Adenovirus Subviral Particles and Cores Can Support Limited DNA Replication

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(Accepted 18 August 1989)

SUMMARY

Adenovirus type 2 cores can function effectively as templates in an in vitro replication system. Viral DNA replication assays using cores as templates do not differ in their requirements to the well characterized assays using DNA-complex templates, i.e. there is a dependence on terminal protein precursor (pTP), DNA polymerase and DNA binding protein and the assay is greatly stimulated by certain host transcription factors. The products of initiation and limited elongation are easily distinguishable and, in the system described, there is specific proteolysis of the pTP adducts as a function of the adenovirus-coded protease, present in the nuclear extracts from infected cells, or the core templates. Substitution of Mn$^{2+}$ ions for Mg$^{2+}$ ions in the replication assay has a dramatic effect on the nature of the replication events, in most cases resulting in the stimulation of initiation without elongation. Similar results can be achieved by utilizing subviral particles as templates, obtained by dialysis of purified adenovirus in a hypotonic buffer at pH 6.4. Restriction enzyme analysis of the replicated products confirmed that DNA synthesis proceeds from the adenovirus termini using both the core and subviral templates. By adding an ATP-regenerating system elongation can be further stimulated, particularly in the case of the subviral templates. Quantification of nucleotide incorporation into the appropriate restriction fragments indicates that for the subviral templates replication can proceed for a least 2000 to 3000 bases from either terminus. These results suggest that the adenovirus genome is packaged in the virion in a conformation readily available for at least the initial replication events. Such a conformation might also be appropriate for early transcription.

INTRODUCTION

The virion of adenovirus consists of an icosahedral protein capsid surrounding a nucleoprotein core, which contains a linear double-stranded DNA genome (35937 bp in adenovirus type 2) with inverted terminal repeats. Attached to each of the $\overline{5}$ termini is a virus-coded 55K terminal protein (TP). This protein is synthesized as an 80K precursor (pTP), which is processed by a virus-coded protease to the mature protein at later stages of infection. Closely associated with the genome are the virus-coded basic proteins VII (of $M_r$ 19K) and V (of $M_r$ 46K) and a minor virus protein IVa$_2$ (of $M_r$ 52K) (Russell & Precious, 1982). These so called core proteins are made late in infection and at least one of these (VII) is also synthesized as a precursor protein, which is processed by the virus-coded protease during virus maturation. Cores of virus DNA with covalently attached TP and associated proteins can be readily prepared from purified virus preparations by gentle disruption of virus, using a pulse of heat (56 °C) in the presence of 0.5% sodium deoxycholate (Russell et al., 1971). In addition, very mild disruption of purified virus particles by dialysis against a hypotonic buffer can produce subviral particles which have lost their apical penton capsomeres (Prage et al., 1970) and thus presumably contain core components in a configuration which is accessible to external stimuli and may be nearer to the in vivo situation, where the virus is 'uncoated' prior to genome activation. The precise mechanisms for this latter process are, as yet, still unclear.
Many of the steps in adenovirus DNA replication have been analysed in molecular detail, following the development of soluble in vitro systems (for a review see Hay & Russell, 1989). Using as templates either virus DNA complexed with TP, or plasmids linearized to expose the viral origin of DNA replication, it has been shown that DNA replication initiates at the termini of the genome by the formation of an ester linkage between the phosphate of 5' dCMP and the β-OH of a serine residue in the pTP (Lichy et al., 1981; Challberg & Kelly, 1981). pTP is intimately associated with the virus-coded DNA polymerase (Pol) and the evidence suggests that a complex of pTP and Pol is involved in initiation, before participation of Pol in elongation of nascent DNA strands (Lichy et al., 1982; Stillman et al., 1982).

Studies of the in vitro systems have also shown that cellular factors can stimulate replication. Two sequence-specific DNA-binding proteins (NFI and NFIII) increase the efficiency of the initiation reaction (Nagata et al., 1982; Pruijn et al., 1986) and a cellular topoisomerase (NFII) is required for the synthesis of genome-length DNA strands (Nagata et al., 1983). Another virus-coded protein (DBP), which binds preferentially to single-stranded DNA, plays an essential role in the elongation of the nascent chains (Krevolin & Horwitz, 1987; Chase & Williams, 1986) and also facilitates initiation events (Kenny & Hurwitz, 1988; Cleat & Hay, 1989a). DBP has an apparent Mr in SDS-PAGE of 72K, but contains 529 amino acids with a total Mr 59K, the difference being attributed to the proline-rich nature of the polypeptide and to post-translational modifications such as phosphorylation (Kruijer et al., 1981). With the exception of two reports (Goding & Russell, 1983; Harris & Hay, 1988) all of the studies on in vitro DNA replication have utilized either linearized plasmids or DNA–TP complexes as templates, and indeed one investigation (Korn & Horwitz, 1986) suggested that core proteins must be removed from the template before replication can proceed. In this latter study core proteins VII and V were purified from guanidine–HCl-disrupted virus particles. After dialysis against phosphate buffer at pH 5.6 it was noted that the (apparently) renatured proteins completely inhibited viral replication in vitro on a template of DNA–TP complex. On the basis of these studies it was concluded that the core proteins synthesized late in infection attach to viral templates, thus inhibiting replication and concomitantly initiating packaging of virus genomes. These conclusions are therefore compatible with a basic model for initiation of replication during infection, in which the virus genome is stripped of all its core proteins before replication can proceed and ‘late’ events ensue. However, the studies described in this paper indicate that initiation and some significant elongation of virus DNA can be achieved using both cores and subviral particles as templates. The data suggest that these templates may be utilized at some stages during infection and that ‘naked’ templates may therefore not be the only participants in replicative events.

METHODS

Cells and virus. HeLa cells were grown in suspension in Glasgow S-MEM medium supplemented with 7% newborn calf serum at a density of between 3 × 10⁵ and 6 × 10⁵ per ml. Adenovirus type 2 (Ad2) was grown in suspension cells, purified by multiple density gradient centrifugation and titrated as previously described (Russell et al., 1967; Winters & Russell, 1971).

Enzymes and radiochemicals. Restriction enzymes were obtained from either Bethesda Research Laboratories or New England Biolabs and all radiochemicals were purchased from Amersham.

Purification of templates. Adenovirus deproteinized DNA, DNA–protein complex and viral cores were prepared as previously described (Goding & Russell, 1983). Purified virus was disrupted by dialysing virus obtained from CsCl equilibrium gradients against a large volume of 10 mm-Tris–maleate buffer pH 6.4, at 4°C with vigorous stirring and changes of buffer (Prage et al., 1970).

Viral DNA concentration. The concentration of viral DNA was determined spectrophotometrically. Samples of viral DNA and core DNA were diluted in 10 mm-Tris–HCl pH 7.5, whereas dialysed virus was diluted into 10 mm-Tris–HCl pH 7.5, 0.5% SDS. The concentration was calculated assuming that 1 A₂₆₀ is equivalent to 50 μg/ml of DNA.

Protein concentration. The concentrations of protein in cell extracts were estimated by the method of Bradford (1976) using bovine serum albumin to construct a standard curve of known protein concentrations. Column fractions were assayed for protein by spotting 2 μl onto Whatman 3MM paper and staining with Coomassie Brilliant Blue.
Oligonucleotide synthesis. Oligonucleotides were synthesized using an Applied Biosystems 381A DNA Synthesizer with 0.2 μmol scale (50 nm) long chain alkylamino controlled pore glass columns (Cruachem). Double-stranded oligonucleotides used in this study contained either an NFI binding site, GAGCTCACCGAGATGCTGGCCGATC or part of the simian virus 40 (SV40) enhancer containing the binding site for EBPI (SV-1), GAGCTCACCGAGATGCTGGCCGATC (Clark et al., 1988).

Hybridization of oligonucleotides. Equimolar amounts of the oligonucleotides (2 mg/ml) were diluted into 10 mM-Tris–HCl pH 7.5, 1 mM-EDTA and 0.3 m-NaCl, boiled for 2 min, cooled to 50 °C and incubated at this temperature for 3 to 4 h. Double-stranded oligonucleotides were then stored at −20 °C.

Densitometry. Band densities from autoradiograms were measured using a Shimadzu Dual-Wavelength Flying Spot Scanner CS9000 set on a drift line to eliminate background values.

Purification of replication factors. Ten 1 of suspended HeLa cells at a density of approx. 5 x 10⁵ cells/ml were concentrated by centrifugation (500 g, 10 min), resuspended in 1/10 th volume of medium without calf serum and infected with Ad2 at a multiplicity of 100 p.f.u./cell. After 1 h adsorption at 37 °C the cells were resuspended in the original volume with medium containing 2% calf serum and after a further 1 h at 37 °C, hydroxyurea (Sigma) was added to 10 mM. At 21 h after infection at 37 °C cytoplasmic extracts and nuclei were prepared (Challberg & Kelly, 1979).

The adenovirus-encoded replication proteins DBP and pTP/Pol were purified essentially as described by Rosenfeld et al. (1987) with a few modifications. Prior to centrifugation (10000 g for 1 h) the cytoplasmic extract was adjusted to pH 6 with 8.5% orthophosphoric acid, 150 mM-NaCl, 1 mM-DTT and 1 mM-EDTA. The supernatant was loaded on a phosphocellulose column (Whatman P11) that had been pre-equilibrated with buffer B (10 mM-sodium phosphate buffer pH 6, 10% sucrose, 10% glycerol, 0.01% NP40, 1 mM-EDTA, 1 mM-PMPSF) containing 150 mM-NaCl. The column was washed with buffer B containing 150 mM-NaCl (2 column volumes) and proteins were eluted with a linear gradient from 150 mM to 1 M-NaCl in buffer B (5 column volumes). Fractions containing the pTP/Pol were identified by their associated aphidicolin-resistant DNA polymerase activity as described below. Active fractions were pooled, dialysed for 4 h at 4 °C into 150 mM-NaCl in buffer C (25 mM-sodium phosphate buffer pH 6, 10% glycerol, 0.1% NP40, 1 mM-EDTA, 0.1 mM-PMPSF) and loaded on a single-stranded calf thymus DNA-Sepharose (CNBr-Sepharose 4B, Pharmacia) column. The column was washed with buffer C containing 150 mM-NaCl (2 column volumes) and the proteins were eluted with a linear gradient from 150 mM- to 560 mM-NaCl in buffer C (5 column volumes). Fractions were again assayed for their aphidicolin-resistant DNA polymerase activity. Active fractions (0.015 units/μl) were stored at −70 °C before being concentrated (0.5 units/μl) and desalted into buffer C containing 25 mM-NaCl, using a Centricron 30 microconcentrator (Amicon) to give a final 20- to 40-fold purification of pTP/Pol. Adenovirus DBP was eluted from the ssDNA column with 1 M-NaCl and fractions were assayed for protein content.

Fractions containing the major protein peak were subjected to SDS–PAGE analysis to confirm the presence of DBP, before being pooled and dialysed at 4 °C against 25 mM-Tris–HCl pH 8, 100 mM-NaCl, 0.1 mM-PMPSF and 50% glycerol and stored at −20 °C (1 mg/ml). Two major bands of Mr 72K and 46K were observed, consistent with the presence of DBP and its principal breakdown product (Ariga et al., 1980; Russell et al., 1989).

DNA polymerase assays. Column fractions were assayed for aphidicolin-resistant polymerase activity by adding 2.5 μl to a reaction mixture (25 μl) containing 50 mM-Tris–HCl pH 8, 7 mM-MgCl₂, 1 mM-DTT, 200 μg/ml activated DNA, 40 μM-dNTPs, 40 μg/ml aphidicolin and 1 μCi [α-32P]dCTP (approx. 3000 Ci/mmol). After incubation for 1 h at 37 °C, acid-insoluble 32P radioactivity was determined by standard procedures. One unit of DNA polymerase activity is defined as the incorporation of 1 nmol of dCMP into acid-insoluble DNA in 1 h at 37 °C.

In vitro DNA replication assay. Cytoplasmic and nuclear extracts were prepared from adenovirus-infected and uninfected HeLa cells as described above.

Formation of the 80K pTP–dCMP initiation complex was assayed in reaction mixtures (35 μl) containing 150 to 300 ng Ad2 template DNA, 6 to 12 μl of infected cell extract, 5 μCi [α-32P]dCTP (approx. 3000 Ci/mmol), 25 mM-HEPES pH 7.5, 5 mM-MgCl₂, 4 mM-EDTA, 3 μM-ATP. If uninfected nuclear extracts (2.5 μg/ml) were used instead of infected extracts (nuclear extract 5 mg/ml, cytoplasmic extract 10-5 mg/ml) the assay mixture was supplemented with partially purified pTP/Pol (2 units) and DBP (2 μg). After incubation for 90 min at 30 °C the product was digested with micrococcal nuclease (Pharmacia, 5 units) in the presence of 10 mM-CaCl₂ for 30 min at 37 °C. Reactions were stopped by addition of one-third volume 4% SDS, 200 mM-Tris–HCl pH 7, 3 m-2-mercaptoethanol, 20% glycerol, with bromophenol blue and boiled for 3 min. Products were analysed by SDS (10%)-PAGE, followed by autoradiography.

The assay for initiation followed by limited elongation used reaction mixtures (35 μl) and incubations as above, with the following changes; 35 μM each of dATP, dTTP and dGTP were added and digestion with micrococcal nuclease was omitted. In some experiments an ATP regeneration system consisting of 5 mM-creatine phosphate (Sigma) and 5 μg/ml creatine phosphokinase (Sigma) was utilized.

Analysis of replication products. Elongation assays were as described above, except that dATP and dTTP concentrations were increased to 85 μM, ddGTP was replaced by dGTP at 85 μM, 2 μM-dCTP was added and [α-32P]dCTP increased to 20 μCi. After incubation for 2 h at 30 °C the product was treated with proteinase K (Boehringer, et al., 1988).
50 μg/ml in 0.5% SDS, 1 mM-EDTA, 10 mM-HEPES pH 7.5) for a further 2 h at 56 °C, extracted with phenol and chloroform, ethanol-precipitated, washed in 70% ethanol and dried. Resuspended DNA was cleaved with PvulI or KpnI using conditions specified by the manufacturer and DNA fractionated by electrophoresis in a 2% agarose gel. The gel was fixed in 10% acetic acid for 1 h, dried and autoradiographed. KpnI and PvulI restriction patterns were derived from standard databanks for Ad2 and confirmed by direct analysis on purified viral DNA.

RESULTS

Initiation and limited elongation using cores as templates

Previous studies demonstrated that adenovirus cores were capable of acting as in vitro templates for DNA replication and indeed appeared to function in this respect more effectively than DNA–protein complexes (Goding & Russell, 1983). In the studies described here we have confirmed and extended these observations.

Initiation of adenovirus DNA replication involves formation of a covalent complex between the viral 80K preterminal protein pTP and a dCMP residue; this complex is then used as a primer for further DNA synthesis. Transfer of [32p]dCMP on to the pTP was therefore used as an assay for the initiation reaction. Fig. 1 (a) demonstrates the initiation complex formation using cores as templates with infected nuclear extracts supplying the replication factors. Reactions also containing the chain terminator ddGTP with dATP and dTTP result in complex formations corresponding to the pTP-dCMP complex and its 26 base elongation product (pTP-26). (The first dG residue is at nucleotide 26 in Ad2.)

Fig. 1 (b) demonstrates the complex formations using cores as templates, with infected nuclear extracts at optimal and suboptimal concentrations (Fig. 1 b, lanes 1 and 2). Fig. 1 (b), lane 3 shows that replication with suboptimal concentrations of extracts can be further promoted by adding additional purified DBP. Similar effects were achieved using infected cytoplasmic extracts (Fig. 1 c). In another experiment (data not shown) we observed that the efficacy of the cytoplasmic extracts can be significantly enhanced by adding extracts from uninfected cellular nuclei, illustrating the relative importance of nuclear factors in the replication events. Fig. 1 (d) indicates that with uninfected nuclear extracts and partially purified pTP/Pol in the absence of DBP some initiation can be observed although there is significant background labelling (Fig. 1 d, lane 1). However, addition of DBP significantly improves the replication reaction in terms of initiation and elongation while also reducing the background (Fig. 1 d, lane 2). Omission of pTP/Pol completely prevented the reaction (Fig. 1 d, lane 3). Similar studies were also carried out without nuclear extracts and it was evident that the core templates alone with pTP/Pol in the absence of DBP could allow initiation (Fig. 1 e, lane 2). On adding DBP, as above, initiation and elongation (with reduced background) were noted (Fig. 1 e, lane 1).

It is interesting that although these particular cell extracts (infected and uninfected) function well in the replication reaction with cores as templates they have proved unsatisfactory with linearized plasmids (data not shown), suggesting that the terminal protein serves as an important defence against endogenous exonucleases.

These observations indicate that adenovirus cores can successfully act as in vitro templates and have the same requirements as those described for plasmid and DNA–protein complex templates (Hay & Russell, 1989).

Proteolytic breakdown of the preterminal protein

Previous studies have shown that the 80K pTP is a precursor of the 55K TP present on virus DNA (Challberg & Kelly, 1981). The enzyme that catalyses the cleavage is a virus-encoded protease and the conversion apparently involves an intermediate fragment of 62K (Smart & Stillman, 1982).

Fig. 1 (b) clearly shows these breakdown products of the pTP and pTP-26 complexes. On analysis of these components in comparison to those in Fig. 1 (a) (where only the pTP–dCMP complex and its cleavage products are visible) and analyses of many other similar products (not shown), the bands observed appear to correspond to the product after elongation to the 26th nucleotide (pTP-26), to the pTP initiation complex, to a specific intermediate degradation product of the pTP-26 complex (ipTP-26), to a specific intermediate product from pTP (ipTP),
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Fig. 1. Formation of the $^32$P-labelled complexes after initiation (a) and limited elongation (b, c, d and e) with cellular extracts and/or partially purified proteins from Ad2-infected cells. Extracts were incubated in the presence of [$\alpha$-$^32$P]dCTP, using virus cores as template and optimal concentrations of MgCl$_2$ and ATP as described in the methods section. Polypeptides were separated by PAGE and labelled species detected by autoradiography. Assay mixtures included the following. (a) Infected nuclear extracts: lane 1, 33 µg; lane 2, 66 µg. (b) Infected nuclear extracts: lane 1, 33 µg; lane 2, 66 µg; lane 3, 33 µg. DBP (2 µg) was added to lane 3. (c) Infected cytoplasmic extracts: lanes 1 and 2, 84 µg. DBP (2 µg) was added to lane 2. (d) Uninfected nuclear extracts: lanes 1, 2 and 3, 17 µg, with partially purified pTP/Pol in lanes 1 and 2 (2 units) and DBP in lanes 2 and 3 (2 µg). (e) No cellular extracts; only pTP/Pol in lanes 1 and 2 (2 units) and DBP in lane 1 (2 µg).

to a band of similar mobility corresponding to the final cleaved product from pTP-26 (TP-26) and to a fainter band corresponding to the final cleaved product from pTP (TP). Closer examination of a number of these electrophoretic patterns showed that a double band may also be apparent in the ipTP-26 region; in some other cases one of the bands in the doublet corresponding to ipTP and TP-26 appears stronger (e.g. Fig. 1b and 3a). This would be consistent with a double intermediate cleavage in the pTP protein and is in agreement with other studies in this laboratory on peptide pTP substrates for the adenovirus protease and on
investigations of the direct effect of the adenovirus protease on labelled pTP (Webster et al., 1989a, b). It was evident in the case of infected cytoplasmic extracts (Fig. 1 c) that specific proteolysis was very much reduced, suggesting that the virus-encoded protease had mainly partitioned into the nuclear extracts.

**Effect of divalent ions on in vitro replication**

As noted by previous investigators it was found that initiation and elongation of adenovirus DNA required Mg$^{2+}$ ions in the concentration range 2 to 5 mM (Harris, 1987). Other divalent ions such as Ni$^{2+}$, Zn$^{2+}$ and Ca$^{2+}$ were unable to replace Mg$^{2+}$ over a similar concentration range. 

Fig. 2. Initiation and limited elongation assays using Ad2 cores as templates. (a) Titration of MnCl$_2$ in the standard assay, replacing MgCl$_2$, using infected nuclear extracts (33 μg) in lanes 1 to 7; 1 μM, 10 μM, 100 μM, 1 mM, 2 mM, 4 mM, 8 mM respectively. (b) Uninfected nuclear extracts (17 μg) with pTP/Pol (2 units) and DBP (2 μg): lane 1, 5 mM-MgCl$_2$; lane 2, 1 mM-MnCl$_2$; (c) as (b) except only initiation was assayed. (d) Infected cytoplasmic extracts (84 μg) with additional DBP (2 μg) in lanes 3 and 4; lanes 1 and 3 with 5 mM-MgCl$_2$; lanes 2 and 4 with 1 mM-MnCl$_2$. (e) No cellular extracts, only pTP/Pol (2 units), DBP (2 μg) in lanes 1 and 2; lanes 2 and 3 with 5 mM-MgCl$_2$; lanes 1 and 4 with 1 mM-MnCl$_2$. 


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range. The addition of Co(NH₃)₆³⁺ ions in concentrations known to alter DNA conformation (Leith et al., 1988) was also unable to support the reactions. However, the effect of Mn²⁺ ions was remarkable. Fig. 2(a) shows that Mn²⁺ ions can replace Mg²⁺ ions over a rather narrow range (1 to 2 mM) of concentration and, in the presence of cell extracts, had the effect of preventing elongation and stimulating initiation (Fig. 2a). Fig. 2(b) and (c) show a comparison of assays using Mg²⁺ (lane 1) or Mn²⁺ ions (lane 2) in the presence of uninfected nuclear extracts and partially purified pTP/Pol and DBP. Fig. 2(b) clearly shows that elongation is prevented and initiation is stimulated by Mn²⁺ ions. Stimulation of initiation was also evident with Mn²⁺ in the absence of elongation (Fig. 2c), indicating that there was a direct effect, rather than a build-up of initiation complexes following inability to elongate.

Infected cytoplasmic extracts also gave similar effects (Fig. 2d, lanes 1 and 2) and Fig. 2(d) lanes 3 and 4 show that when replication was boosted by adding additional DBP in the presence of Mn²⁺ ions initiation was stimulated and elongation, although greatly reduced, was detected at a low level, thus indicating that additional DBP could partly overcome the Mn²⁺ block to elongation (Fig. 2d, lane 4). When using assays which did not contain crude cell extracts, i.e. only core templates with partially purified pTP/Pol and DBP, Mn²⁺ ions stimulated both initiation and elongation (Fig. 2e, lanes 1 and 2). This result was in contrast to Fig. 2(b) where uninfected nuclear extracts were present and elongation was prevented. In the absence of elongation (Fig. 2e, lanes 3 and 4) Mn²⁺ ions stimulated initiation indicating (as with Fig. 2c) that there was a direct effect on the initiation event. A similar effect of Mn²⁺ ions on elongation and initiation could be demonstrated using DNA–protein complexes as templates (data not shown).

Role of cellular factors in the in vitro reaction

Previous studies (reviewed by Hay & Russell, 1989) had shown that cellular factors such as nuclear factor I (NFI) can increase replication efficiency very dramatically. NFI is known to be a sequence-specific DNA-binding protein that interacts with a site located between nucleotides 19 and 39 on the viral genome. To determine the role of NFI in this system, double-stranded oligonucleotides were synthesized which encompassed this binding domain, added to the replication reactions described above and the efficiency of replication was assessed with respect to another control oligonucleotide (SV1 oligo) of similar length. Fig. 3(a and b) shows that inhibition of initiation and elongation were noted with the specific oligonucleotide in cell extracts (the presumed major source of NFI) and also with the more defined system where there were no additional cell extracts (Fig. 3c). This latter result suggested that NFI was present in the partially purified fractions, or was already attached to the core templates.

In all the systems tested as much as 250 ng of oligonucleotide was required to achieve significant inhibition, possibly, in part, a reflection of interactions with non-specific DNA-binding proteins in the extract. Specificity of the inhibiting action of the NFI double-stranded oligonucleotide was demonstrated by the lack of inhibition shown with the control oligonucleotide. These results therefore are consistent with NFI playing a role in these in vitro reactions, but indicate that other degradative and inhibitory factors may also be operating in this system.

Initiation and elongation using subviral particles as templates

By dialysing purified virus into a hypotonic buffer, virus particles can be obtained which have lost their penton capsomeres. Such preparations were utilized as templates for DNA replication in vitro and Fig. 4 shows that although replication was about five- to ten-fold reduced compared to core templates using infected nuclear extracts (lane 6), both initiation and elongation could be readily detected. Fig. 4 lanes 1 and 2 compare the effect of adding additional DBP to suboptimal concentrations of infected nuclear extracts; as with core templates the extra DBP stimulates both initiation and elongation. An assay without cell extracts (Fig. 4, lane 3) did not give sufficient replication to be easily detected, but replacing the Mg²⁺ ions with Mn²⁺ ions resulted in a stimulation of initiation to a detectable level, although no elongation was noted (Fig. 4, lane 4). This apparent prevention of elongation was in contrast to the result with core template in
Fig. 3. Competition for nuclear factor 1 binding in Ad2 replication assays. Double-stranded oligonucleotides (oligos) containing either an NFI binding site or part of the SV40 enhancer (SV-1) were included in the assay, using virus cores as template. (a) Infected nuclear extract (33 μg) with NFI oligo; lanes 1 to 5, 0 ng, 1 ng, 10 ng, 100 ng, 500 ng respectively; (b) uninfected nuclear extract (17 μg) with pTP/Pol (2 units) and DBP (2 μg) with NFI oligo; lane 1, 0 ng, lane 2, 250 ng; lane 3, 250 ng of SV-1 oligo; (c) no cellular extracts, only pTP/Pol (2 units) and DBP (2 μg) with NFI oligo lane 1, 100 ng; lane 2, 250 ng; lane 3, 250 ng of SV-1 oligo.

Fig. 4. Initiation and limited elongation assays using Ad2 subviral particles as templates with: lane 1, infected nuclear extract (33 μg); lane 2, infected nuclear extract (33 μg) and additional DBP (2 μg); lane 3, no cellular extract, pTP/Pol (2 units) and DBP (2 μg); lane 4, as lane 3 except MgCl₂ replaced by 1 mM-MnCl₂; lane 5, as lane 1 except MgCl₂ replaced by 1 mM-MnCl₂; lane 6, infected nuclear extract (33 μg) with adenovirus cores as template.
assays without cell extracts where elongation was stimulated (Fig. 2e). When Mn$^{2+}$ ions were used with an infected nuclear extract, as with the core template, stimulation of initiation accompanied by inhibition of elongation was observed (Fig. 4, lane 5).

**Analysis of the products of the in vitro reactions**

Previous studies using adenovirus cores as templates had shown that replication could proceed well beyond the 26th nucleotide (Goding & Russell, 1983) although it was not possible to ascertain the extent of elongation. In the experiments described here elongation on the core templates was allowed to proceed in the presence of sufficient unlabelled dCTP to facilitate the reaction and to retain sufficient sensitivity to label the product DNA. Cleavage of the products with KpnI indicated that only the terminal fragments of 2 kb (left) and 1.9 kb (right) were substantially labelled (Fig. 5a, lane 1), indicating that although initiation and limited elongation had taken place it had not extended beyond 2 kb, since fragments distal or adjacent to these fragments were not labelled. Using uninfected cell extracts instead of infected extracts, no labelling of the terminal fragments (Fig. 5a, lane 2) was obtained.

To determine the extent of elongation, replicated DNA was cleaved with Pvull to generate smaller terminal fragments. The labelling patterns obtained were consistent with the label being incorporated from both termini into proximal restriction fragments as well (Fig. 5b). Fig. 5(b) lane 1 shows the pattern using core templates with infected nuclear extract. The left terminal fragment of 451 bases (L1) is clearly labelled and its adjacent fragment of 171 bases (L2) is also labelled; the adjacent fragment to this of 1866 bases (L3) is not significantly labelled, indicating that elongation had not extended beyond the L2 fragment, i.e. a maximum of 622 bases in total. The right terminal fragment of 1135 bases (R1) is also clearly labelled, but its adjacent fragment, 1155 bases (R2) is only slightly labelled, indicating elongation may have extended just beyond the right terminal 1135 bases. Uninfected extracts did not demonstrate labelling of the terminal fragments (Fig. 5b, lane 2). The core templates with uninfected nuclear extracts and purified pTP/Pol and DBP also show labelling of the terminal fragments (Fig. 5b, lane 3), although no labelling of the proximal fragments is evident. In another experiment the effect of adding an ATP-regenerating system was assessed and it was evident that significant extension of labelling was achieved. Fig. 6, lanes 1 and 2 show the effect of the regenerating system on an assay using core templates and infected nuclear extracts. A marginal improvement on extension is apparent (with label becoming more prominent in the R2 fragment). However, in assays using subviral particles as templates and infected nuclear extracts (Fig. 6, lanes 3 and 4) a marked increase in extension was achieved with the regenerating system. Labelling was evident in fragments even further from the termini, e.g. the L3 fragment of 1866 bases adjacent to the left terminal fragment (Fig. 6, lane 4).

To quantify these labelling patterns the radioactive contents of the corresponding bands on the autoradiogram were estimated using a microdensitometer and a value, Q, derived for each restriction fragment by dividing this nominal area (after subtracting background) by the total number of cytidines in the replicating strand of the restriction fragment. Fig. 7 illustrates the Q values for the four lanes in Fig. 6 corresponding to the products of the reaction using cores as templates (a) without and (b) with the regenerating system and, using subviral particles as template, (c) without and (d) with the regenerating system. If it is supposed that radioactivity incorporated into fragments can result from initiation, premature termination and elongation up to the restriction site then it will be evident that the much larger Q values associated with the smaller left-hand terminal fragment (L1) is consistent with this fragment containing many initiation events without successful elongation, in agreement with the results shown in Fig. 1 to 5. It is also of interest that fragments proximal to this one (i.e. L2) have similar Q values in both (a) and (b), i.e. reflecting the incorporated label resulting from elongation. In confirmation of this observation it should be noted that the Q values for L2 and L3 fragments in (d) where replication has appeared to proceed further are the same. Using these stable Q values the approximate lengths of the replicated products encompassing the internal fragments L3, L4 and R2 were then assessed using the derived areas and the number of cytidines in the appropriate fragments, giving the extent of replication from the two termini in terms of
Fig. 5. Restriction endonuclease analysis of Ad2 DNA synthesized in vitro using virus cores as templates. DNA fragments were fractionated by electrophoresis on 2% agarose and labelled species detected by autoradiography. (a) Labelled fragments after using Kpol enzyme: lane 1, with infected nuclear extract (33 μg) and DBP (2 μg); lane 2, with uninfected nuclear extract (17 μg) and DBP (2 μg). (b) Labelled fragments using PvulI enzyme: lane 1, with infected nuclear extract (33 μg) and DBP (2 μg); lane 2, with uninfected nuclear extract (17 μg) and DBP (2 μg); lane 3, with uninfected nuclear extract (17 μg), pTP/Pol (2 units) and DBP (2 μg). Figures shown are number of bases.

Fig. 6. Restriction endonuclease (PvulI) analysis of Ad2 DNA synthesized in vitro in the presence (lanes 2, 4) or absence (lanes 1, 3) of an ATP regeneration system. Adenovirus cores (lanes 1, 2) or subviral particles (lanes 3, 4) were used as templates with infected nuclear extracts (33 μg) and DBP (2 μg). Figures shown are number of bases. (In lane 4, a band can be detected at L2 in the original autoradiogram.)

nucleotides, as shown in Fig. 7. It will be evident that replication has proceeded much more efficiently when using subviral particles as a template in the presence of an ATP-regenerating system and although stimulation was also observed with the core templates, the extent of replication was significantly less.
DISCUSSION

The experiments described here have confirmed and extended the previous observations on the utility of adenovirus cores as templates for DNA replication. These templates behaved in a similar fashion to the well characterized DNA–complex templates in that there was dependence on pTP/Pol, evidence of stimulation by cellular factors and initiation as well as elongation events were modulated by addition of DBP. Cytoplasmic extracts from infected cells also provided a source of replication factors and it was interesting that there was very little evidence of specific proteolysis from this source, an observation consistent with previous findings that most of the protease activity was located in nuclei from infected cells (Bhatti & Weber, 1979). However, it was noted that protease activity was evident using cores as templates in the absence of cell extracts (Fig. 1 e), thus cytoplasmic extracts may contain an inhibitor of the protease activity. Our results also suggested that proteolysis of pTP occurred in stages, with defined
intermediates of about 65K between the 80K precursor and the mature 55K product (see also Challberg et al., 1982; Webster et al., 1989b). It was interesting to note that pTP-26 appeared in some cases to be more susceptible to processing than the pTP, perhaps as a reflection of differences in the conformation of the pTP as a result of elongation of the attached nucleotides. It was also notable that replication could be detected with the partially purified pTP/Pol and DBP components in the absence of added cellular factors and, since significant inhibition of the reaction could be achieved by adding the NFI-specific oligonucleotide, it seemed probable that the NFI protein was available in the reaction. Although it is not possible to rule out that the cores could contain NFI already bound to the DNA template [NFI being primarily a transcription factor (see Hay & Russell, 1989) and as such may have been bound to the template before maturation] it is more likely that NFI copurified with the pTP/Pol, particularly as a published purification procedure for NFI also used column chromatographic procedures (Leegwater et al., 1985) similar to those used in our studies.

The relative insensitivity of the inhibitory action of the oligonucleotides may also be related to unavailability of NFI (already bound to template) (Cleat & Hay, 1989b), or result from them being digested by the exonucleases apparently present in these reactions, as evidenced by the inability of plasmids to function effectively. The role of Mn$^{2+}$ in significantly increasing initiation and preventing elongation is not immediately evident, especially since other divalent ions (at the concentrations examined) failed to support initiation. A similar phenomenon where initiation was increased in response to Mn$^{2+}$ ions was noted by Harris (1987) using extracts from adenovirus type 4 (Ad4)-infected cells and plasmid DNA containing the Ad4 origin of replication as a template. Effects on DNA conformation which might be important in presentation of template for initiation, on complex formation with dNTPs, on the viral polymerase requirements and in inhibiting other factors involved in processing should therefore be considered.

The finding that templates prepared by mildly disrupting the virus to remove the apical capsomeres were effective for both initiating and elongating virus DNA was somewhat surprising and suggests that the template is already available for initiation in the packaged virion. Indeed, initiation of replication could be detected even in the absence of crude cellular extracts when Mn$^{2+}$ ions were present (Fig. 5).

Analysis of the replication products and estimates of the extent of elongation indicated that, even in the presence of core proteins, replication proceeded in the cores to about nucleotides 660 at the left terminal and 1210 at the right terminal. It was notable that stimulation of elongation occurred on adding the ATP-regenerating system, indicating that utilization of ATP in the assay is one critical feature of the process. Although initiation efficiency was about fivefold less when using subviral particles as templates (compare Q values for L1 fragments in Fig. 6, lanes 1 and 3) the extent of elongation was considerably greater, particularly with the ATP regeneration system. Products consistent with replication to about nucleotide 3150 from the left terminal and nucleotide 2390 from the right terminal could be detected (Fig. 7).

It is interesting that these results were obtained in the presence of aphidicolin (which significantly inhibited background repair synthesis), since it has been previously reported that this drug inhibits the later stages of elongation when using DNA–protein complexes as templates (Nagata et al., 1983). The same study reported that topoisomerase availability is required in the later stages of replication and no attempt has been made in our experiments to supplement the cell extracts with additional enzyme; indeed, it may be that our assay is not optimal to allow full-length elongation. The relatively greater efficiency of the subviral particles in elongation may be a reflection of their conformational integrity, since core templates must suffer a degree of denaturation in their preparation, being heated to 36 °C for 90 s.

The suggestion by Korn & Horwitz (1986) that cores can function as templates because of a small percentage of completely uncoated DNA molecules seems extremely unlikely. Thus our previous studies (Goding & Russell, 1983) indicated that cores were more efficient templates in this system than DNA–protein complexes and there seems little doubt that removal of the core proteins from viral DNA can only be accomplished in vitro using very harsh and non-physiological conditions. Nevertheless our studies do not characterize the active template in
these in vitro reactions using the dialysed virus, since it seems possible that a variety of intermediates ranging from pentonless nucleocapsids to cores could be present. However, the studies by Prage et al. (1970) have analysed such dialysed virus preparations by electron microscopy and polypeptide gel electrophoresis and have clearly shown that the major population consists of virus particles devoid of pentons and also of peripentonal hexons and that removal of the remaining hexons from the core is achieved only by extensive freezing and thawing of the preparations.

These findings therefore provide the first evidence even in subviral particles, and presumably therefore in mature particles, that on first ‘uncoating’ the template DNA is readily available for limited replication. Such utilization of the template may arise because core proteins are not attached to this portion of the DNA, or it may be that elongation can proceed even in the presence of these basic proteins. However, in vivo such a template availability might be more applicable to early transcription rather than to replication, especially since the template regions apparently available for replication at the termini almost cover the entire ‘early’ E1 and E4 genes. Thus the concept of complete uncoating of the genome as a necessary early stage of transcription might have to be reconsidered in the light of these observations and it cannot be ruled out therefore that the critical templates for the primary events in vivo might also involve core proteins, perhaps in a different conformation due to the transcriptional events that have occurred on the ‘early’ genes. A recent electron microscopic analysis by Wong & Hsu (1989) on bis-psoralen cross-linked adenovirus type 5 virion DNA has suggested that the genomic DNA is arranged in eight supercoiled loops, excluding the two termini which appear to be exposed. These observations are consistent with our findings indicating that the termini are available for replication.

We wish to thank Margaret Bell and Margaret Wilson for typing and Bill Blyth for photography. This work was supported by MRC Grant No. 48420312GA.

REFERENCES


(Received 5 June 1989)