
19. Lichy, J. H., J. Field, M. S. Horwitz, and J. Hurwitz. 1982. Separation of the adenovirus terminal protein precursor from its associated DNA polymerase: role of both proteins in the initiation of adenovirus DNA replication. *Proc. Natl. Acad. Sci. USA* **79**:5225–5229.
20. Lindenbaum, J. D., J. Field, and J. Hurwitz. 1986. The adenovirus DNA-binding protein and adenovirus DNA polymerase interact to catalyse elongation of primed DNA templates. *J. Biol. Chem.* **261**:10218–10227.
21. Matthews, J. R., W. Kaszubski, G. Turcatti, T. N. C. Wells, and R. T. Hay. 1993. Role of cysteine₆₂ in DNA recognition by the P50 subunit of NF- κ B. *Nucleic Acids Res.* **21**:1727–1734.
22. Mendez, J., L. Blanco, J. A. Estaban, B. Bernad, and M. Salas. 1992. Initiation of ϕ 29 DNA replication occurs at the second 3' nucleotide of the linear template: a sliding-back mechanism for protein-primed DNA replication. *Proc. Natl. Acad. Sci. USA* **89**:9579–9583.
23. Monaghan, A., A. Webster, and R. T. Hay. 1994. Adenovirus DNA binding protein: helix destabilising properties. *Nucleic Acids Res.* **22**:742–748.
24. Mul, Y. M., C. P. Verrijzer, and P. C. van der Vliet. 1990. Transcription factors NF1 and NFIII/oct-1 function independently, employing different mechanisms to enhance adenovirus DNA replication. *J. Virol.* **64**:5510–5518.
25. Mul, Y. M., and P. C. van der Vliet. 1992. Nuclear factor I enhances adenovirus DNA replication increasing the stability of preinitiation complex. *EMBO J.* **11**:751–760.
26. Mul, Y. M., and P. C. van der Vliet. 1993. The adenovirus DNA binding protein effects the kinetics of DNA replication by a mechanism distinct from NF1 or Oct-1. *Nucleic Acids Res.* **21**:641–647.
27. Pettit, S. C., M. S. Horwitz, and J. A. Engler. 1989. Mutations in the precursor to the terminal protein of adenovirus serotypes 2 and 5. *J. Virol.* **63**:5344–5250.
28. Pronk, R., and P. C. van der Vliet. 1993. The adenovirus terminal protein influences binding of replication proteins and changes origin structure. *Nucleic Acids Res.* **21**:2293–2300.
29. Pruijn, G. J. M., W. van Driel, and P. C. van der Vliet. 1986. Nuclear factor III, a novel sequence-specific DNA binding protein from HeLa stimulating adenovirus DNA replication. *Nature* **322**:656–659.
30. Randall, R. E., C. Newman, and R. W. Honess. 1984. Isolation and characterisation of monoclonal antibodies to structural and nonstructural herpesvirus saimiri proteins. *J. Gen. Virol.* **52**:872–883.
31. Randall, R. E., D. F. Young, K. K. A. Goswami, and W. C. Russell. 1987. Isolation and characterisation of monoclonal antibodies to simian virus 5 and their use in revealing antigenic differences between human, canine and simian isolates. *J. Gen. Virol.* **68**:2769–2780.
32. Rijnders, A. W. M., B. G. M. van Bergen, P. C. van der Vliet, and J. S. Sussenbach. 1983. Specific binding of adenovirus terminal protein precursor-DNA polymerase complex to the origin of DNA replication. *Nucleic Acids Res.* **11**:8777–8789.
33. Roovers, D. J., P. F. Overman, X. Q. Chen, and J. S. Sussenbach. 1991. Linker mutation scanning of the genes encoding the adenovirus type 5 terminal protein precursor and DNA polymerase. *Virology* **180**:273–284.
34. Roovers, D. J., F. M. van der Lee, J. van der Wees, and J. C. Sussenbach. 1993. Analysis of adenovirus type 5 terminal protein precursor and DNA polymerase by linker insertion mutagenesis. *J. Virol.* **67**:265–276.
35. Salas, M. 1991. Protein-priming of DNA replication. *Annu. Rev. Biochem.* **60**:39–71.
36. Schaak, J., W. Y. Ho, P. Freimuth, and T. Shenk. 1990. Adenovirus terminal protein mediates both nuclear matrix association and efficient transcription of adenovirus DNA. *Genes Dev.* **4**:1197–1208.
37. Schagger, H., and G. von Jagow. 1987. Tricine sodium dodecyl-sulphate polyacrylamide-gel electrophoresis for the separation of proteins in the range from 1-kDa to 100-kDa. *Anal. Biochem.* **166**:368–379.
38. Smart, J. E., and W. Stillman. 1982. Adenovirus terminal protein precursor. *J. Biol. Chem.* **257**:13499–13506.
39. Stanglmaier, M., and E. L. Winnacker. 1996. Cloning, sequence determination and functional expression of the genes encoding adenovirus type-4 polymerase and the terminal protein-precursor. *Gene* **168**:177–182.
40. Stuiver, M. H., and P. C. van der Vliet. 1990. The adenovirus DNA-binding protein forms a multimeric protein complex with double-stranded DNA and enhances binding of nuclear factor I. *J. Virol.* **64**:379–386.
41. Temperley, S. M., and R. T. Hay. 1992. Recognition of the adenovirus type 2 origin of DNA replication by the virally encoded DNA polymerase and preterminal proteins. *EMBO J.* **11**:761–768.
42. Tucker, P. A., D. Tsernoglou, A. D. Tucker, F. E. J. Coenjaerts, H. Leenders, and P. C. van der Vliet. 1994. Crystal structure of the adenovirus DNA binding protein reveals a hook-on model for cooperative DNA binding. *EMBO J.* **13**:2994–3002.
43. van der Vliet, P. C. 1991. The role of cellular transcription factors in enhancement of adenovirus DNA replication. *Semin. Virol.* **2**:271–280.
44. Webster, A., S. Russell, P. Talbot, W. C. Russell, and G. D. Kemp. 1989. Characterisation of the adenovirus protease: substrate specificity. *J. Gen. Virol.* **70**:3225–3234.
45. Webster, A., R. T. Hay, and G. D. Kemp. 1993. The adenovirus protease is activated by a virus-coded disulphide-linked peptide. *Cell* **72**:97–104.
46. Webster, A., I. R. Leith, and R. T. Hay. 1994. Activation of adenovirus-coded protease and processing of the preterminal protein. *J. Virol.* **68**:7292–7300.
47. Zaballos, A., and M. Salas. 1989. Functional domains in the bacteriophage ϕ 29 terminal protein for interaction with the ϕ 29 DNA polymerase and with DNA. *Nucleic Acids Res.* **17**:10353–10366.
48. Zhao, L. J., and R. Padmanabhan. 1988. Nuclear transport of adenovirus polymerase is facilitated by interaction with preterminal protein. *Cell* **55**:1005–1015.
49. Zijdeveld, D. C., and P. C. van der Vliet. 1994. Helix-destabilising properties of the adenovirus DNA-binding protein. *J. Virol.* **68**:1158–1164.