Replication of adenovirus type 4 DNA by a purified fraction from infected cells

Simon M. Temperley and Ronald T. Hay*
Department of Biochemistry and Microbiology, University of St Andrews, Irvine Building, North Street, St Andrews, Fife KY16 9AL, UK

Received April 4, 1991; Revised and Accepted May 15, 1991

ABSTRACT
An extract from Adenovirus type 4 infected HeLa cells was fractionated by ion-exchange and DNA affinity chromatography. One fraction, which bound tightly to single stranded DNA, contained predominantly a protein of apparent molecular weight 65,000 and three less abundant proteins. Immunological cross-reactivity with adenovirus type 2 proteins confirmed the presence of preterminal protein and indicated that the abundant species was the virus coded DNA binding protein. This fraction contained an aphidicolin resistant DNA polymerase activity and in the presence of a linearised plasmid containing the adenovirus type 4 origin of DNA replication efficient transfer of dCMP onto preterminal protein, indicative of initiation, was observed. Furthermore, addition of all four deoxyribonucleotide triphosphates and an ATP regenerating system resulted in the elongation of initiated molecules to generate plasmid molecules covalently attached to preterminal protein. Adenovirus type 4 DNA binding protein was extensively purified from crude adenovirus-4 infected HeLa extract by immunoaffinity chromatography using a monoclonal antibody raised against adenovirus type 2 DNA binding protein. A low level of initiation of DNA replication was detected in the fraction depleted of DNA binding protein but activity was restored by addition of purified DNA binding protein. DNA binding protein therefore plays an important role in the initiation of Ad4 DNA replication.

INTRODUCTION
Human adenoviruses contain linear duplex DNA genomes of approximately 35kb with inverted terminal repeats (ITR's) (1) and a 55kDa terminal protein (TP) covalently bound to each 5' end (2, 3). Initiation of DNA replication occurs at the termini by a protein priming mechanism in which dCMP, the terminal nucleotide is covalently attached to an 80kDa precursor of the terminal protein (pTP) in a template dependent reaction. The nascent strand is elongated by viral DNA polymerase using the 3'-OH of the pTP-dCMP complex as a primer resulting in the displacement of the non-template strand which can then act as a template for a second round of DNA synthesis (4). There is evidence that the second round of synthesis occurs on a partially duplex 'panhandle' template formed by base pairing of the ITR's of the displaced strand (5, 6, 7).

The development of cell free systems, initially by Challberg and Kelly (8), in which adenovirus DNA can be synthesised from template molecules of viral or plasmid origin in vitro has played a key role in the identification of the sequences important for origin function and the isolation and purification of the proteins involved in DNA replication. The replication of Ad2 and Ad5 DNA has been the most extensively studied and has been shown to require three virus encoded proteins, the 80kDa pTP, the 140kDa Ad DNA polymerase (Ad pol) and the 72kDa DNA binding protein (DBP) as well as two host proteins, nuclear factor I (NFI) (9, 10, 11, 12, 13) and nuclear factor III (NFIII) (14, 15, 16, 17) which on binding to their cognate sites in the origin of replication can stimulate initiation of DNA replication up to 100-fold. In vitro studies have shown that the origins of Ad2 and Ad5 consist of a core domain comprising the terminal 18bp of the genome which alone is only capable of supporting a low level of initiation (18, 19), and an auxiliary region encompassing nucleotides 19-50 which contains the recognition sequences for NFI and NFIII. The sequence requirements of the Ad2 origin have also been defined in vivo where deletion analysis has demonstrated that the terminal 45bp are required for efficient replication of adenovirus mini chromosomes co-transfected with helper Ad2 DNA (20), and similarly that virus genomes with deletions extending into the terminal 45bp where rendered non-infectious (21). Transfection assays carried out using Ad4 as helper virus showed that unlike Ad2 only the terminal 18bp of the genome, which in Ad2 constitute the core origin, were required for efficient DNA replication in vivo (22). This has been borne out in vitro where it was demonstrated that linearised plasmid containing only the terminal 18bp of an adenovirus ITR could support initiation of DNA replication as effectively as a template containing a complete Ad4 ITR (23, Temperley et al., manuscript in preparation). Correspondingly the protein requirements for Ad2/5 differs from Ad4 in that Ad4 shows no dependence on NFI or NFIII for efficient DNA replication.

* To whom correspondence should be addressed
Ad4 inverted terminal repeat does not contain a NFI recognition site and whilst it does have a binding site for NFIII neither site is required for DNA replication in vivo and the purified proteins are incapable of stimulating DNA replication in vitro even when the templates contain the cognate binding site (24). The mechanisms by which host factors increase the efficiency of DNA replication has yet to be properly defined but it has been demonstrated that the DNA binding domains of NFI and NFIII alone are sufficient to stimulate adenovirus Ad2 DNA replication (25, 26, 27). Recent evidence of a direct interaction between NFI and Ad2 polymerase has led to the suggestion that NFI increases the rate of initiation by stabilising a pre-initiation complex with pTP and pol via its interaction with pol (28, 29, 30).

The likelihood is that host proteins play auxiliary roles which enhance the functions of the viral replicative machinery and it is possible that Ad4 is able to efficiently replicate its DNA in the absence of host proteins because viral DNA replication proteins can adequately fulfill such roles themselves. To study the proteins required for Ad4 DNA replication we fractionated crude Ad4 infected HeLa cell extract which was capable of in vitro DNA replication by a combination of ion exchange and DNA affinity chromatography. A fraction eluted from DNA-Sepharose with 1M NaCl containing 4 polypeptide species was sufficient for initiation and elongation of Ad4 DNA replication in vitro. Immunological and biochemical experiments confirmed the presence of Ad4 DBP, pol and pTP in these fractions. Using a single step immunoaffinity purification, we isolated near homogeneous DBP from crude Ad4 infected HeLa extract and demonstrated the important role of this protein in the initiation reaction.

**MATERIALS AND METHODS**

**Cells and virus**

HeLa S3 spinner cells were used to prepare virus stock and, after infection with Ad4, as a source of virus infected cell extract. Cells were grown in Spinner medium containing 50 units/ml of penicillin, 50µg/ml of streptomycin and 5% new-born calf serum. A549 cells were used to titrate virus by the method of Williams (31). Cells were grown in monolayer in Glasgow modified minimal essential medium containing penicillin, streptomycin, sodium bicarbonate and 10% new-born calf serum.

**Templates**

Plasmid p4A2 contains the adenovirus type 4 ITR and is identical to plasmid p4A1 (20). pMDC10 pm18 (pm18) contains the terminal 69bp of the Ad5 ITR carrying a single base transition in the 18th base pair from the terminus (10). Plasmid DNA was prepared by two rounds of CsCl/ethidium bromide centrifugation. Ethidium bromide was removed by repeated extraction with caesium chloride saturated iso-butanol and the DNA was desalted by ultrafiltration in a centriicon-30.

**Preparation of cell extracts**

1 litre of HeLa S3 cells were infected with 100 plaque forming units/cell of adenovirus type 4. After 90min medium containing 2% calf serum plus 10mM hydroxyurea was added. Cells were incubated for 22 hours at 37°C and extract was prepared as described (8). Extract was depleted of nucleic acids by passage over DEAE-Sepharose in 0.2M NaCl and concentrated by precipitation with ammonium sulphate as described (32). The precipitate was resuspended in 2ml of 20mM Hepes (pH7.5), 5mM KCl, 0.5mM MgCl$_2$ and 0.5mM DTT per $3\times10^8$ cells, dialysed against the same buffer and stored in small aliquots at $-70^\circ$C.

**Purification of Ad4 DNA replication proteins**

**DNA affinity chromatography.** Crude cytoplasmic extract was prepared from Ad4 infected HeLa S3 spinner cells as described. NaCl was adjusted to 50mM and the extract was applied to DEAE-Sepharacel equilibrated in 50mM NaCl. The column was washed with two volumes of buffer containing 50mM NaCl, and then with two volumes of buffer containing 0.2 M NaCl. Fractions were collected, assayed for in vitro DNA replication activity by pTP-dCMP complex formation assay and their protein concentration determined by the method of Bradford (33). Activity recovered in the 0.2M eluate was applied to a denatured calf thymus DNA-Sepharose column equilibrated in buffer containing 0.2M NaCl. The column was washed with two volumes of buffer containing 0.2M NaCl and 5 volumes of a concentration gradient from 0.2M to 2M NaCl. Fractions were collected, assayed for their ability to initiate adenovirus DNA replication in vitro and their protein concentration was determined. Active fractions were dialysed against 20mM HEPES (pH8.0), 5mM KCl, 0.5mM MgCl$_2$, 5mM DTT and 1mM PMSF and stored at $-70^\circ$C in small volumes.

**Immunoaffinity Purification.** Mouse ascitic fluid containing 5mg of monoclonal antibody a72k B6-10 raised against Ad2 DBP (34) was collected and bound to 1ml of protein G-Sepharose (Sigma) by direct coupling in 3M NaCl as described (35) and used to prepare a column. Crude extract from Ad4 infected HeLa cells was prepared as described and dialysed against 10mM sodium phosphate. The extract was applied to the antibody column and unbound protein was removed by extensive washing with 10mM sodium phosphate, 0.05% NP40. Bound protein was eluted by increasing stepwise washes of 10, 20, 30, 40, 50 and 100mM NaCl and fractions were analysed by SDS-PAGE. DBP was eluted in a near homogeneous state at 50–100mM MgCl$_2$.

**Assay for the transfer of dCMP to pTP**

1–12µg of Ad4 infected HeLa cell cytoplasmic extract or purified replication proteins were incubated in a 30µl reaction containing 25mM Tris/HCl (pH8), 3mM MgCl$_2$, 2mM DTT, 3mM ATP, 3µCi [γ$^32$P] dCTP (specific activity 3000Ci/mole) and varying

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (µg)</th>
<th>Total Units</th>
<th>% Yield</th>
<th>Specific Activity (units/µg)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>149.2</td>
<td>1798</td>
<td>100</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>29.5</td>
<td>983</td>
<td>55</td>
<td>33</td>
<td>2.75</td>
</tr>
<tr>
<td>Denatured DNA-Sepharose</td>
<td>1.1</td>
<td>220</td>
<td>12</td>
<td>200</td>
<td>16.6</td>
</tr>
</tbody>
</table>

* 1 unit corresponds to the amount of protein required to transfer 1fmol of dCMP to pTP.
amounts of restriction enzyme cleaved template DNA for 90 min at 37°C. Reactions were stopped by heating at 70°C for 5 min and treated with micrococcal nuclease at 33 units/ml in the presence of 2 mM CaCl₂ for 30 min at 37°C. 12 μl of gel loading buffer containing 20% glycerol (v/v), 5% SDS (w/v), 570 mM 2-mercaptoethanol, 33 mM Tris pH 6.7 and 0.2% bromophenol blue (w/v) was added and the samples denatured by heating to 100°C for 2 min. Reaction products were resolved on a 10% SDS-polyacrylamide gel at 35 mA for 4 hours, fixed in 10% acetic acid, dried and then subjected to fluorography at -70°C in the presence of an intensifying screen.

Assay for elongation of DNA initiated in vitro

Restriction enzyme cleaved template DNA was pre-incubated in a total volume of 7 μl for 30 minutes at 30°C with 1 - 3 μl of Ad4 infected HeLa cell cytoplasmic extract or purified viral replication proteins in a buffer containing 25 mM Hepes-KOH (pH 7.5), 4 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA and 0.2 mM aphidicolin. The reaction was started by addition of 7 μl of a buffer containing 80 μM each of dTTP, dATP, and dGTP, 5 μM dCTP, 0.3 μ Ci [α³²P] dCTP (specific activity 3000 Ci/mole), 4 mM ATP, 10 mM creatine phosphate, 10 μg/ml creatine phosphokinase, 0.1 mg/ml BSA, 25 mM Hepes-KOH pH 7.5, 4 mM MgCl₂ and 1 mM DTT to give a total reaction volume of 14 μl. The total reaction was incubated at 30°C for 90 minutes. Reactions were stopped by addition of 6 μl of buffer containing 5% SDS, 50% glycerol, 100 mM EDTA and bromophenol blue and heated at 70°C for 5 minutes. Reaction products were resolved on a 2% agarose gel containing 0.1% SDS at 35 mA for 4 hours which was fixed in 10% acetic acid, dried and subjected to fluorography at -70°C in the presence of an intensifying screen.

Assay for DNA polymerase activity

2 - 10 μg of protein sample were incubated with 10 μg of activated calf thymus DNA, 100 μM dTTP, dGTP, dCTP, 20 μM dATP, 1 μCi [α³²P] dATP (specific activity 3000 Ci/mole), 5 mM Tris pH 8.0, 7 mM MgCl₂, 10 mM DTT and 100 μM aphidicolin in a total volume of 50 μl for 1 hour at 37°C. Reactions were terminated by addition of 10% TCA, 0.5% sodium pyrophosphate and insoluble radioactivity measured by scintillation counting.

Western Blot analysis

All blots were carried out using semi-dry Western blot equipment according to the manufacturer's directions. Filters were blocked by incubation with 10% w/v powdered non-fat milk in PBS and, after washing in PBS, were probed by incubating with 200 μl of immune serum in 5 ml of PBS, at 37°C for 1 hour. Antibody-antigen complex was detected by incubation of the filter with [¹²⁵I] protein A at 2 μg/ml and after extensive washing with PBS, 0.05% NP40, labelled species were detected by exposure to X-ray film at -70°C.

RESULTS

Purification of a fraction from Ad4 infected HeLa cells capable of DNA replication in vitro

Previous studies in vivo and in vitro have established that Ad4 has a unique and very simple origin of DNA replication compared to the more intensively studied Ad2/Ad5. Accordingly, the protein requirements differ markedly, in that Ad4 is able to replicate its DNA without the need for cellular factors NFI/C-TF and NFm/Oct-1.

To examine the proteins involved in Ad4 DNA replication we fractionated Ad4 infected HeLa extract which was capable of in vitro DNA replication. Crude Ad4 infected extract was prepared from 10 litres of HeLa suspension cells as described (see methods). Soluble extract was adjusted to 50 mM NaCl and applied to DEAE-Sepharose equilibrated with 50 mM NaCl, followed by extensive washing with 50mM NaCl. Bound proteins

Figure 1. Purification of a fraction capable of replication of adenovirus DNA in vitro. A. Proteins bound to denatured calf thymus DNA-Sepharose were eluted with a 0.2 M to 2 M NaCl gradient. The protein concentration of each fraction was determined and the two major peaks designated P1 and P2 (white squares). 2 μl of each fraction was incubated in a standard DNA polymerase reaction (see methods) and the activity determined (black diamonds). B. Plasmid p4A2 (50 ng) containing a copy of the Ad4 ITR was cleaved with EcoRI and incubated with 8 μl of each fraction (dialysed against 0.1 M NaCl buffer) from DNA-Sepharose in a standard assay for initiation of DNA replication (see methods) containing [α³²P] dCTP (3000 Ci/mole) and 100 μM aphidicolin. After incubation at 32°C for 90 minutes and micrococcal nuclease digestion polypeptides were fractionated by SDS polyacrylamide gel electrophoresis and labelled species were detected by autoradiography.
were eluted with 0.2M NaCl and fractions were collected. The protein concentration of each fraction was determined and the peak fractions were assayed for their ability to catalyse pTP-dCMP complex formation using EcoRI cleaved p4A2 as template. This step gave an overall 5-fold increase in specific activity with 50% yield and freed the extract of nucleic acids (Table 1). Peak fractions were pooled and applied to denatured calf thymus DNA-Sepharose equilibrated with 0.2M NaCl. After extensive washing with 0.2M NaCl bound proteins were eluted with a linear gradient of 0.2-2M NaCl. Fractions were collected and the protein concentration determined. One peak of protein eluted between 0.5-0.8M NaCl (P1) followed by a second peak eluting at 1.0-1.2M NaCl (P2) (Figure 1A). P1 corresponded to a single peak of protein obtained when extract of uninfected cells was fractionated under similar conditions (data not shown). Individual fractions were tested for their ability to initiate DNA replication in vitro by assaying the efficiency with which they were able to catalyse the transfer of dCMP onto pTP using EcoRI cleaved p4A2 as template. Only fractions in P2 were capable of initiation (Figure 1B). The purification procedure resulted in a 16 fold increase in the specific activity with a 12% overall yield (Table 1).

An aphidicolin resistant DNA polymerase co-eluted from the denatured DNA-Sepharose column with the template dependent pTP-dCMP transfer activity (Figure 1A).

**Analysis of proteins involved in Ad4 DNA replication**

To examine the proteins present at each stage of the purification samples of crude Ad4 infected extract, 0.2M eluate from the DEAE-Sephacel, flowthrough from the denatured DNA-Sepharose and eluates P1 and P2 from the denatured DNA-Sepharose were analysed by SDS polyacrylamide gel electrophoresis. Proteins were visualised by staining with Coomassie brilliant blue. All fractions analysed, with the exception of P2, contained multiple species. P2 apparently contained only one species of apparent molecular weight 65kDa which from its relatively high abundance and elution characteristics was likely to be viral DBP (Figure 2A). Since this fraction was capable of initiation of DNA replication in vitro it was clear there must be other replication proteins present. To further analyse fraction P2 we examined various quantities of P2 by SDS PAGE and used silver staining to detect the fractionated polypeptides. This revealed the presence of three additional species of apparent molecular weights approximately 70kDa, 85kDa and 95kDa (Figure 2B, lane 1; 2, 3 and 4).

**Elongation of initiated molecules in vitro by P2**

Although it had been demonstrated that the fraction containing the four polypeptide species was capable of initiation of replication it was not clear if the fraction could also carry out elongation of initiated molecules. To compare the activity of crude and fractionated extracts two different templates were used; p4A2 which as described (22) contains one copy of an Ad4 ITR and pM18 containing an Ad5 ITR carrying a C to T base change at position 18 which has been shown to severely reduce template efficiency in vitro (Temperley et al., manuscript in preparation). Templates were cleaved with EcoRI and AvaII to give a 1.6kb fragment with the origin sequence at the EcoRI terminus and a 0.9 kb fragment containing only plasmid sequences and incubated with various protein samples, dNTP’s (including [α-32P]dCTP), optimal MgCl₂ and an ATP regeneration system. Reaction products were fractionated by electrophoresis in an agarose gel containing SDS and replicated templates which had incorporated [32P]dCMP were detectable by autoradiography. Correctly initiated products have pTP linked at the 5’ end which results in a lower electrophoretic mobility (Fig 3, black arrow) than input template molecules which incorporate [32P]dCTP at a low level (Fig 3, open arrow). Crude extract gave efficient elongation on p4A2 but greatly reduced elongation on pM18, consistent with its previously observed compromised ability to support in vitro DNA replication (Figure 3, lanes 1 and 2). The DEAE-Sephacel eluate and DNA-Sepharose eluate fraction P2 both gave efficient elongation on p4A2 but utilised the pM18 template inefficiently (Figure 3, lanes 3 to 6). DNA-Sepharose eluate P1 was unable to initiate molecules.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Protein analysis of peak fractions. A. 5μg each of crude extract, eluate from DEAE-Sephasel, flowthrough from DNA-Sepharose, and peak fractions P1 and P2 eluted from DNA-Sepharose were denatured by heating at 100°C in SDS and mercaptoethanol and the polypeptides present fractionated by electrophoresis. Species were visualised by silver staining. B. Samples of 0.1μg, 0.2μg, 0.5μg and 1μg of fraction P2 eluate from DNA-Sepharose were denatured in SDS and mercaptoethanol and the polypeptides present fractionated by electrophoresis. Species were visualised by silver staining.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Elongation of initiated templates by fraction P2. Plasmid templates p4A2 and pM18 (100ng) were cleaved with EcoRI/AvaII and incubated with 3μg of crude Ad4 infected extract (lanes 1 and 2), 3μg of DEAE-Sephasel eluate (lanes 3 and 4), 2μg of DNA-Sepharose eluate P2 (lanes 5 and 6), 2μg of DNA-Sepharose eluate P1 (lanes 7 and 8) and a mixture of 2μg each of P1 and P2 (lanes 9 and 10) in a standard elongation reaction containing [α-32P]dCTP (see methods). After incubation at 30°C for 90 minutes reaction products were resolved by electrophoresis in a 2% agarose gel containing 0.1% SDS. Labelled species were detected by autoradiography. Correctly initiated molecules have pTP attached (black arrow head) and so have a lower electrophoretic mobility than input template (white arrow head) which is labelled at a low level due to repair synthesis. A second fragment containing only plasmid sequences is run out of the gel.
to elongate either template consistent with its inability to initiate DNA replication in vitro (Figure 3, lanes 7 and 8). Although fraction P1 itself was unable to replicate adenovirus DNA in vitro the presence of factors involved in DNA replication could not be ruled out. To determine if fraction P1 contained factors important for DNA replication P1 and P2 were incubated together with templates p4A2 and pm18 in a standard elongation reaction (Figure 3, lanes 9 and 10). The combined fractions did not support DNA replication at a significantly higher level than fraction P2 alone. Various combinations of flowthrough, P1 and P2 were examined under initiation conditions but no combination gave initiation levels in excess of that obtained with P2 alone.

These data demonstrate that all the proteins necessary for Ad4 DNA replication in vitro were present in fraction P2 from the DNA-Sepharose column. The presence of viral polymerase and pTP was inferred by the ability of P2 to initiate on and elongate from input template.

Western blot analysis of P2

To identify the polypeptide species present in P2 samples were fractionated by SDS-PAGE, transferred to nitrocellulose and incubated with antisera raised against Ad2 replication proteins. Antibody-antigen complexes were detected with [I125] protein-A followed by autoradiography. A polyclonal guinea-pig antiserum raised against Ad2 DBP strongly reacted with the 65kDa species (Figure 2B, lane 1 and Figure 4A; lane 1) confirming the identity of this species as DBP. Antiserum raised in rabbits against purified Ad2 pTP and pol respectively were also used to probe blots of P2. The antiserum against Ad2 pTP recognised only the second highest molecular weight species (Figure 2B, lane 3) and Figure 4A, lane 2). The antiserum against Ad2 polymerase however failed to recognise any of the species present (Figure 4A, lane 3). The presence of Ad4 pol in the fraction however was implied by the data from both DNA polymerase and elongation assays. Since the antisera has been shown to detect Ad2 pol (Figure 4B, lane 2) this result suggests that differences in the DNA polymerases of the two serotypes prevented cross reaction with Ad4 pol.

These data confirmed the identity of two of the species present in P2. As predicted, the most abundant protein corresponded to DBP and the presence of pTP, as predicted from pTP-dCMP transfer assay was confirmed.

Immunoaffinity purification of Ad4 DBP

To fractionate DBP from the other components present in P2 a different approach was adopted. A column was prepared using protein G-Sepharose linked to mouse monoclonal antibody directed against an epitope present in Ad2 DBP (34) as described (35). Crude extract from Ad4 infected HeLa cells was prepared as described (8) and the soluble extract freed from nucleic acids by passage over DEAE-Sepharose in 0.2M NaCl. The buffer was changed by dialysis against 10mM NaPO4 pH7.2 and applied to the antibody column equilibrated in 10mM NaPO4 pH7.2. After extensive washing with 10mM NaPO4 pH7.2 and 0.05% NP40 bound proteins were eluted with increasing stepwise concentrations of 10mM, 20mM, 30mM, 50mM and 100mM MgCl2. Fractions were collected, samples analysed in an SDS polyacrylamide gel and proteins visualised by Coomassie staining.

Figure 4. Western blot analysis of P2. Samples containing 10µg each of the second peak eluted from the DNA-Sepharose (P2), Ad2 pTP and Ad2 pol were denatured in SDS and mercaptoethanol and incubated with antisera raised against Ad2 replication proteins. Antibody-antigen complexes were detected with [I125] protein-A followed by autoradiography.

Figure 5. Immunoaffinity purification of Ad4 DBP. A. A crude extract from Ad4 infected HeLa cells was applied to a column containing α72K monoclonal antibody against Ad2 DBP linked to protein-G Sepharose. Bound protein was eluted by application of increasing concentrations of MgCl2. Proteins present in each fraction were analysed by electrophoresis in an acrylamide gel containing SDS followed by staining with Coomassie blue. B. DBP peak fraction (no. 14) was incubated at 1µg, 2µg and 4µg alone (lanes 1−3) in a standard initiation assay with 50ng of EcoRI cleaved p4A2 as template. In addition, 2µg each of immunoaffinity column flowthrough and crude extract were incubated alone (lanes 4 and 7) and with 0.5µg and 1µg respectively of fraction 14 (lanes 5 & 6 and 8 & 9) under standard initiation conditions.
The majority of the protein remained unbound and was washed from the column with 0.05% NP40 (Figure 5A lane 2). DBP was eluted from the column at 50mM to 100mM MgCl₂ (Figure 5A lanes 14 to 18) at about 98% purity. Approximately half of the DBP was not bound to the matrix. Because of the observed heterogeneity of DBP it was thought that the unbound portion may represent modified states of the protein which the monoclonal antibody was unable to recognise. To test this possibility the flowthrough was re-applied to the antibody matrix and a series of increasing stepwise elutions of MgCl₂ were used to elute bound protein as described. The DBP remaining in the flowthrough after the first round of purification bound to the column and was eluted at 50–100mM MgCl₂ (data not shown). These data demonstrated that the monoclonal antibody recognised all DBP present in the crude extract. The binding capacity of the antibody-Sepharose matrix was reached during the application of the crude extract leading to the observed presence of DBP in the flowthrough.

To test the effect of purified DBP on DNA replication, DBP in the peak fraction (Figure 5A, lane 14) was added to standard pTP-dCMP complex formation assays containing [α³²P]dCTP and 50ng of EcoRI cleaved p4A2 as template. The fraction containing DBP (14) alone was incapable of in vitro initiation of DNA replication (Figure 5B, lanes 1 to 3). The flowthrough from the second round of immunoaffinity purification which was depleted of DBP was capable of initiation of DNA replication only at a very low level (figure 5B, lane 4), however addition of DBP (fraction 14) resulted in an approximately 20-fold stimulation of initiation activity (Figure 5B, lanes 5 and 6). Initiation in crude extracts was stimulated by addition of DBP (fraction 14) at 0.5 ug but was inhibited at 1ug (Figure 5, lanes 8 and 9).

These data show that by immunoaffinity purification active and near homogeneous Ad4 DBP could be isolated from crude infected extract in a single step, separating it from viral polymerase and pTP. The data obtained from initiation assays suggest that DBP plays an important role in the initiation of DNA replication.

**DISCUSSION**

Two different approaches were used to isolate and characterise the proteins required for Ad4 DNA replication. In the first a fraction containing only four detectable polypeptides able to both initiate DNA replication and elongate initiated molecules in vitro was purified. In the second, near homogeneous DBP was isolated from crude Ad4 infected cell extract. The data obtained indicates that there are important differences in the viral replication proteins between Ad4 and the more extensively characterised Ad2.

Initially a combination of ion exchange and single strand DNA affinity chromatography was employed with the intention of purifying DBP as described in the method of Schechter et al. (36). Surprisingly, a fraction whose major component was DBP, but which contained three detectable higher molecular weight species was isolated. Furthermore this fraction was capable of initiation of DNA replication and elongation in vitro implying the presence of pTP and viral DNA polymerase in the fraction. Two of the species, which were visualised by SDS PAGE, were identified as DBP and pTP by probing of a Western blot with anti Ad2 pTP antiserum and anti Ad2 DBP antiserum respectively. However an antiserum against Ad2 pol, which recognised Ad2 pol in Western blots, failed to recognise any species present in the fraction. Sequencing data reveals that there is considerable variation in the region near the amino termini of the DNA polymerases of Ad2, Ad7 and Ad12 (37, 38). Although no sequence data for Ad4 pol exists the variation that has been observed in the DNA polymerases from the subgroups thus far analysed suggests that perhaps there is insufficient homology between the DNA polymerases of Ad2 and Ad4 for serum raised against Ad2 pol to recognise that of Ad4. In agreement with this data it has been observed that the same antiserum is unable to recognise native Ad4 pol in immuno-fluorescence of Ad4 infected HeLa cells (J. Boshier, unpublished observation).

As stated previously pTP and pol co-elute with DBP from denatured DNA-Sepharose with 1M NaCl. This differs markedly from Ad2 where denatured DNA-Sepharose chromatography separates pTP/pol and DBP: pTP/pol is eluted with 0.36M NaCl whilst 1.0M NaCl is required to elute DBP (39, 40). Thus the requirement for 1.0M NaCl to elute the Ad4 pTP/pol complex may reflect a higher affinity of the Ad4 proteins for single stranded DNA or alternatively may be a result of a direct interaction between the Ad4 pTP/pol complex and DBP. However, under the conditions used for immunoaffinity purification DBP was separated from pTP/pol in low ionic strength buffer suggesting that the formation of such a complex forms, it may be DNA dependent. Another possibility is that bound DBP alters the structure of the DNA template resulting in higher affinity binding by Ad4 pTP/pol. This latter possibility is reminiscent of the effect of the Ad2 DBP on the DNA binding properties of NFI: DBP increases the affinity of NFI for its recognition site in the Ad2 origin of replication although no direct interaction between the two proteins could be detected (41, 42). Although Ad2 pTP/pol and DBP do not co-elute from DNA-Sepharose, the thermostability and processivity of pol is increased in the presence of DBP (43), suggesting the existence of functionally significant interactions between the proteins. Specific interactions between single stranded DNA binding proteins and DNA polymerases have previously been reported in prokaryotic phage systems such as T4 and T7 (44, 45). Such an interaction may play a critical functional role in light of the independence of Ad4 DNA replication from transcription cellular factors. The recent observation that NFI and Ad2 pol interact (28, 29, 30) and the implied role of NFI in the formation of a preinitiation complex suggests that such a function in Ad4 may be fulfilled by the viral replication proteins themselves. Thus Ad4 pTP and/or pol may themselves recognise and bind to the origin of replication with higher affinity than their Ad2 counterparts.

A marked stimulation of initiation of DNA replication was observed in the presence of the immunoaffinity purified fraction of DBP indicating the important role of this protein in initiation of Ad4 DNA replication. While there is ample biochemical and genetic evidence for the role of Ad2 DBP in elongation (46), its role in initiation is less clear cut, although under certain conditions it too can stimulate the initiation of Ad2 DNA replication (41, 42).

The relatively simple protein requirements for Ad4 DNA synthesis may well reflect the evolutionary development of a more autonomous system of viral self replication, where the reliance on host factors is reduced. This may well prove to be advantageous to the investigation of the underlying mechanisms of DNA replication. Further fractionation of the proteins will be needed to facilitate a more detailed functional analysis of each component.
ACKNOWLEDGEMENTS

We would like to thank Bill Blyth and Dave Roche for photography. We are indebted to A. Levine for the gift of the mouse hybridoma line α72K B6-10 which was used for the production of anti-DBP monoclonal antibody. S.M.T was the recipient of a SERC studentship award.

REFERENCES