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Recognition of the adenovirus type 2 origin of DNA replication by the virally encoded DNA polymerase and preterminal proteins

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Initiation of adenovirus DNA synthesis is preceded by the assembly of a nucleoprotein complex at the origin of DNA replication containing three viral proteins, preterminal protein, DNA polymerase and DNA binding protein, and two cellular proteins, nuclear factors I and III. While sequence specific interactions of the cellular proteins with their cognate sites in the origin of DNA replication are well characterized, the question of how the viral replication proteins recognize the origin has remained unanswered. Preterminal protein and DNA polymerase were therefore purified to homogeneity from recombinant baculovirus infected insect cells. Gel filtration demonstrated that while DNA polymerase existed in monomeric and dimeric forms, preterminal protein was predominantly monomeric and when combined the proteins formed a stable heterodimer. In a gel electrophoresis DNA binding assay each of the protein species recognized DNA within the origin of DNA replication with unique specificity. Competition analysis and DNase I protection experiments revealed that although each protein could recognize the origin, the heterodimer did so with enhanced specificity, protecting bases 8-17 from cleavage with the nuclease. Thus the highly conserved 'core' of the origin of DNA replication, present in all human adenoviruses, is recognized by the preterminal protein-DNA polymerase heterodimer.

Key words: adenovirus/DNA replication/DNA-protein interaction/DNA polymerase/preterminal protein

Introduction

The initiation of DNA replication is a tightly regulated and highly specific event in the life of a cell or the multiplication of an infecting virus. In both eukaryotes and prokaryotes much of the specificity inherent in this process derives from the many DNA-protein and protein-protein interactions that are required to form the large nucleoprotein complexes that have been detected at the origins of DNA replication (Kornberg, 1988; Tsurimoto *et al.*, 1990). It is likely that the principles governing the assembly of these replication complexes will be equally relevant to other cellular processes such as transcription and recombination. The replication of adenovirus DNA represents an excellent system in which to study the network of molecular interactions that take place at a eukaryotic origin of DNA replication as the viral genome can be replicated *in vitro* by the action of three viral proteins, DNA binding protein (DBP), preterminal protein (pTP) and DNA polymerase (pol), and two cellular proteins; nuclear factor I (NFI) and nuclear factor III (NFIII). Initiation of adenovirus DNA replication takes place at either terminus of the linear 36 kbp viral genome by the formation of a covalent linkage between the α-phosphoryl group of the terminal residue, dCMP, and the β-OH group of a serine residue in pTP. The 3'-OH group of the pTP-dCMP complex then serves as a primer for synthesis of the nascent strand by pol which proceeds displacing the non-template strand. Displaced single strands can form partial duplexes by base pairing of the inverted terminal repeats (ITRs) on which a second round of DNA synthesis may be initiated (for recent reviews see Challberg and Kelly, 1989; Hay and Russell, 1989; Stillman, 1989).

Located within the the ITRs are the *cis*-acting DNA sequences which define the origin of DNA replication. Covalently attached to each 5' end of the DNA is a terminal protein (TP) which is likely to be an additional *cis*-acting component of the replication origin. While removal of the TP reduces the efficiency of adenovirus type 2 (Ad2) and adenovirus type 4 (Ad4) DNA replication *in vitro* it does not abolish replication and plasmid templates, provided that the origin has been exposed by restriction enzyme cleavage, can function *in vivo* and *in vitro* (Harris and Hay, 1988; Hay *et al.*, 1984; Tamanoi and Stillman, 1982; van Bergen *et al.*, 1983). Extensive mutational analysis on such templates has defined four regions within the terminal 51 bp of the Ad2 genome that contribute to origin activity *in vitro* and *in vivo*. The terminal 18 bp appear to be necessary, but alone can support only limited initiation (Challberg and Rawlins, 1984; Guggenheimer *et al.*, 1984; Hay 1985a; Lally *et al.*, 1984; Tamanoi and Stillman, 1983; van Bergen *et al.*, 1983; Wides *et al.*, 1987). This DNA sequence appears to represent a minimal origin of DNA replication, since the same 18 bp region constitutes a fully functional origin of DNA replication for the related Ad4 (Hay, 1985b; Harris and Hay, 1988). Separated from the minimal origin by a precisely defined spacer region, where sequence changes are tolerated but insertions and deletions are not (Adyha *et al.*, 1986; Bosher *et al.*, 1990; Wides *et al.*, 1987), is the recognition site for the cellular protein NFI or CAAT transcription factor (Jones *et al.*, 1987; Meisterernst *et al.*, 1989; Paonessa *et al.*, 1988; Santoro *et al.*, 1988). Occupancy of the recognition site by NFI increases the frequency of initiation of viral DNA replication both *in vivo* and *in vitro* (Nagata *et al.*, 1983; Rawlins *et al.*, 1984; Guggenheimer *et al.*, 1984; deVries *et al.*, 1985; Hay, 1985a,b; Schneider *et al.*, 1986). Immediately adjacent to the NFI recognition site is the binding site for another cellular DNA binding protein, NFIII or octamer binding protein (Oct I) (O'Neill and Kelly, 1988; Pruijn *et al.*, 1986; Rosenfeld *et al.*, 1987; Sturm *et al.*, 1988; Wides *et al.*, 1987). While addition of NFIII to an

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Ad2 *in vitro* system results in the stimulation of DNA replication (Mul *et al.*, 1990) it should be noted that reconstructed viruses which contain an origin of DNA replication lacking an NFIII binding site replicate their DNA with wild type kinetics (Hay and McDougall, 1986). The mechanisms by which these host factors increase the efficiency of DNA replication has yet to be established but in each case it has been demonstrated that the DNA binding domain of the protein is sufficient to stimulate DNA synthesis (Santoro *et al.*, 1988; Mermod *et al.*, 1989; Gounari *et al.*, 1990; Verrijzer *et al.*, 1990). Genes for the three viral replicative proteins pTP, pol and DBP have all been identified and the proteins expressed in a variety of heterologous systems thus facilitating large scale purification and analysis (Klessig *et al.*, 1984; Pettit *et al.*, 1988; Shu *et al.*, 1987; Stunnenberg *et al.*, 1988; Watson and Hay, 1990). DBP is a 68 000 Dalton protein, which is expressed at high levels in infected cells and involved at multiple stages of DNA replication. During elongation of nascent DNA chains, DBP functions by increasing the processivity of pol and by coating displaced single strands (Lindenbaum *et al.*, 1986). Prior to initiation, DBP binds to template molecules which results in NFI having a higher affinity for its recognition site in the replication origin and leading to an increase in the frequency of initiation of viral DNA replication (Cleat and Hay, 1989; Kenny and Hurwitz, 1988; Stuiver and van der Vliet, 1990). A direct interaction between NFI and pol can then target the pTP–pol heterodimer to the replication origin (Bosher *et al.*, 1990; Chen *et al.*, 1990; Mul *et al.*, 1990). Although further recognition of the replication origin by pTP–pol has not been reported, the strict spatial arrangement of the NFI binding site and the minimal origin of DNA replication suggests the existence of such an interaction. To test this possibility pTP and pol were overexpressed in insect cells and the purified proteins assessed for sequence specific DNA binding activity. Gel electrophoresis DNA binding and DNase I footprinting experiments revealed that while each protein could specifically recognize DNA sequences within the terminal 18 bp of the viral genome, the pTP–pol heterodimer bound to this region with enhanced specificity protecting base pairs 8–17 from DNase I cleavage. Thus the highly conserved DNA sequence, present in all human adenoviruses, that is located within the minimal origin of DNA replication is recognized by the pTP–pol heterodimer.

Results

Purification of preterminal protein and DNA polymerase from recombinant baculovirus infected Spodoptera frugiperda cells

Cell extracts were prepared from two 500 ml batches of insect cells infected with recombinant baculovirus containing the pTP and pol genes respectively as described (Materials and methods). Both proteins were purified by the same, two column, chromatographic procedure in which the proteins were successively bound to, and eluted from, denatured DNA–Sepharose and hydroxylapatite. Samples of crude extract, DNA–Sepharose eluate and hydroxylapatite eluate were denatured in mercaptoethanol and SDS and analysed by SDS–PAGE followed by staining with Coomassie brilliant blue. Prominent polypeptide species representing over-expressed pTP and pol could be distinguished in the crude extract (Figure 1, lanes 1 and 2). In each case 80–90% of the total protein eluted from the DNA–Sepharose was either pTP or pol (Figure 1, lanes 3 and 4

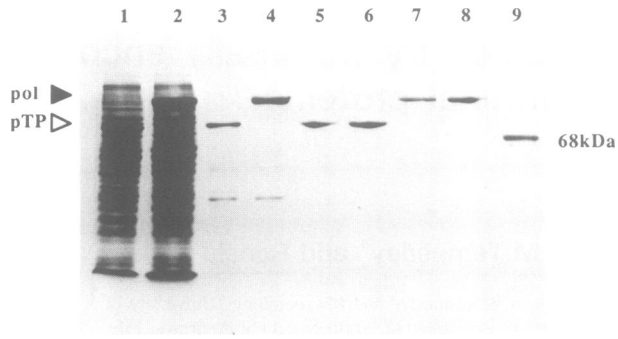

Fig. 1. Purification of preterminal protein and DNA polymerase. *Spodoptera frugiperda* cells were infected with recombinant baculoviruses containing the genes for pTP and pol. At 72 h post infection, cell extracts were prepared and the proteins purified by column chromatography on denatured DNA–Sepharose and hydroxylapatite. Fractions from each stage of the purification were analysed on 8% SDS polyacrylamide gels and proteins were stained with Coomassie brilliant blue. 1, 15 μg pTP cell extract. 2, 15 μg pol cell extract. 3, 3 μg pTP DNA–Sepharose fraction. 4, 4 μg pol DNA–Sepharose fraction. 5, 3 μg. 6, 5 μg pTP hydroxylapatite fraction. 7, 3 μg. 8, 5 μg pol hydroxylapatite fraction. 9, 5 μg BSA.

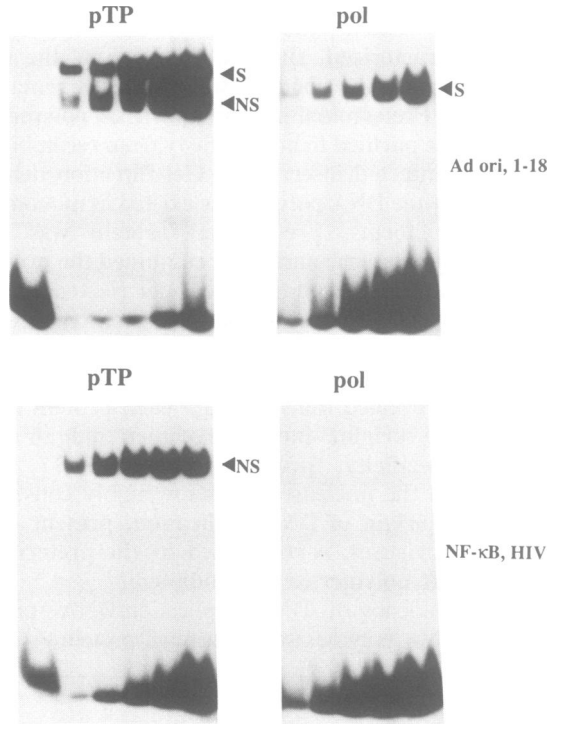

Fig. 2. Interaction of pTP and pol with the 1–18 sequence from the Ad2 origin of DNA replication. Purified adenovirus pol (140 ng, 1 pmol) and pTP (80 ng, 1 pmol) were incubated under conditions described in Materials and methods with increasing concentrations of ^{32}P end-labelled double-stranded oligonucleotides (1, 2, 4, 6 and 10 fmol, 2×10^3 c.p.m./fmol) containing the terminal 18 bp sequence of the adenovirus origin (Ad ori, 1–18, upper panels) and an unrelated HIV enhancer sequence (NF-κB, HIV, lower panels). DNA–protein complexes (black arrowheads; S, specific and NS, non-specific) were resolved from free DNA by electrophoresis in an 8% native polyacrylamide gel.

respectively). The eluate from hydroxylapatite consisted of essentially homogeneous pTP and pol (Figure 1, lanes 5 + 6 and 7 + 8) with overall yields of 6.5 and 10 mg per litre respectively. Both proteins were active in an Ad2 *in vitro* replication system when tested in initiation or elongation assays (data not shown).

Preterminal protein and DNA polymerase form DNA–protein complexes with the terminal 18 bp of the adenovirus origin of DNA replication

Recognition of a specific sequence in the origin of DNA replication by pTP and pol would provide one of the simplest routes to position the viral DNA replication proteins at the origin of DNA replication prior to initiation of DNA synthesis, but direct evidence supporting the existence of such an interaction has not been forthcoming. To examine the specific DNA binding properties of pTP and pol, a gel electrophoresis DNA binding assay was employed in which purified pTP and pol were incubated separately with increasing amounts of a double-stranded oligonucleotide containing the terminal 18 bp of the Ad2 origin of DNA replication (1–18) and an oligonucleotide of equal size but unrelated sequence derived from the HIV enhancer (NF-$\varkappa$B binding site). Incubation of pTP with the 1–18 DNA resulted in the formation of two DNA–protein complexes (Figure 2, upper left) whereas incubation of the same protein with the unrelated NF-$\varkappa$B DNA resulted in the formation of only one DNA–protein complex (Figure 2, lower left) which corresponded in electrophoretic mobility to the faster migrating species detected with the 1–18 DNA. Thus the more slowly migrating species was assumed to result from a specific DNA–protein interaction (Figure 2, S) whereas the faster migrating species was a likely result of non-specific DNA binding (Figure 2, NS). Binding of pol to the 1–18 DNA resulted in the appearance of a single DNA–protein complex whereas no DNA–protein complex was formed in the presence of the NF-$\varkappa$B DNA. Thus pol specifically recognizes sequences present within the terminal 18 bp of the viral origin of DNA replication.

Formation and DNA binding properties of a preterminal protein–DNA polymerase heterodimer

To determine the oligomeric state of pol and pTP that is active in DNA binding and to detect formation of a pTP–pol heterodimer, the two purified proteins were analysed separately or in combination by high resolution gel filtration. Samples of pTP, pol or a mixture of the two proteins were injected onto the column and the protein concentration of the eluate was monitored (OD_{280}). Fractions were collected and assayed for specific DNA binding, DNA polymerase activity and polypeptide composition. Prior to analysis of pTP and pol the column was calibrated with a series of proteins of known molecular weight from which the apparent molecular weights of pol and pTP could be calculated. pTP was eluted from the column as a sharp peak at 9.01 min (Figure 3) which corresponds to an apparent molecular weight of 89 000, close to the molecular weight calculated from its sequence i.e. 80 000. Therefore under the conditions employed in this analysis pTP is predominantly monomeric in solution. Fractions collected between 8 and 10 min were incubated with 1–18 DNA and DNA binding activity was analysed in a gel electrophoresis DNA binding assay. DNA binding activity responsible for the previously observed

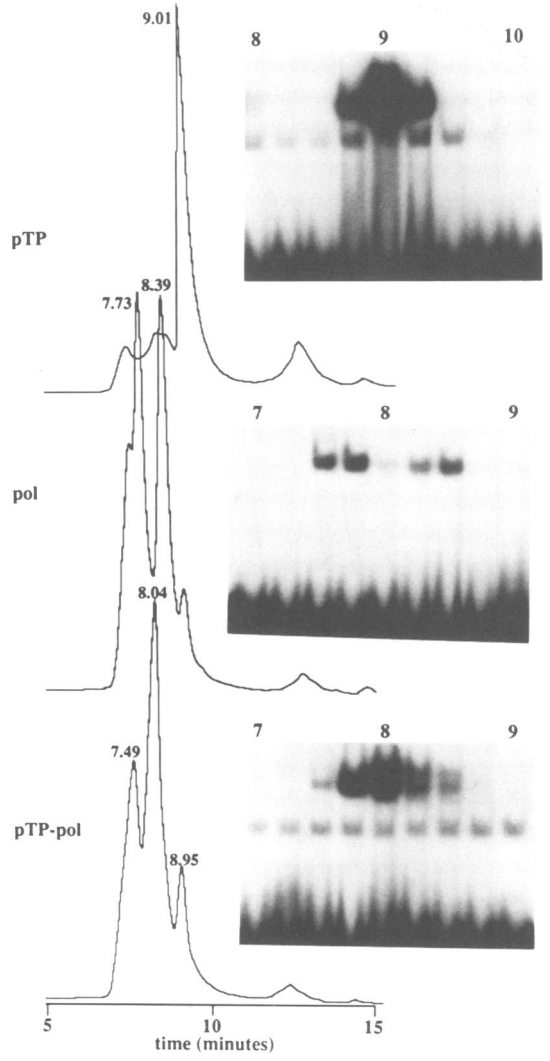

Fig. 3. Formation and DNA binding properties of a pTP–pol heterodimer. Purified pTP (100 μg), pol (100 μg) or a mixture of the two, pTP–pol (60 μg pTP and 105 μg pol preincubated at 21°C for 30 minutes), were analysed by gel filtration as described in Materials and methods. Protein samples were injected onto a Zorbax GF-250 column and eluted isocratically at 1 ml/min. The eluate was monitored continuously at 280 nm and retention times are noted above the relevant protein peaks. Retention coefficients (V_e/V_o) were 1.302 (pTP), 1.212 and 1.117 (pol) and 1.162 (pTP–pol). Fractions were collected every 12 s and specific DNA binding activity was determined by incubation of 1 μl samples with ^{32}P-labelled 1–18 oligonucleotide followed by electrophoresis in a native polyacrylamide gel. Sample collection time, in minutes, is indicated above each autoradiograph. The column was calibrated by plotting the retention coefficient (V_e/V_o against the $\log_{10}$ of the molecular weight of a set of standards of known molecular weights (Blue Dextran, thyroglobulin, apoferritin, β-amylase, bovine serum albumin, ovalbumin and cytochrome *c*).

(Figure 2) specific DNA–protein complex eluted from the column coincidentally with the elution of monomeric pTP (Figure 3). In contrast to pTP two major species of pol were detected eluting at 7.73 and 8.39 min (Figure 3) which would correspond to apparent molecular weights of 175 000 and 310 000. As pol has a molecular weight of 140 000, the protein appears to exist in solution as a mixture of monomeric and dimeric forms, although an even higher oligomer, thought to be a trimer (apparent molecular weight 420 000) can also be detected as a shoulder on the dimer peak

(Figure 3). Analysis of the fractions in the gel electrophoresis DNA binding assay revealed two peaks of specific DNA binding activity that co-eluted with the monomer and dimer fractions of pol. Although monomeric and dimeric pol exist in solution, the DNA–protein complex formed with both species has the same electrophoretic mobility (Figure 3). Incubation of an equimolar mixture of pol and pTP prior to analysis resulted in disappearance of the pTP peak at 9.01 min and the major pol peaks at 7.73 and 8.39 min, but accompanied by the appearance of a new peak eluting at 8.04 min that was associated with DNA binding activity and the formation of a DNA–protein complex of slightly different electrophoretic mobility from that observed independently with pTP or pol (Figure 3). The apparent molecular weight of this new species is 235 000 which is consistent with the formation of the pTP–pol heterodimer of molecular weight 220 000 (80 000 pTP plus 140 000 pol).

To establish that this species did indeed represent the pTP–pol heterodimer, the DNA polymerase activity present in individual fractions was determined. In the pol fraction DNA polymerase activity is associated with both the monomeric and dimeric form of the protein, but after addition of pTP a new single peak of DNA polymerase activity is observed eluting after 8 min, suggesting the formation of the pTP–pol heterodimer (Figure 4). This was confirmed by analysis of fractions in SDS–polyacrylamide gels followed by silver staining. The new peak eluting at 8 min does indeed contain equimolar amounts of pTP and pol (Figure 4). Thus the pTP and pol proteins are associated with specific DNA binding activity that is also present when the two proteins interact to form a heterodimer. After mixing of pTP and pol, residual uncomplexed monomeric pTP was detected eluting at 8.95 min, but this material did not display DNA binding activity (Figure 3, lower panel) and may therefore represent an inactive or modified form of pTP that can neither interact with pol nor bind DNA. Similarly the trimeric pol fraction that was detected as a shoulder on the dimeric peak (Figure 3, middle panel) was not reduced in intensity when pol and pTP were mixed and can be observed eluting as a separate peak at 7.49 min (Figure 3, lower panel; Figure 4, lower panel) indicating that it was not complexed to pTP.

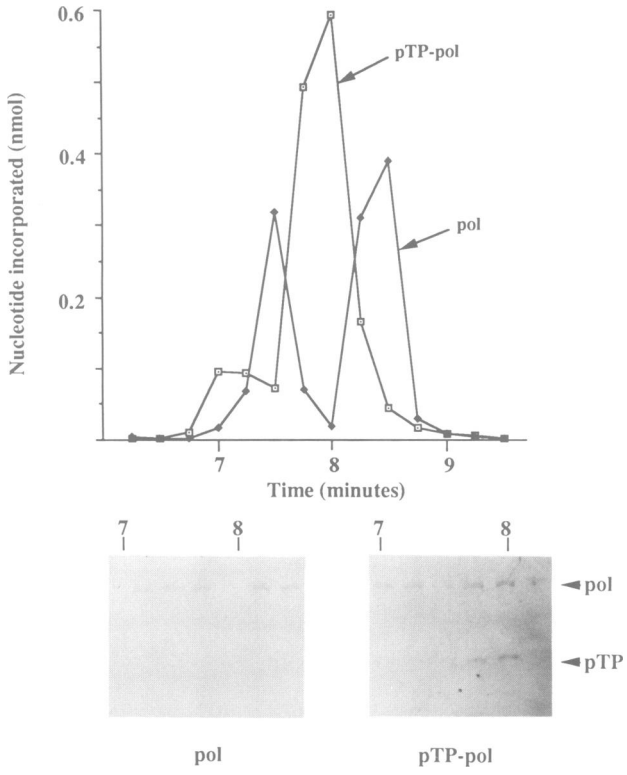

Fig. 4. DNA polymerase activity and polypeptide composition of the pTP–pol heterodimer. Fractions (10 μl) from the gel filtration analysis of pol and pTP–pol described in the legend to Figure 3 were assayed for DNA polymerase activity in the presence of activated DNA and [α-^{32}P]dATP. Total nucleotide incorporation was determined from quantification of acid insoluble radioactivity. Polypeptide composition of individual fractions was determined by silver staining after electrophoresis of samples (10 μl) in 8% polyacrylamide gels containing SDS. The position of pol and pTP are indicated by arrowheads and the elution time shown (minutes) corresponds to the traces shown in Figure 4.

DNA binding specificity of DNA polymerase, preterminal protein and the heterodimer

To determine the specificity with which pTP, pol and the pTP–pol heterodimer bind to the Ad2 origin of DNA replication, the purified proteins were incubated with ^{32}P-

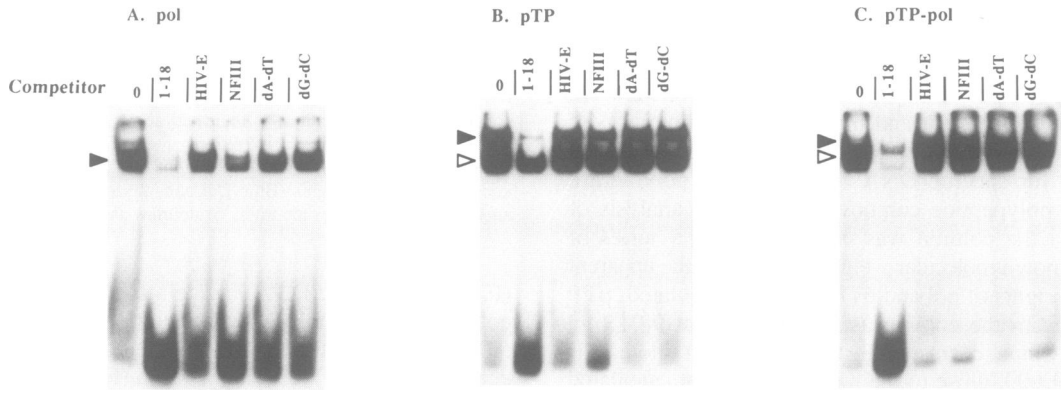

Fig. 5. DNA binding specificity of pol (**A**), pTP (**B**) and the pTP–pol heterodimer (**C**). Purified pol (140 ng, 1 pmol), pTP (80 ng, 1 pmol) and preformed pTP–pol heterodimer (220 ng, 1 pmol) were incubated with ^{32}P-labelled 1–18 oligonucleotide (0.1 ng, 6 fmol, 1.2 × 10^4 c.p.m.) in the absence (0) or presence of a 100-fold excess (10 ng) of unlabelled DNA prior to electrophoresis in a native polyacrylamide gel. Competitors used were the homologous 1–18 oligonucleotide (1–18), an oligonucleotide from the HIV enhancer containing the binding site for NF-κB (HIV-E), an oligonucleotide from the Ad4 ITR containing the binding site for NFIII (NFIII) and the alternating copolymers poly(dA–dT)·poly(dA–dT) and poly(dG–dG)·poly(dG–dC). Arrows on the autoradiogram indicate the position of DNA–protein complexes.

labelled 1−18 DNA in the presence of a 100-fold excess of various unlabelled DNAs. In the absence of competitor, pol binds most of the labelled DNA but in the presence of unlabelled homologous competitor DNA, the labelled DNA−protein complex is eliminated. In contrast, challenge with HIV (NF-κB binding site), pol(dA−dT)·poly(dA−dT) or poly(dG−dC)·poly(dG−dC) had little effect on the quantity of labelled DNA−protein complex formed (Figure 5A). However, inclusion of a double-stranded oligonucleotide containing the recognition site for NFIII in the binding reaction reduced binding by ~50% (Figure 5A, NFIII) but the sequences AATAA (8−12) and TAATAT (10−15) from the Ad2 origin are also present in the NFIII oligonucleotide. Incubation of pTP with 1−18 DNA resulted in formation of specific and a non-specific DNA−protein complexes as previously noted. The more slowly migrating complex was efficiently competed for by unlabelled 1−18 DNA, but not by HIV DNA, NFIII DNA, poly(dA−dT)·poly(dA−dT) or poly(dG−dC)·poly(dG−dC) (Figure 5B). None of the DNAs tested competed for formation of the more rapidly migrating DNA−protein complex, confirming that this DNA−protein complex resulted from a non-specific protein−DNA interaction whereas the slowly migrating DNA−protein complex was a result of a specific protein−DNA interaction. To examine the binding of the pTP−pol complex to the 1−18 DNA, an equimolar mixture of pTP and pol was preincubated at 21°C for 20 min to allow the proteins to interact. Samples of heterodimer were then allowed to interact with labelled 1−18 DNA in the absence or presence of unlabelled DNA as described above. In the absence of unlabelled DNA, pTP−pol forms two DNA−protein complexes, that although similar in electrophoretic mobility to those formed with pTP and pol alone were nonetheless distinguishable. Both DNA−protein complexes appeared to result from specific DNA−protein interactions: unlabelled homologous 1−18 competes for DNA binding whereas other unlabelled DNA does not (Figure 5C). The heterodimer clearly has different DNA binding properties from either pol or pTP alone. NFIII DNA does not compete for binding, as is the case with pol alone and the non-specific DNA−protein complex observed with pTP alone is no longer present: virtually all of the labelled DNA−protein complex formed is competed with the unlabelled 1−18 DNA. Thus it appears that the pTP−pol heterodimer has greater DNA binding specificity than either of the components alone.

Interaction of DNA polymerase, preterminal protein and the heterodimer with the intact Ad2 origin of DNA replication

To investigate the mode of interaction of pol, pTP and the pTP−pol heterodimer with the intact origin of DNA replication, the location and extent of DNA binding were determined by DNase I footprint analysis. A 3′-^{32}P-labelled

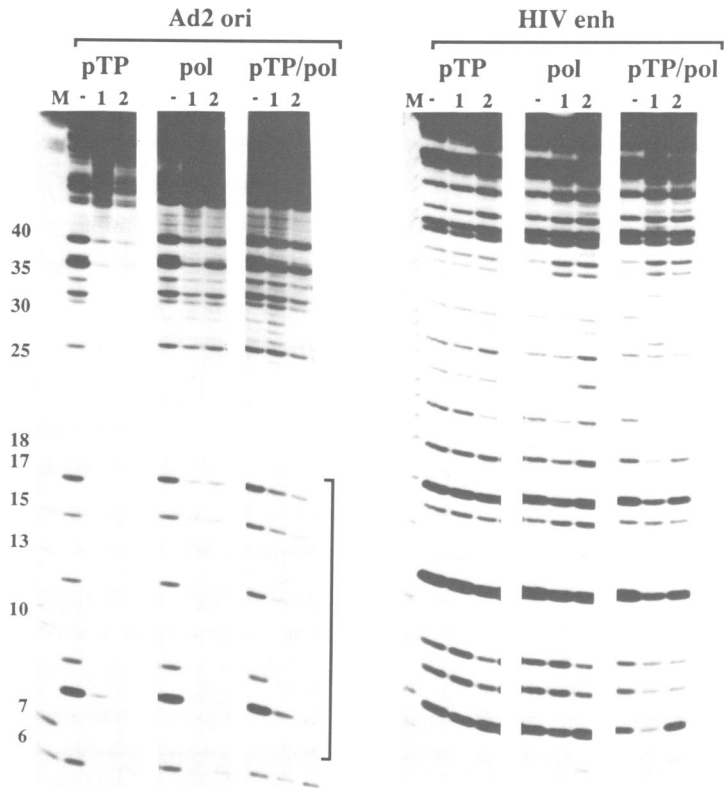

Fig. 6. DNase I footprint analysis of pol, pTP and pTP−pol DNA binding specificity. A 3′-^{32}P-labelled DNA fragment containing the complete Ad2 origin of DNA replication (Ad2 ori, 5 fmol, 6.5 × 10^4 c.p.m.) or a control DNA fragment containing the NF-κB site in the HIV enhancer (HIVenh, 5 fmol, 6.5 × 10^4 c.p.m.) was incubated in the absence (−) or presence (1,2) of pTP (30 ng, 375 fmol), pol (125 ng, 892 fmol) or preformed heterodimer pTP−pol (82 ng, 375 fmol) either with (2) or without (1) the addition of ATP to 3 mM. After incubation on ice for 30 min, 1 U of DNase I was added and digestion was allowed to proceed for 1 min at 20°C. The reaction was terminated and DNA was isolated by organic extraction followed by ethanol precipitation. DNA was resuspended in formamide, fractionated by electrophoresis on a 20% polyacrylamide gel in the presence of urea and the cleavage products were visualized by autoradiography. G+A specific cleavage of the labelled fragment (Ad2 ori) was used to generate DNA markers (M) which were analysed in parallel.

DNA fragment containing the fully functional origin of DNA replication was incubated with pol, pTP or the heterodimer in the presence or absence of ATP. As a control the proteins were also incubated with a similarly sized and labelled DNA fragment containing the NF-κB binding site in the HIV enhancer. After 30 min on ice, DNA–protein complexes were treated with DNase I and the DNase I cleavage products were analysed on a polyacrylamide gel. Incubation of pTP with the DNA fragment containing the origin of DNA replication, either in the absence or presence of ATP, results in extensive protection of DNA from nuclease cleavage (Figure 6) extending from the terminus of the DNA fragment up to at least position 40 in the origin of replication. Under identical conditions the DNA fragment containing the NF-κB recognition site was not protected from DNase I cleavage by pTP (Figure 6). Titration of pTP into the binding reaction following by DNase I cleavage revealed that at low concentrations pTP bound at, or close to, the end of the origin-containing fragment but, as the concentration of protein was increased, binding extended in from the end of the DNA fragment (data not shown). A similar situation was evident with pol, in that the origin-containing fragment was protected and the control DNA fragment was not protected from cleavage with DNase I (Figure 6). In this case, however, significant binding was not apparent further into the origin-containing DNA. Interaction of the pTP–pol heterodimer with the origin-containing DNA resulted in yet another pattern of DNase I protection: bp 6 is not protected, but bp 8–17 are protected from DNase I cleavage, particularly in the presence of ATP (Figure 6, bracketed). No protection outside this region is observed but under identical conditions of DNA and protein concentration, pTP–pol does cause some alteration in the DNase I cleavage pattern of the control DNA; this is less apparent in the presence of ATP (Figure 6, compare lanes 1 and 2, pTP/pol, HIVenh). The sequence of the HIV enhancer (5′-GGATC-CTGGAAAG-3′) in this region does not demonstrate any extensive homology with sequences present in the Ad2 origin of DNA replication. It should be noted that addition of pol to pTP blocks the extensive binding observed with pTP alone and restricts binding to the 8–17 region. This appears to correspond to the situation observed in the gel electrophoresis DNA binding assays where the non-specific DNA–protein complex observed with pTP alone was eliminated upon formation of the pTP–pol heterodimer (Figure 5).

Discussion

Replication of Ad2 DNA *in vitro* requires the presence of three viral proteins, pol, pTP and DBP, two cellular DNA binding proteins, NFI and NFIII, and the viral origin of DNA replication. Although this establishes the protein and DNA requirements for DNA replication, the precise sequence of events that precedes initiation of DNA replication has yet to be determined. Clearly multiple DNA–protein and protein–protein interactions are required for formation of the preinitiation complex at the origin of DNA replication. Given the concentration of Ad2 DBP in the infected cell nucleus, it is likely that all of the viral DNA acting as template in viral DNA replication will be coated with DBP. A consequence of the template being coated with DBP is that it increases the affinity of NFI for its recognition site in the Ad2 origin of DNA replication (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). Recent experiments indicating that NFI interacts directly with Ad2 pol (Bosher *et al.*, 1990; Chen *et al.*, 1990; Mul *et al.*, 1990) suggest that the function of NFI is to facilitate incorporation of the pTP–pol heterodimer into a nucleoprotein complex at the origin of DNA replication. However, it has been argued (Bosher *et al.*, 1990) that because of the symmetrical nature of the NFI–DNA interaction (de Vries *et al.*, 1987) and presumably the NFI–pol interaction, binding of pol to NFI is insufficient to position the pTP–pol heterodimer correctly on the origin of DNA replication. Although the simplest solution to this problem would be for pTP–pol to recognize specifically the origin of DNA replication, direct evidence for such an interaction has not previously been reported. Experiments utilizing partially duplex templates consisting of a double-stranded NFI binding site and a single-stranded extension containing portions of the conserved 1–18 core region demonstrated that pTP–pol bound to the single-stranded region (Kenny and Hurwitz, 1988; Chen *et al.*, 1990). The implication of this observation, however, is that the origin must be unwound prior to binding of pTP–pol. To date, no evidence has been reported that would suggest that NFI and DBP alone could accomplish this. The experiments reported here were therefore designed to determine if pTP–pol could recognize the double-stranded form of the Ad2 origin of DNA replication. Since it seemed likely that this might be a rather weak interaction it was imperative that pTP and pol could be produced in large amounts free from associated adenoviral and human proteins. This was achieved by high level expression of the proteins in insect cells and a two column purification procedure. Gel filtration analysis of the proteins indicated that both pol and pTP were associated with DNA binding activities which were specific for DNA sequences within the terminal 18 bp of the Ad2 origin of DNA replication. While pTP was monomeric in solution, pol was present as a mixture of monomer and dimer with both forms giving rise to DNA–protein complexes of similar electrophoretic mobility. When pTP and pol were combined prior to gel filtration analysis, the monomeric pTP and both forms of pol were replaced by heterodimeric pTP–pol. Since a complex containing two molecules of pol and one of pTP was not detected, this suggests that the surfaces of pol that interact with pTP in the heterodimer are the same surfaces that interact to form the pol dimer. Gel electrophoresis DNA binding assays indicated that while pol and pTP could both recognize DNA sequences within the terminal 18 bp of the Ad2 genome, formation of the pTP–pol heterodimer was accompanied by an increase in DNA binding specificity. This was evident from competition analysis (Figure 5) and DNase I footprinting (Figure 6) where pTP–pol protected base pairs 8–17 from cleavage with DNase I. This covers the area from 9–18 that is perfectly conserved in all human adenoviruses sequenced to date and is regarded as the 'core' of the Ad2 replication origin. In isolation the terminal 18 bp of the Ad2 genome supports a low, but detectable, level of DNA synthesis *in vitro* (Challberg and Rawlins, 1984; de Vries *et al.*, 1985; Guggenheimer *et al.*, 1984; Lally *et al.*, 1984; Rawlins *et al.*, 1984; Tamanoi and Stillman, 1983; Wides *et al.*, 1987) which is presumably independent of NFI. NFI independent initiation of Ad2 DNA replication is also evident in a highly purified replication system, particularly in the presence of an elevated concentration of

pTP−pol (Mul *et al.*, 1990). Under conditions where the concentration of Ad2 origin DNA is held at 140 pM and the pTP−pol concentration is 1.5 nM (0.3 U/ml), Mul *et al.* (1990) observed a 50-fold increase in replication efficiency in the presence of saturating levels of NFI. However, at pTP−pol concentrations of 46 nM and 150 nM, the stimulation observed with NFI was reduced to 4.6- and 1.5-fold respectively. It is under very similar conditions, i.e. 50 nM pTP−pol and 300 pM 1−18 DNA (Figure 5), that we detect sequence specific interactions between pTP−pol and 1−18 DNA. It is therefore likely that the function of NFI is to direct pTP−pol to the origin of DNA replication, thus locally increasing the concentration of pTP−pol and allowing recognition of the 1−18 sequence. In the presence of high concentrations of pTP−pol, the requirement for NFI is overcome as the heterodimer displays its potential for sequence specific DNA recognition.

In fact, an interaction between pTP−pol and a 110 bp fragment containing the Ad2 origin of DNA replication has been suggested on the basis of chemical cross-linking and immunoprecipitation experiments (Mul *et al.*, 1990). Possession of a DNA binding activity by pol is also expected from inspection of the protein sequence which reveals the presence of two potential 'zinc fingers'. Mutagenesis of one of these zinc fingers eliminates the ability of the protein to bind to single-stranded DNA and to participate in viral DNA replication, but not to interact with NFI (Chen *et al.*, 1990).

NFI and NFIII independent initiation of DNA replication is also evident with adenovirus type 4 (Ad4) where the terminal 18 bp of the genome constitute a fully functional origin of DNA replication *in vivo* (Hay, 1985b) and *in vitro* (Harris and Hay, 1988; Temperley and Hay, 1991). Furthermore, certain mutations in the conserved 9−18 region, which represents the binding site for pTP−pol, also have a severe deleterious effect on the efficiency of DNA replication (Temperley *et al.*, 1991). One possible explanation for the simple origin construction of Ad4 is that the pTP−pol heterodimer has an intrinsically higher affinity for its binding site in the 1−18 region, making it independent of NFI.

The relatively high concentrations of pTP−pol that are required to observe sequence specific DNA binding indicate that although the binding of pTP−pol is specific it is also rather weak. However, this DNA−protein interaction is just one of a large number of potential interactions that may stabilize the preinitiation complex. This is reminiscent of the situation at higher eukaryotic promoters where the general transcription factor TFIIB displays rather weak binding to the promoter (Buratowski *et al.*, 1989) but its binding is stabilized by the presence of a sequence specific DNA binding protein bearing an acidic activating domain (Lin and Green, 1991). Thus the multiple weak interactions that characterize the formation of many macromolecular assemblies are equally evident in the assembly of preinitiation complexes at eukaryotic replication origins and promoters.

Materials and methods

Purification of pTP and pol

Spodoptera frugiperda insect cells were grown in liquid culture in TC-100 medium supplemented with 5% fetal calf serum at 28°C and infected with 10 p.f.u./cell of recombinant *Autographa californica* nuclear polyhedrosis viruses containing the genes of pTP and pol (Watson and Hay, 1990; Bosher *et al.*, 1990). Infected cells were incubated for a further 72 h at 28°C in TC-100 medium supplemented with 5% fetal calf serum and collected by centrifugation at 2500 r.p.m. for 15 min. After washing in phosphate buffered saline the cells were resuspended in ice cold 25 mM HEPES−KOH pH 8.0, 5 mM KCl, 0.5 mM $MgCl_2$ and 0.5 mM DTT, incubated at 4°C for 10 min, then disrupted by 10 strokes in a Dounce homogenizer using a type-B pestle. NaCl was added to bring the final concentration to 0.2 M and the extract was incubated at 4°C for 30 min. Cell debris was removed by centrifugation at 15 000 *g* for 5 min and the extract was clarified by further centrifugation at 100 000 *g* for 15 min. An identical method was used to purify pTP and pol, all procedures were conducted at 4°C. Clarified cell extract in 0.2 M NaCl was applied to denatured calf thymus DNA−Sepharose equilibrated with 25 mM HEPES−NaOH pH 8.0, 1 mM EDTA, 2 mM DTT, 0.2 M NaCl and 10% glycerol. After extensive washing with the equilibration buffer, bound proteins were eluted with the same buffer containing 0.6 M NaCl. Fractions were collected and their protein concentration was determined by the method of Bradford (1976). Peak fractions were pooled and dialysed against two changes of 0.4 M KCl, 5 mM KPO_4 (pH 7.0), 1 mM DTT and 10% glycerol for 8 h and applied onto a column of hydroxylapatite equilibrated in the same buffer. After extensive washing with the equilibration buffer, bound proteins were eluted with 100 mM KPO_4 (pH 7.0), 1 mM DTT, 0.4 M KCl and 10% glycerol. The polypeptide composition of each fraction was monitored throughout the purification procedure by SDS−polyacrylamide gel (8%) electrophoresis. Fractions containing pTP and pol were dispensed in small amounts and stored at −70°C. pTP−pol heterodimers were formed by mixing molar equivalents of the two proteins in storage buffer (100 mM KPO_4 buffer pH 7.0, 1 mM DTT, 0.4 M KCl and 10% glycerol) and incubating for 20 min at 21°C.

Gel filtration analysis

pTP, pol and the preformed pTP−pol heterodimer were injected onto a 9.4 × 250 mm Zorbax G-250 hydrophilic gel filtration column installed in a Waters 600E HPLC system. The column was equilibrated in buffer containing 100 mM KPO_4 (pH 7.0), 400 mM KCl, 1 mM DTT and eluted with the same buffer at a flow rate of 1 ml/min. Absorbance at 280 nm was continuously monitored on a Waters 484 tunable detector and fractions were collected every 12 s. The void volume of the column was determined with Blue Dextran and the column calibrated using the retention coefficient (V_e/V_o) and molecular weights of the following proteins: thyroglobulin (mol. wt 669 000, V_e/V_o 1.023); apoferritin (mol. wt 443 000, V_e/V_o 1.098); β-amylase (mol. wt 200 000, V_e/V_o 1.166); BSA (mol wt 66 000, V_e/V_o 1.295); ovalbumin (mol. wt 48 000, V_e/V_o 1.378); cytochrome *c* (mol. wt 12 400, V_e/V_o 1.670).

Gel electrophoresis DNA binding assay

Two complementary oligonucleotides containing the 1−18 sequence from the Ad2 origin of DNA replication were synthesized on an Applied Biosystems 381A: 5′-GATCCATCATCAATAATATACCG-3′ (top strand) and 5′-GATCCGGTATATTATTGATGATG-3′ (bottom strand). The oligonucleotides were annealed and labelled with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP and dCTP, and the labelled fragment was purified on an 8% polyacrylamide gel. DNA was eluted from the gel by passive diffusion into 10 mM Tris−HCl pH 7.5, 100 mM NaCl and 1 mM EDTA and stored at 4°C. A double-stranded oligonucleotide containing the NF-κB binding site in the HIV-1 enhancer has been described previously (Clark *et al.*, 1990). Purified proteins were diluted to the desired concentration in storage buffer containing 1 mg/ml BSA and were then incubated with ^{32}P end-labelled double-stranded oligonucleotide for 30 min at 21°C in a total volume of 20 μl containing 25 mM HEPES−KOH pH 7.5, 1 mM DTT, 1 mM EDTA, 20 mM KCl and 1 mg/ml BSA. At the end of the incubation period, glycerol was added to 5% and the samples were loaded onto an 8% polyacrylamide gel containing 50 mM Tris−borate 1 mM EDTA pH 8.3 which was run at 10 V/cm for 60 min. Labelled species were visualized by exposure of the dried gel to X-ray film at −70°C in the presence of an intensifying screen.

DNase I footprinting

Plasmids pHR54Δ (Hay, 1985), containing the terminal 54 bp of the Ad2 origin of DNA replication, and pHIV-R (Clark *et al.*, 1990), containing the NF-κB site from the HIV-1 enhancer were cleaved with *Eco*RI and *Pst*I and the *Eco*RI site was labelled by incubation with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP. Labelled DNA fragments were gel purified and eluted as described above. Purified proteins were incubated with ^{32}P-labelled DNA in a 50 μl reaction containing 25 mM HEPES−KOH pH 7.5, 1 mM DTT, 20 mM KCl, 4 mM $MgCl_2$ and 1 mg/ml BSA. After 30 min on ice samples were placed at 20°C for 1 min then digested with 1 U DNase I (Amersham) for a further 1 min at 20°C. Digestion was terminated by the addition of an equal volume

of buffer containing 0.3 M sodium acetate, 20 mM EDTA pH 8.0, 1% SDS, 0.1 mg/ml salmon sperm DNA and the DNA was isolated by organic extraction and ethanol precipitation. DNA was resuspended in formamide, heated at 100°C for 2 min and analysed in a 20% polyacrylamide gel containing urea. The wet gel was covered with Saran Wrap and exposed to X-ray film at −70°C in the presence of an intensifying screen.

Assay for DNA polymerase activity

Protein samples were incubated with 10 μg of activated calf thymus DNA, 100 μM dTTP, dGTP, dCTP, 20 μM dATP, 1 μCi [α-^{32}P]dATP (specific activity 3000 Ci/mmol), 50 mM Tris pH 8.0, 7 mM $MgCl_2$, 10 mM DTT and 100 μM aphidicolin in a total volume of 50 μl for 1 h at 37°C. Reactions were terminated by addition of 10% TCA, 0.5% sodium pyrophosphate and the acid insoluble radioactivity retained on a glass fibre filter was determined by liquid scintillation counting.

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