The EMBO Journal Vol.2 No.3 pp.339-344, 1983

Adenovirus cores can function as templates in *in vitro* DNA replication

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Communicated by A.E.Smith
Received on 22 November 1982, revised on 31 December 1982

Adenovirus cores prepared by gentle disruption of virus by heating at 56°C in the presence of deoxycholate were able to function as templates in an *in vitro* DNA replication system, allowing both initiation, indicated by the formation of terminal protein-dCMP complex, and elongation of > 300 nucleotides. Using both cores and DNA-protein complexes as templates, it was also demonstrated that novobiocin, an inhibitor of DNA gyrase, inhibited *in vitro* DNA replication by preventing formation of the initiation complex.

Key words: adenovirus/DNA replication/cores/novobiocin

Introduction

The adenovirus type 5 (Ad5) genome is a linear double-stranded DNA molecule of ~35 000 bp possessing an inverted terminal repetition of ~100 bp. A viral-coded protein of 55 000 mol. wt. (Tp) is covalently linked to the 5′ termini, forming the so-called adenovirus DNA-protein complex (Rekosh *et al.*, 1977; Stillman *et al.*, 1981). DNA replication takes place in the nucleus and proceeds by a displacement mechanism with initiation apparently occurring at either end of the DNA molecule with equal frequency (Horwitz, 1971; Sussenbach and Kuijk, 1977).

Recently, soluble *in vitro* systems capable of initiating and elongating adenovirus DNA replication from an exogenous template of adenovirus DNA-protein complex have been described (Challberg and Kelly, 1979; Kaplan *et al.*, 1979). Using these systems, it had been shown that an early step in initiation of adenovirus DNA replication was the covalent attachment of a dCTP molecule to an 80 000-90 000 (80-90 K) precursor to the terminal protein (pTP) to form a pTP-dCMP complex (Lichy *et al.*, 1981; Challberg *et al.*, 1980). The latest evidence suggests that this initiation reaction is catalysed by a polymerase of apparent mol. wt. 140 K which is viral coded (Enemoto *et al.*, 1981; Lichy *et al.*, 1982). Another viral-coded product, the single-stranded DNA-binding protein of apparent mol. wt. 72 K also plays an essential role in replication (Enemoto *et al.*, 1981; Challberg *et al.*, 1982). However, efficient initiation (and elongation) of replication is also apparently dependent on factors supplied by uninfected cells (Kaplan *et al.*, 1979; Nagata *et al.*, 1982). The nature of these cellular factors is unclear but they may play some role in processing nucleotides and template nucleic acid, e.g., nucleases, topoisomerases, ATPases, etc.

It is also clear that the presence of intact terminal protein on an otherwise protein-free template is an important factor in allowing replicative, rather than repair-type DNA synthesis to be catalysed by these *in vitro* systems. The DNA-protein complexes are prepared by completely disrupting purified virus with guanidinium hydrochloride (GuHCl) followed by sucrose or caesium chloride gradient centrifugation in the presence of 4 M GuHCl (Sharp *et al.*, 1976) or chromatography on Sepharose in the presence of 4 M GuHCl. However, the viral genome *in vivo* is also closely associated with at least two other proteins, V and VII, to form the viral 'core' (for review, see Nermut, 1983) although it is not known whether one or both of these proteins are released from the DNA during transcription or replication, or whether the viral DNA retains a chromatin-like conformation, possibly also involving cellular histones. In contrast to the DNA-protein complex, viral cores can be readily prepared by gentle disruption of virus using a combination of detergent and heat (Russell *et al.*, 1971; Nermut *et al.*, 1975) and it seems possible that they can retain, to a large extent, the original conformation of the DNA present in the virion.

In this communication we demonstrate that viral cores, comprising the viral genome associated with the core proteins V, VII (and IVa2: Goding *et al.*, 1983), are capable of functioning as templates for initiation and elongation of DNA replication *in vitro*. Moreover, we have utilised this sytem to analyse nuclear extracts for other indications of enzymatic activities and show that an event leading to the formation of the pTP-dCMP complex is sensitive to novobiocin, an inhibitor of bacterial DNA gyrase.

Results

Preliminary experiments were undertaken to determine whether cores were capable of acting as *in vitro* templates for the formation of the pTP-dCMP complex which is characteristic of initiation of adenovirus DNA replication. *In vitro* replication reactions were therefore carried out in the presence of [α-^{32}P]dCTP and ddGTP using both DNA-protein complexes and cores as templates. [Inclusion of the ddGTP allows elongation only as far as the first dG residue – nucleotide 26 in Ad5 DNA; Tolun *et al.* (1979).] Analysis of the products of these reactions by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography revealed two major labelled species with electrophoretic mobilities intermediate between marker adenovirus viral polypeptides II and III (Figure 1). These bands were present using either cores or DNA-protein complex as templates and possessed properties characteristic of the pTP-dCMP complex and its 26 base elongation product (pTP-26) (Lichy *et al.*, 1981; Tamanoi and Stillman, 1982). Thus they were apparent only if an infected cell nuclear extract was used, and were not seen using pronase-treated Ad5 DNA template or in the absence of added template, and were pronase-sensitive.

The efficiency of replication of cores (calculated from the incorporation of ^{32}P into the labelled bands) was consistently as good as the most efficient preparations of DNA-protein complex and the ability of the cores to function as templates was not diminished by several weeks storage at 4°C. Moreover, all preparations of cores were invariably capable of acting as templates whereas, in contrast, ~60% of the preparations of DNA-protein complex were inactive in this

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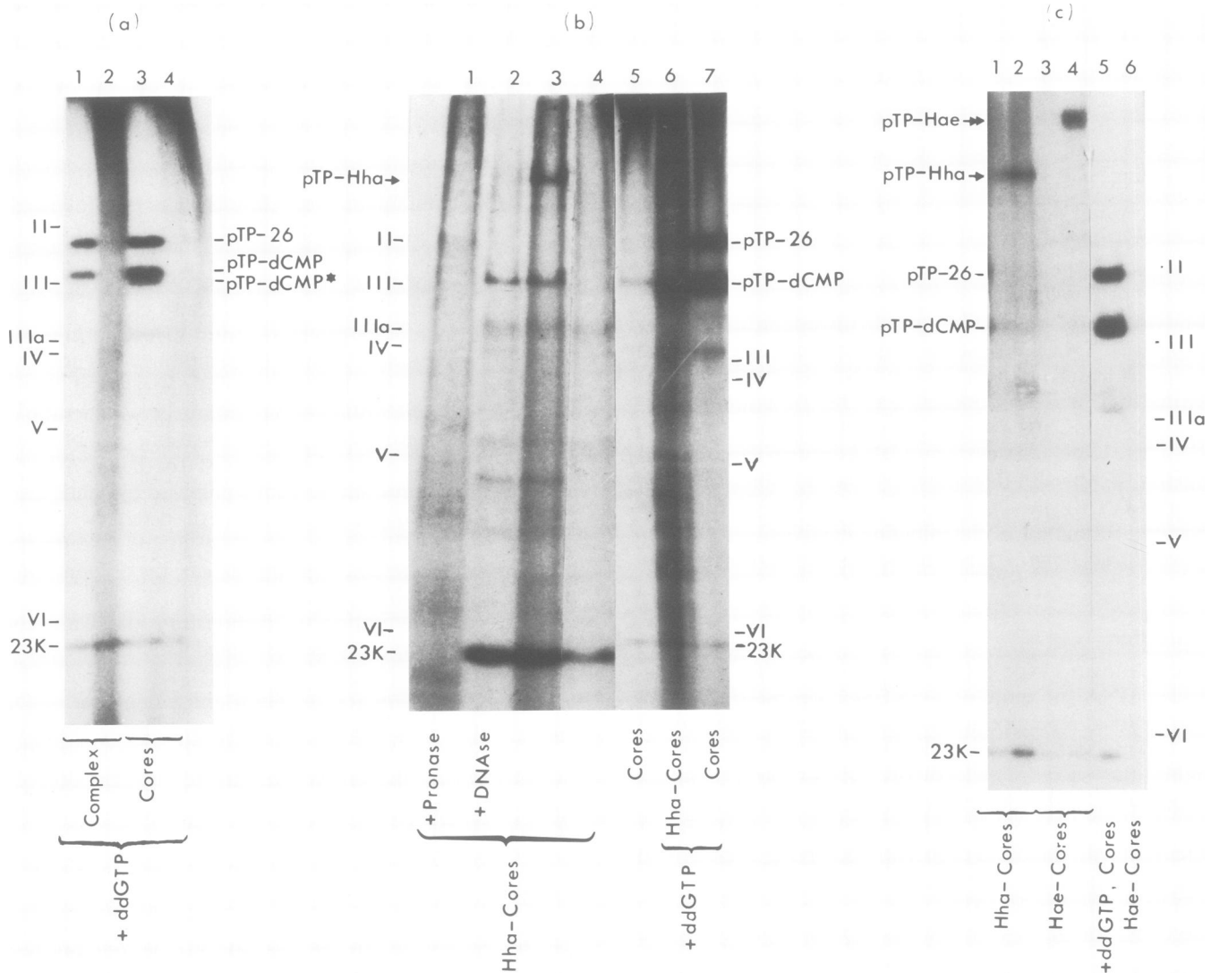

Fig. 1. Products of *in vitro* replication reactions analysed by SDS-PAGE and autoradiography. All reactions were performed using infected cell nuclear extracts except those shown in **lanes 2** and **4** in **(a)** and **lane 4** in **(b)** which used uninfected cell nuclear extracts. The label used was [α-^{32}P]dCTP in all reactions except that shown in **lane 2** in **(c)** which used [α-^{32}P]dATP. The templates (DNA-protein complex, cores or restricted cores) are indicated as are those reactions in which ddGTP was added. Reaction mixtures treated with 1 mg/ml pronase/1% SDS or 50 μg/ml DNase for 30 min at 37°C prior to analysis as indicated in **(b)** and the reaction products shown in **lanes 3** and **5** in **(c)** were similarly treated with DNase and that in **lane 6(c)** with pronase. Marker adenovirus polypeptides (using ^{125}I- or ^{35}S-labelled virus) are indicated by roman numerals (Rekosh *et al.*, 1977).

respect. In addition to the pTP-dCMP and pTP-26 complexes, a third dCTP-labelled species (pTP-dCMP*) with a slightly higher mobility on SDS-polyacrylamide gels could be seen (Figure 1). pTP-dCMP* was also pronase sensitive and could only be demonstrated using a template of cores (although it was not apparent in all experiments). The identity of pTP-dCMP*, perhaps an altered form of the pTP-dCMP complex or some other similarly labelled protein, or possible replication intermediate, remains unclear. A labelled pronase-sensitive species of apparent mol. wt. ~23 K could also be distinguished (Figure 1). This protein was also present in nuclear extracts of uninfected cells and labelled independently of added template with all four dNTPs. It may be similar to a polypeptide with similar labelling characteristics of apparent mol. wt. 30 K, reported by Pincus *et al.* (1981). This protein was not investigated further but it is interesting that an activity present in uninfected cells is apparently capable of attaching nucleotides to a substrate protein in a reaction with obvious similarities to that of the formation of the pTP-dCMP complex.

In an attempt to determine how far into the cores replication could proceed, preparations of cores were cut separately with two restriction endonucleases prior to use as templates. Only terminal fragments possessing TP would be expected to replicate under these conditions. The enzymes used were *Hha*I, which cuts within the terminal repeats at ~70 bp from each end of the genome and *Hae*III, which cleaves at ~270 bp from the left terminus (Bos *et al.*, 1981), and 320 bp from the right terminus (Steenbergh and Sussenbach, 1979). The products of *in vitro* replication using these pre-restricted templates were analysed by SDS-PAGE and autoradiography. It was assumed that if replication on these restricted templates could proceed this would result in labelled DNase-sensitive, pronase-sensitive bands corresponding to pTP linked to the replicated *Hae* or *Hha* terminal fragments and that the mobilities of these bands would be dependent on the length of the DNA attached to the pTP. The results shown in Figure 1 are consistent with replication in this manner. The

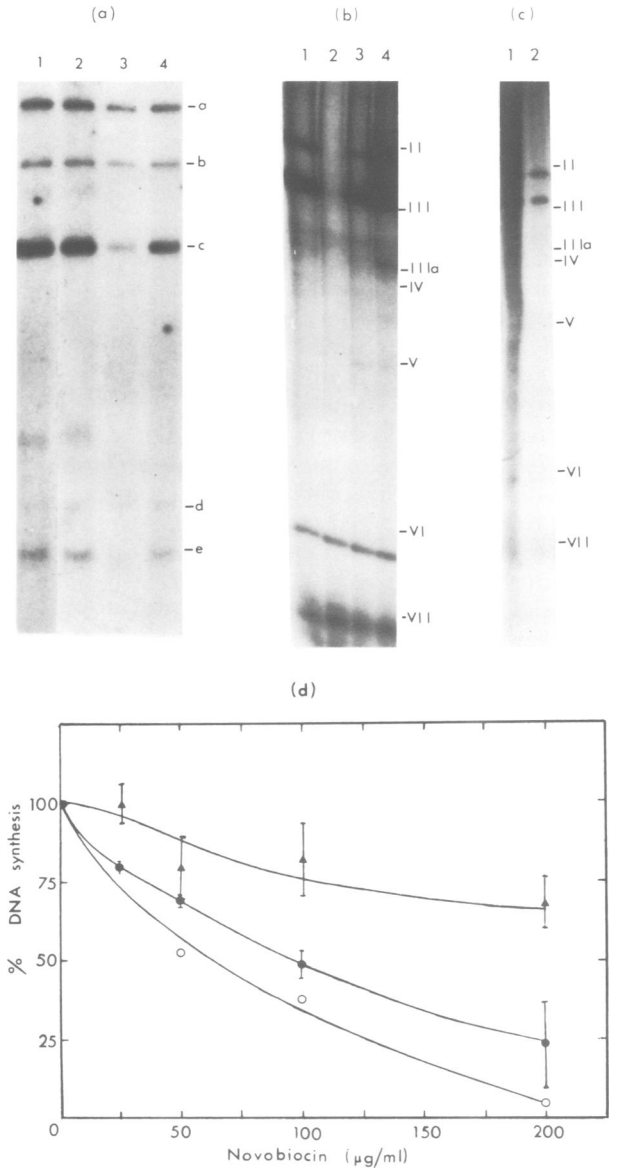

Fig. 2. Effects of novobiocin on *in vitro* replication. **(a)** Products of *in vitro* replication reactions using a template of *Xba*I pre-restricted DNA-protein complex analysed by SDS-agarose gel electrophoresis and autoradiography. Reactions were performed in the presence of: **lane 1**, 500 μg/ml nalidixic acid; **lane 2**, no added inhibitor; **lane 3**, 200 μg/ml novobiocin; **lane 4**, 100 μg/ml novobiocin. **(b)** Products of *in vitro* replication reactions performed in the presence of ddGTP and using [α-^{32}P]dCTP as label analysed by SDS-PAGE and autoradiography. Novobiocin was added to a concentration of: **lane 1**, 50 μg/ml; **lane 2**, 200 μg/ml; **lane 3**, 100 μg/ml; **lane 4**, no added inhibitor. **(c)** As for **(b)**. **Lane 1**, no inhibitor added; **lane 2**, 50 μg/ml aphidicolin. **(d)** Inhibition of *in vitro* replication by novobiocin. Values were obtained from densitometer tracings of autoradiograms. (○——○) formation of pTP-dCMP complex with values determined from autoradiogram shown in **(b)**. *In vitro* reactions using an infected cell nuclear extract and an *Xba*I pre-restricted template of DNA-protein complex giving values for fragment C (●——●) (replication) and fragment B (▲——▲) (repair). Points are the means of the three separate experiments with standard deviations indicated.

labelled pTP-70 bp complex (pTP-*Hha* in Figure 1) has a lower mobility than the pTP-26 complex and the broad band labelled pTP-*Hae* (presumably corresponding to the pTP-270 and pTP-320 complexes) has a significantly lower mobility than the pTP-*Hha* complex. These bands were only present if infected cell nuclear extracts were used and were both DNase- and pronase-sensitive. It was unlikely that they were the result of repair-type synthesis since such bands were not observed using uninfected cell nuclear extracts which elaborated repair synthesis by other criteria. Using a pre-restricted template in the presence of ddGTP, the pTP-dCMP complex was formed but a range of DNase-sensitive, pronase-insensitive bands were also apparent (Figure 1). These bands were presumably products of non-replicative DNA synthesis on the digestion products of the cores since incorporation into internal restriction fragments of DNA-protein complex or into DNA lacking the TP has previously been demonstrated to be a result of random repair-type DNA synthesis (Challberg and Kelly, 1979). It was also noted in these experiments that, in the absence of ddGTP, the degree of labelling of the pTP-dCMP complex using an unrestricted template was substantially reduced although label was present in high mol. wt. DNase-sensitive material (lane 5 Figure 1) which probably corresponded to elongation products of the pTP-dCMP complex. This suggests that the presence of ddGTP induces multiple abortive initiations resulting in excessive production of non-elongated pTP-dCMP complex. In addition, both the pTP-dCMP and pTP-26 complexes were resistant to DNase, implying that the 26 nucleotide elongation product of the pTP-dCMP complex was protected from nuclease action, possibly by the pTP and its associated 140-K DNA polymerase activity.

Having established that replication could proceed at least as far as the 26th and probably beyond the 320th nucleotide, attempts were then made to demonstrate more extensive elongation. Similar assays to those performed using core templates digested with *Hae*III and *Hha*I were attempted using restriction enzymes with sites further from the termini. However, *Xba*I, *Hind*III, *Bam*HI and *Eco*RI failed to cut the core DNA although adenovirus DNA-protein complex was efficiently restricted under the same conditions (data not shown). Subjection of such replicated and restricted cores to alkaline agarose gel electrophoresis and autoradiography revealed labelled single-stranded DNA of up to 8000 bases in length (data not shown). However, unreplicated cores analysed on SDS-agarose gels and stained with ethidium bromide showed a similar size pattern of DNA, implying that the core template DNA contained a significant number of single-strand breaks. As a result, it was not feasible by this method to determine how far into the cores replication could proceed. Attempts to determine a gradient of radioactivity (Pincus *et al.*, 1981) by restricting core templates after the reaction were similarly obscured by a significant background of repair-type synthesis.

Sensitivity of in vitro replicating systems to novobiocin

Having established that cores could act at least as a limited template, the sensitivity of *in vitro* replication to novobiocin was examined. Previous studies by D'Halluin *et al.* (1980) had shown that adenovirus DNA replication *in vivo* was sensitive to this inhibitor and although their results, using DNA-negative temperature-sensitive mutants, suggested that initiation of replication was affected, the novobiocin-sensitive step was not unambiguously determined. We used a template of DNA-protein complex pre-restricted with *Xba*I and analysed by electrophoresis in an SDS-agarose gel and autoradiography; replication of the terminal fragments, indicated by the higher incorporation into terminal fragments C and E compared with the internal fragments A, B and D, was shown to be sensitive to novobiocin but not to nalidixic acid,

an inhibitor of the B subunit of DNA gyrase (Figure 2a). The inhibition of DNA replication by novobiocin was also revealed by examining [^{35}S]methionine incorporation into adenovirus polypeptides in infected cells. In the presence of novobiocin (200 μg/ml) incorporation into late viral polypeptides, and presumably therefore DNA synthesis, was effectively inhibited while synthesis of early polypeptides was not diminished (data not shown). The sensitivity of adenovirus DNA replication to novobiocin was more closely examined by analysing the products of *in vitro* replication reactions labelled with [α-^{32}P]dCTP in the presence of cores or DNA-protein complex templates. Although only the level of the labelled pTP-dCMP complex was used for quantitation, it is clear that novobiocin at 200 μg/ml effectively inhibits both the formation of the pTP-dCMP complex and the formation of the pTP-26 complex (Figure 2b and d).

The major target proteins for novobiocin in eukaryotic cells are postulated to be type II topoisomerase (Hsieh and Brutlag, 1980; Baldi *et al.*, 1980) and DNA polymerase α (Edenberg, 1980); indeed inhibition of SV40 DNA replication by novobiocin can largely be explained by its effect on polymerase α (Edenberg, 1980). To assess the involvement of this enzyme in the initial events of adenovirus *in vitro* replication the sensitivity of the formation of the pTP-dCMP complex to aphidicolin, a specific inhibitor of polymerase α, was examined. In agreement with the results of others (Pincus *et al.*, 1981; Lichy *et al.*, 1981) aphidicolin at concentrations of up to 50 μg/ml failed to prevent the formation of the pTP-dCMP complex (Figure 2) while incorporation into DNase-sensitive material seen as a background to the pTP-dCMP complex was significantly reduced. Since aphidicolin at concentrations of <1 μg/ml efficiently inhibits cellular DNA synthesis it is unlikely, therefore, that polymerase α is involved in initiation of adenovirus replication or that it is the target for novobiocin.

In view of the results obtained using novobiocin, the *in vitro* replication system was examined for the presence of topoisomerase activity. Using a substrate of supercoiled plasmid (pAT 153), an ATP, Mg^{2+}-independent type I topoisomerase was identified in extracts derived from both infected and uninfected cells (data not shown). However, the activity of this enzyme was insensitive to both novobiocin and nalidixic acid and its presence could not therefore be related to adenovirus DNA replication

Discussion

The state of the viral genome during replication and transcription *in vivo* is not known with any certainty and studies on the susceptibility of intranuclear parental viral DNA to micrococcal nuclease suggest that up to 50% of the viral DNA is in nucleosome-like structures 6 h post-infection (Tate and Philipson, 1979) and at late times after the onset of viral DNA replication (Sergeant *et al.*, 1979). The observation that cores can support the initial events in *in vitro* replication therefore implies that removal of the core proteins is not a prerequisite for *in vivo* replication. Moreover, the structure of cores produced by the heat-deoxycholate method has recently been shown by c.d. analysis to be highly condensed, as in the virion itself (Boulanger and Loucheux-Lefebre, 1982). This suggests that the folding is ordered in a way allowing access to the ends of the virus template by factors involved in initiation of replication and that elongation can proceed, at least as far as a few hundred nucleotides. It should be pointed out that the presence of core proteins is not an absolute requirement for viral DNA replication or transcription since 'naked' adenovirus DNA-protein complex is infectious, albeit very inefficiently (Sharp *et al.*, 1976). *In vitro*, however, it seems unlikely that the core proteins, which remain associated with the viral DNA throughout treatment with mild detergent and glycerol gradient centrifugation, are removed during replication. Analysis of proteins by SDS-PAGE and staining with Coomassie blue did not reveal any significant changes in the protein pattern, suggesting that the core proteins remained largely undegraded (data not shown). This is not to say that the highly folded state characteristic of cores freshly isolated from virions is retained (Nermut *et al.*, 1975). ADP-ribosylation of core proteins (Goding *et al.*, 1982), together with the type I topoisomerase present in the replication system, might serve to relax the core structure to facilitate replication (c.f., Poirier *et al.*, 1982). An explanation for the apparent ability of some restriction enzymes (*Hha*I and *Hae*III) to cut DNA inside cores while others (*Hind*III, *Bam*HI, *Xba*I and *Eco*RI) cannot, may be that enzymes (*Hha* and *Hae*) with many sites on the genome are more likely to have an available restriction site on the surface of the cores, and the subsequent cleavage results in a more relaxed core structure which in turn exposes more sites.

The presence of single-strand nicks in the core DNA was surprising and may imply that core preparations contain some associated nuclease activity. Although such nicks may preclude studies on extensive elongation it is clear that such cores can act as efficient templates for at least the initial events in *in vitro* replication.

The apparent sensitivity of adenovirus DNA replication to novobiocin is intriguing. It is possible that, in the presence of ddGTP, the band corresponding to the pTP-dCMP complex represents a product incapable of elongation and that novobiocin does not inhibit 'true' initiation. However, this is unlikely since in the absence of ddGTP very little unelongated pTP-dCMP was observed (lane 5, Figure 2b) and novobiocin similarly affected the formation of the limited elongation product, the pTP-26 complex. It has previously been reported that novobiocin can affect a range of eukaryotic enzymes including DNA polymerase α, type II topoisomerases (Hsieh and Brutlag, 1980; Baldi *et al.*, 1980) and yeast DNA polymerases I and II (Nakayama and Sugino, 1980) and at much higher levels, protein and RNA synthesis (Edenberg, 1980), yeast topoisomerase type I (Nakayama and Sugino, 1980) and yeast tRNA synthetases (Wright *et al.*, 1981). Inhibition of any of the latter group of enzymes is unlikely at the concentrations employed and use of the *in vitro* replicating system (in which the proteins necessary for replication are already present and hence no synthesis is required) also implies that novobiocin affects an alternative target. Moreover, the results with aphidicolin suggest that this target is not DNA polymerase α. It cannot be ruled out that novobiocin binds non-specifically to factors involved in initiation of replication notwithstanding that it does not appear to interact with DNA (Smith and Davies, 1965) although one possible target for this drug could be the 140-K polymerase that appears to catalyze the addition of dCTP to the pTP (Lichy *et al.*, 1982). Unsuccessful attempts were made to demonstrate a type II topoisomerase using a decatenation reaction with a kinetoplast DNA substrate (Borst and Hoeijmakers, 1979) and chromatography of the nuclear extract on a phosphocellulose column followed by a relaxation assay (data not shown). Such an en-

zyme could facilitate unwinding of the termini of the genome, placing the DNA in a conformation acceptable to the proteins involved in initiation of replication. This latter proposition is consistent with the observation of Tamanoi and Stillman (1982) who showed that initiation of replication was possible on a template of single-stranded adenovirus DNA lacking a terminal protein. In addition to the protein priming model of Rekosh *et al.* (1977), a model involving a topoisomerase activity, based on ϕX174, has been proposed (Pearson *et al.*, 1982). If this model is valid then it is possible that the nicking-priming event prior to and necessary for formation of the pTP-dCMP complex would be inhibited by novobiocin. It is pertinent to note that circular adenovirus DNA molecules have recently been isolated from infected cells although it was not established whether these molecules were complete or products of recombination between the inverted repeats (Ruben *et al.*, 1983). The type I topoisomerase activity present in nuclear extracts could not be demonstrated to be directly related to adenovirus DNA replication *in vitro* but it cannot be ruled out that such an enzyme may have some function in preparing the DNA template or maintaining it in a conformation to facilitate DNA replication.

In light of the work presented here it is evident that adenovirus cores can provide convenient and reliable templates for examining both initiation and limited elongation of replication using the *in vitro* system and at the same time may provide a means of relating these events to DNA chromatin structure.

Materials and methods

Cells and virus

HeLa cells and KB cells (Flow) were grown in suspension in Joklik's MEM F14 supplemented with 7% newborn calf serum (NCS) at a density between 3 x 10^5 and 6 x 10^5/ml. Ad5 was grown in suspension cells, titrated and purified as previously described (Russell *et al.*, 1967; Winters and Russell, 1971). HeLa cells in monolayer were grown in H21 supplemented with 5% NCS and labelling of cells with [^{35}S]methionine was performed as described by Russell *et al.*, 1981.

Enzymes and radiochemicals

Restriction enzymes were obtained from either Bethesda Research Laboratories or New England Biolabs. All radiochemicals were purchased from Amersham International.

Purification of templates

Adenovirus DNA was prepared from purified desalted virus in 10 mM Tris/HCl pH 7.8 by addition of SDS to 1% and EDTA to 25 mM followed by incubation with pronase (1 mg/ml) at 37°C for 2 h. The DNA was phenol-extracted, ethanol-precipitated and resuspended in 10 mM Tris/HCl pH 7.8. The DNA-protein complex was obtained by disruption of purified virus with an equal volume of 8 M GuHCl (B.D.H.) followed by chromatography on a column of Sepharose 4 B (Pharmacia) equilibrated in 4 M GuHCl. The GuHCl was removed by extensive dialysis against 10 mM Tris/HCl pH 7.8. Viral cores were made by heating purified desalted virus in a small glass vial at 56°C for 45 s immediately after addition of sodium deoxycholate to 0.5%. The disrupted virions were then rapidly cooled on ice before loading on to a 15–60% glycerol gradient and centrifuging (80 000 *g*) for 2.5 h in an M.S.E. 6 x 5.5 rotor to separate the cores from capsomeres (Russell *et al.*, 1971).

In vitro DNA replication

Suspension HeLa cells at a density of ~4.5 x 10^5/ml were concentrated to 1/10 volume and infected at a multiplicity of 100 p.f.u./cell. After 1 h adsorption at 37°C the cells were resuspended to the original volume and after a further 1 h at 37°C, hydroxyurea (Sigma) was added to 10 mM. Preparation and extraction of nuclei from these cells was carried out as described previously by Challberg and Kelly (1979) with the exception that the isolated nuclei were resuspended in an equal volume of buffer prior to freezing in 0.4 ml aliquots and subsequent thawing and extraction. Nuclear extracts were stored at 4°C.

The standard reaction for *in vitro* DNA replication contained 20 ng of template DNA; 10–12 μl of nuclear extract; 5 μl of a buffer containing 250 mM Hepes pH 7.5, 25 mM $MgCl_2$, 2.5 mM dithiothreitol, 100 μM each of dGTP, dATP, dTTP, 50 μM dCTP, 1 μCi [α-^{32}P]dCTP (Amersham), 1.0 mM ATP; 3 μl 1.0 mg/ml creatinine phosphokinase (Sigma), 2 mM creatinine phosphate (Sigma), and the reaction volume made to 30 μl with water. In experiments designed to detect the pTP-26 complex the reactions contained 5 μCi [α-^{32}P]dCTP and the dGTP was replaced by ddGTP.

Reactions were stopped either by addition of 1/10 volume 200 mM EDTA, 1% SDS, 60% sucrose, 0.02% Bromophenol blue and analysed by electrophoresis on 1% agarose gels (Sharp *et al.*, 1973) or by addition of an equal volume of denaturing buffer and electrophoresis on 15% SDS polyacrylamide gels as described by Russell and Blair (1977). Restriction enzyme digestions were carried out using the standard conditions for each enzyme.

Assay for topoisomerase activity

1 μg purified plasmid DNA was added to the *in vitro* replication reaction in place of the adenovirus DNA templates and incubated at 37°C for 5–10 min. In some experiments ATP and Mg^{2+} were omitted from the buffer and the nuclear extracts were dialysed to removed endogenous ATP and Mg^{2+}. Reactions were stopped by the addition of 1/10 volume, 200 mM EDTA, 1% SDS, 60% sucrose, 0.02% Bromophenol blue and analysed by electrophoresis in 1% agarose gels containing 0.1% SDS which were subsequently dried and stained with ethidium bromide and the DNA visualised by u.v. light.

Acknowledgements

We would like to thank Kristina Quade for critically reading this manuscript. C.R.G. was in receipt of a Medical Research Council Scholarship.

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