

Function of adenovirus terminal protein in the initiation of DNA replication

(DNA replication *in vitro*/origin-containing plasmid/DNA-protein complex/piperidine-treated DNA)

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ABSTRACT An early event in the initiation of adenovirus DNA replication is the formation of a covalent complex between the 87,000-dalton adenovirus terminal protein precursor and 5'-dCMP (pTP-dCMP complex). Nuclear extracts prepared from adenovirus-infected HeLa cells catalyzed complex formation in the presence of ATP, Mg²⁺, and adenovirus DNA-protein complex but were not active when Pronase-treated DNA was used as template. The activity has been partially purified by chromatography on denatured DNA-cellulose and used to examine whether the 55,000-dalton terminal protein on adenovirus DNA is required for pTP-dCMP complex formation. Results obtained with either DNA-protein complex or Pronase-treated DNA were identical to those obtained using crude nuclear extracts. However, after treatment with piperidine to remove residual peptides, Pronase-treated DNA supported complex formation with the partially purified activity but not with crude extracts. In addition, when a plasmid containing an origin of adenovirus DNA replication was used as template, the pTP-dCMP complex was formed provided the plasmid was linearized in such a way that the origin was located at the end of the molecule. Neither linearized plasmid DNA with an internal origin nor supercoiled plasmid DNA supported complex formation. Furthermore, after heat denaturation, the linear plasmid DNA still supported complex formation, again provided that the origin was located at the end of the molecule. The partially purified protein fraction supported a limited amount of DNA chain elongation, which permitted exact positioning of the initiation site. These results suggest that enzymes responsible for complex formation recognize a DNA sequence at the origin and that the terminal protein on the template DNA plays a subordinate role.

The genome of adenovirus contains a protein covalently bound to the 5' end of each strand of the linear DNA molecule (1-3). Since adenovirus DNA replication is initiated at the termini of the DNA molecule (4, 5), Rekosh *et al.* (3) proposed that the terminal protein may serve as a primer for initiation of replication. Subsequent studies are broadly consistent with this model. However, the priming function now appears to reside in a precursor form of the terminal protein (pTP) found on the 5' ends of nascent DNA strands replicated *in vitro* (6) and as a component of DNA-protein complexes isolated from virions of the protease-deficient adenovirus serotype 2 (Ad2) mutant *ts1* (7, 8). Stillman *et al.* (8) demonstrated that pTP is encoded by the leftward-transcribed strand of the viral genome and comprises part of a transcription unit that also encodes the single-strand DNA binding protein.

Challberg and Kelly (9) developed a cell-free system to study adenovirus DNA replication and demonstrated that the adenovirus DNA-protein (Ad-protein) complex supports DNA replication whereas Pronase-treated adenovirus DNA does not (9, 10). The same requirement for the Ad-protein complex was seen with a partially fractionated *in vitro* system (11). Recently,

Lichy *et al.* (5) devised an assay to study initiation of adenovirus DNA replication that involves formation of a covalent complex between pTP and dCMP, the 5'-terminal nucleotide of both strands of adenovirus DNA. The formation of this initiation complex also required Ad-protein complex as a template but was not observed when Pronase-treated adenovirus DNA was used instead. Based on these results, they suggested that the terminal protein on the parental DNA serves to facilitate initiation of daughter-strand synthesis at the molecular termini via specific interactions with replication proteins. However, the conclusion that parental terminal protein plays an active role in the initiation cannot be drawn unambiguously from these experiments because Pronase digestion probably leaves a short peptide attached to the 5' end of the DNA (12). In this paper, we report a reexamination of the role of parental terminal protein using protein-free DNA templates.

Studies of the interaction between the DNA template and the enzymes involved in initiation of DNA replication would be greatly simplified if cloned DNA (free of the protein) could be used; hence, we have focused our attention on the use of a plasmid DNA containing the origin of adenovirus DNA replication as a template. Cloning of the origin located at the left end of adenovirus DNA was first accomplished by Stow (13). This was possible by using a dG-dC tailing procedure to circumvent problems associated with the presence of residual amino acids of the terminal protein that remain linked to the 5' terminus of each strand after protease treatment of the viral DNA. Recently, piperidine or alkali has been used to remove the terminal protein (8, 12), leaving blunt ends that can be ligated to DNA linkers and thereby facilitating cloning of the terminal fragments of viral DNA into restriction sites in pBR322 DNA. By using DNAs so constructed, we have shown that a plasmid DNA that contains the origin of DNA replication does indeed support initiation of adenovirus DNA replication. For the plasmid DNA to be functional as a template, it has to be linearized with the restriction cut immediately next to the origin. This system will allow precise determination of the DNA sequences required for initiation of adenovirus DNA replication.

MATERIALS AND METHODS

Materials. The growth of HeLa cells and Ad2 were carried out as described (14). Denatured DNA cellulose was prepared according to Alberts and Herrick (15). [α -³²P]dNTPs (410 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) were purchased from the Radiochemical Centre, Amersham. Aphidicolin was provided by A. Sugino (National Institutes of Health, N. Carolina). 2',3'-dideoxyguanosine triphosphate (ddGTP) was purchased from P.-L. Biochemicals.

Abbreviations: Ad-protein complex, adenovirus with a protein covalently bound to the 5' end of each strand; pTP, terminal protein precursor; ddNTP, 2',3'-dideoxynucleoside triphosphate; Ad2 and Ad5, adenovirus serotypes 2 and 5, respectively.

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Extracts and Enzyme Preparation. Nuclear and cytoplasmic extracts from Ad2-infected cells were prepared as described (8, 9). To prepare the partially purified enzyme fraction, 2 ml of nuclear extract was passed through denatured DNA-cellulose (column vol, 1.5 ml) equilibrated with 0.1 M NaCl/buffer F (25 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/20% glycerol). The column was washed with 2 column vol of the same buffer and the activity was eluted with 2 column vol of 0.2 M NaCl/buffer F. Fractions (1.5 ml) were collected, frozen immediately, and stored at -70°C until use. The protein concentration of the fraction containing the pTP-dCMP complex-formation activity was ≈ 0.5 mg/ml and that of the crude nuclear extract was 8–10 mg/ml.

Viral DNA and Plasmid DNAs. The preparation of DNA-protein complex has been described (14) and Pronase-treated adenovirus DNA was prepared as described (16). Piperidine-treated DNA was prepared by incubating *Kpn* I-digested Pronase-treated adenovirus DNA with 0.5 M piperidine for 2 hr at 37°C . The piperidine was removed by multiple lyophilizations (8), and DNA fragments were reannealed in 10 mM Tris·HCl, pH 8.0/1 mM EDTA/1 M NaCl at 65°C for 4 hr. Annealing was complete as judged by agarose gel electrophoresis. Plasmids pLA1 and *8 were provided by H. Reichl and N. D. Stow, respectively (see Fig. 3 and below). The plasmid DNAs were purified by standard procedures (17). To linearize the plasmid DNA, it was digested with various restriction enzymes, heated at 65°C for 10 min, and chilled.

Formation of the pTP-dCMP Complex. Reaction mixtures (20 μl) contained 25 mM Hepes/KOH, pH 7.5/5 mM MgCl_2 /2 mM dithiothreitol/3 mM ATP/0.85 μM [α - ^{32}P]dCTP in addition to 6–10 μl of enzyme fraction and 80 ng of various DNAs. Where indicated 2', 3'-dideoxyadenosine triphosphate (ddATP) and aphidicolin were added to final concentrations of 40 μM and 40 $\mu\text{g}/\text{ml}$, respectively. After incubation at 30°C , reactions were stopped by addition of 1 μl of 0.2 M EDTA and 21 μl of double-strength Laemmli buffer (18). Where indicated, 2 μl of pancreatic DNase at 1 mg/ml was added and the incubation was continued for 10 min at 30°C . Samples were heated for 3 min at 100°C and analyzed by electrophoresis in 10% polyacrylamide/NaDodSO₄ gels (18) containing 0.13% bisacrylamide. After acid fixation, gels were dried and autoradiographed at -70°C .

RESULTS

Formation of the pTP-dCMP Complex by Nuclear Extracts.

Nuclear extracts prepared from adenovirus-infected cells carry out synthesis of full-length DNA on added Ad-protein complex. The synthesis is initiated at or near the ends of the DNA molecule, mimicking replication *in vivo* (10). It has been proposed that the first step in replication is the formation of a complex between pTP and the 5'-deoxycytidine residue on the DNA and that this complex acts as the primer for elongation (3). Recently, the formation of such a complex has been demonstrated in cytoplasmic extracts of infected cells (5). In our hands, a similar complex is formed in nuclear extracts incubated with [α - ^{32}P]dCTP in the presence of Ad-protein complex, ATP, and Mg^{2+} (Fig. 1A, lanes a–c). The product was analyzed by polyacrylamide/NaDodSO₄ gel electrophoresis followed by autoradiography, which showed a band of ^{32}P at the position of pTP (8) migrating slightly slower than the marker adenovirus protein III, which has a molecular size of 85,000 daltons (Fig. 1). Tryptic peptide analysis has shown that this ^{32}P -labeled protein is related to pTP (unpublished data). Approximately 0.5 fmol of ^{32}P are incorporated into the complex, which corresponds to $\approx 7\%$ of the input DNA acting as template and is similar to the fraction of template that is active for DNA replication *in vitro* (9). When cytoplasmic extracts were used, no radioactive band was de-

tected (Fig. 1A, lanes d–f), and mixing nuclear extracts with cytoplasmic extracts neither increased nor decreased the intensity of the band (lanes g and h). In our experience, the activity forming the pTP-dCMP complex resides solely in the nuclei.

Results obtained with other ^{32}P -labeled nucleoside triphosphates are shown in Fig. 1B. To deplete the nuclear extract of unlabeled triphosphates, it was passed through DEAE-cellulose in the presence of 0.2 M salt and proteins were precipitated with ammonium sulfate (5). When [α - ^{32}P]dATP was added instead of dCTP (Fig. 1B, lane a), much less radioactivity was found at the pTP position. This small amount of label presumably represents formation of a pTP-pdCpdA complex due to a residual amount of unlabeled dCTP in the enzyme preparation. When [α - ^{32}P]TTP was used (lane c), a trace amount of radioactivity was detected at this position, probably because of the formation of a pTP-pdCpdApdT complex, and no band was seen when [α - ^{32}P]dGTP was used (lane b). These data are consistent with the known DNA sequence at the 5' terminus of adenovirus 2 DNA (see Fig. 3). No radioactive band was detected at this position when ^{32}P -labeled (ribo)CTP was used (data not shown). An additional rapidly migrating band was observed when [α - ^{32}P]dATP, [α - ^{32}P]dGTP, or [α - ^{32}P]TTP were used, but not when [α - ^{32}P]dCTP was used, and its appearance was not dependent on the presence of template DNA (not shown). In contrast, production of the radioactive band at the position of pTP specifically required Ad-protein complex; only a small amount of radioactivity was found in the corresponding position when the Ad-protein complex was replaced by Pronase-treated adenovirus DNA (compare Fig. 1B, lanes d and e).

DNA-Cellulose Column Chromatography of the Complex-Forming Activity. To purify the activity that carries out formation of the pTP-dCMP complex, the nuclear extract was ap-

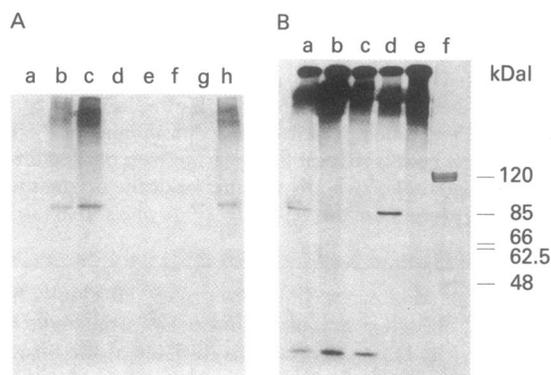


FIG. 1. Detection (A) and characterization (B) of pTP-dCMP complex-forming activity in crude extracts of adenovirus-infected cells. (A) Various amounts of nuclear and cytoplasmic extracts prepared as described (8, 11) were incubated with [α - ^{32}P]dCTP, ddATP, and Ad-protein complex. After 30 min at 30°C , the products were analyzed by electrophoresis in 10% polyacrylamide/NaDodSO₄ gels. Lanes: a–c, 2 μl , 5 μl , and 10 μl , respectively, of nuclear extract; d–f, 2 μl , 5 μl , and 10 μl , respectively, of cytoplasmic extract; g and h, 2.5 μl each and 5 μl each, respectively, of nuclear and cytoplasmic extract. (B) One milliliter of nuclear extract from infected cells was adjusted to 0.2 M NaCl and loaded onto a DEAE-cellulose column (DE52; 2 ml) equilibrated with buffer F/0.2 M NaCl. The column was washed with 2 column vol of the same buffer, and the eluted proteins were precipitated by 2 vol of saturated ammonium sulfate, dissolved in 1 ml of buffer F/0.05 M NaCl, dialyzed against this buffer, and stored at -70°C . Ten microliters of this material was incubated with 20 μl of 25 mM Hepes/KOH, pH 7.5/5 mM MgCl_2 /1 mM dithiothreitol/2 mM ATP containing either 40 ng of Ad-protein complex (lanes a–d) or 40 ng of adenovirus DNA (lane e) and 5 μCi of ^{32}P -labeled dATP (lane a), dGTP (lane b), TTP (lane c), or dCTP (lanes d and e). The reaction was stopped after 30 min at 30°C and the products were separated by electrophoresis on a 10% polyacrylamide/NaDodSO₄ gel. Lane f: ^{35}S -labeled adenovirus marker proteins. kDal, kilodaltons.

plied onto a column of denatured DNA-cellulose. Bound proteins were eluted with an increasing salt concentration, and then each fraction was incubated with [α - 32 P]dCTP in the presence of Ad-protein complex, ATP, and Mg^{2+} . As shown in Fig. 2, the activity bound to the denatured DNA-cellulose and was eluted at 0.2 M salt. This resulted in an \approx 20-fold purification. Adenovirus DNA-binding protein bound more tightly to the denatured DNA-cellulose and was eluted at 0.6 M salt concentration. This suggests that the DNA-binding protein is not involved in formation of the pTP-dCMP complex. The majority of DNA polymerase activity in the extract did not bind to the column.

Cloned Origin of Adenovirus DNA Replication Supports pTP-dCMP Complex Formation. Using enzyme preparation that had been partially purified by denatured DNA-cellulose column chromatography, we asked whether other DNAs could replace the Ad-protein complex as template for formation of the pTP-dCMP complex.

First, plasmid DNAs that contain the origin of adenovirus DNA replication were tested; the structures of two such plasmid DNAs used in this study are shown in Fig. 3. Plasmid pLA1 contains the *Bgl* II fragment of adenovirus serotype 5 (Ad5), 0–9.4 map units, inserted into the vector by using an *Eco*RI linker ligated onto the left end of the viral DNA. Plasmid *8 contains an *Sst* I fragment of Ad2, 0–4.83 map units, and has a dG-dC tail on the left end of the viral DNA. Fig. 3 also shows the DNA sequences at the end of linearized pLA1 or *8 DNA used in this study, and the DNA sequence at the origin of adenovirus DNA replication.

As shown in Fig. 4 (lanes c and d), formation of the pTP-dCMP complex was not seen when either pLA1 or *8 supercoiled DNA was used instead of the Ad-protein complex. However, when pLA1 DNA linearized by *Eco*RI was used, formation of the complex was detected (lane e). pBR322 DNA linearized by *Eco*RI did not support complex formation (lane k), showing that the adenovirus DNA sequence was necessary for template activity. The origin had to be located at the end of the linear DNA since pLA1 DNA linearized by *Sal* I did not support complex formation (lane j). Similarly, *8 DNA linearized by *Pst*

I, in which the adenovirus sequence is \approx 20 nucleotides from the restriction site, did not support complex formation (lane f). The crude nuclear extract was not active for complex formation when plasmid pLA1 DNA linearized with *Eco*RI was used as template (data not shown).

Piperidine-Treated Adenovirus DNA. The results described above show that the pTP-dCMP complex can be formed on a DNA template even though the parental terminal protein is absent. However, as shown in Fig. 4, lane b, Pronase treatment destroyed the template activity of the Ad-protein complex. These apparently contradictory results can be explained by assuming that residual amino acids remaining after Pronase treatment interfere with pTP-dCMP complex formation. If this were the case, we would expect recovery of template activity of double-stranded DNA after removal of the residual amino acids from the Pronase-treated adenovirus DNA by piperidine treatment and reannealing. This prediction was fulfilled, as shown in Fig. 4 (lanes p–r). For this experiment, it was necessary to use *Kpn* I restriction fragments instead of intact adenovirus DNA to obtain complete renaturation of the DNA after reannealing. *Kpn* I-digested Ad-protein complex supported pTP-dCMP complex formation, but *Kpn* I-digested Pronase-treated adenovirus DNA did not (lanes p and q). However, when *Kpn* I-digested Pronase-treated DNA was treated with piperidine and then reannealed, this DNA indeed supported complex formation (lane r). A mixing experiment excluded the possibility that any inhibitory substance was present in the Pronase-treated DNA (data not shown).

Single-Stranded DNA. Current models of adenovirus DNA replication (both type I and type II) show initiation occurring on double-stranded DNA (4). Therefore, it was an unexpected finding that heat-denatured *Eco*RI-linearized pLA1 DNA was active as template for pTP-dCMP complex formation (Fig. 4, lane l). Only trace amounts of the complex were formed with heat-denatured pBR322 DNA (lane n) or simian virus 40 DNA (data not shown). Interestingly, the sequence has to be located at the end of the linear single-stranded DNA, since *Sal* I-linearized pLA1 DNA did not support complex formation, even when heat denatured (lane m). These results suggest that single-stranded adenovirus DNA can also act as template for complex formation. It is possible that the activity observed with heat-denatured *Eco*RI-linearized pLA1 DNA was due to renaturation of the termini during the reaction. However, we consider this unlikely because the activity on heat-denatured DNA was comparable in amount with that on native DNA (at a limiting concentration of native DNA). More significantly, Pronase-treated native adenovirus DNA was not active as a template (lane b) but gained full activity on heat denaturation (data not shown).

Initiation of DNA Replication on Linearized pLA1 DNA. *Eco*RI-Linearized pLA1 DNA has a short extra sequence added to the adenovirus terminus (see Fig. 3), so it was of interest to know on which nucleotide the initiation took place. We first examined the dNTP specificity for complex formation by using the denatured DNA-cellulose fraction. As shown in Fig. 5A, [α - 32 P]dCTP was the predominant triphosphate that formed a complex with pTP.

Second, to determine which deoxycytidine residue is used for initiation, dATP, dTTP, and the chain terminator ddGTP were added to the reaction mixture. Since the first guanosine appears at the 25th nucleotide from the left end of Ad2 DNA (19), these additions should allow a limited amount of elongation. This is shown in Fig. 5B, in which the labeled complex made in the presence of dATP, dTTP, and ddGTP migrated more slowly than the pTP-dCMP complex. This slower migrating band was eluted and treated with piperidine, and the DNA was determined to be 25 nucleotides long on a 12% acryl-

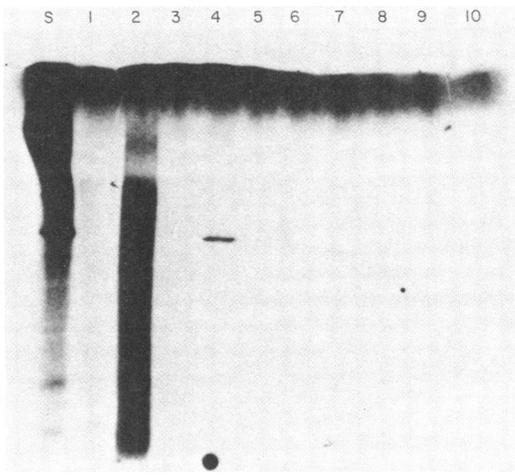
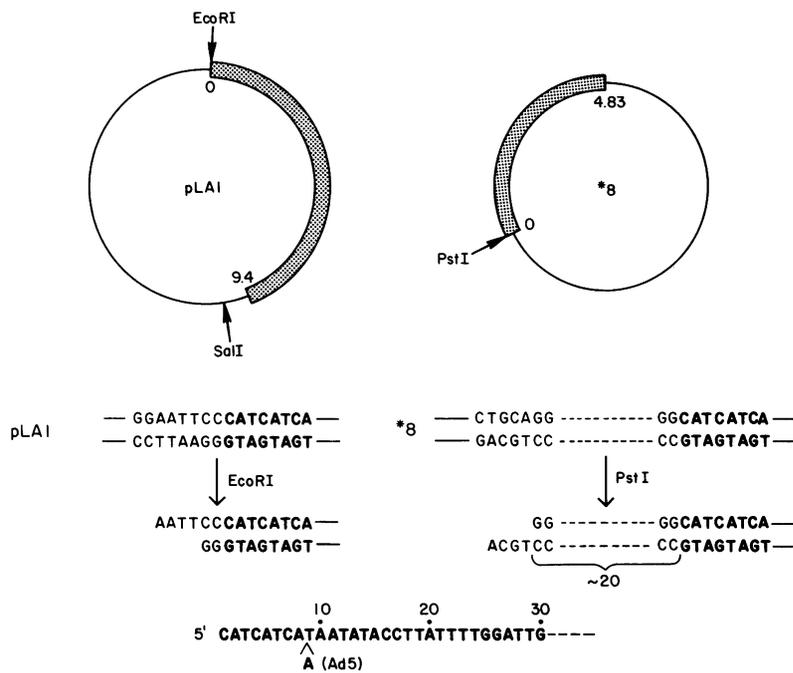


FIG. 2. Denatured DNA-cellulose column chromatography of pTP-dCMP complex-forming activity. Nuclear extract from infected cells were chromatographed on a denatured DNA-cellulose column and 6 μ l of each fraction was assayed for pTP-dCMP complex formation. Ad-protein complex was used as template and ddATP was added; incubation was for 30 min at 30°C, and products were separated on a 10% polyacrylamide/NaDodSO₄ gel. S, Unfractionated extract. Fractions: 1, pass-through volume; 2 and 3, wash; 4 and 5, 0.2 M NaCl eluate; 6 and 7, 0.4 M NaCl eluate; 8 and 9, 0.6 M NaCl eluate; 10, 0.8 M NaCl eluate.



amide/7 M urea sequence analysis gel (Fig. 5C). A similar result was obtained with piperidine-treated double-stranded Ad2 DNA (data not shown). However, when linearized pLA1 DNA was used, two slow-migrating DNA-protein complexes were observed and the corresponding DNAs were 23 and 26 nucleotides long. Ad5 DNA has an insertion of one deoxyadenosine residue compared with Ad2 DNA (see Fig. 3; ref. 20). Therefore, initiation on pLA1 DNA occurs at the sequence corresponding to the 5'-terminal nucleotide of Ad5 DNA as well as at an additional start point four nucleotides on the 3' side of the Ad5 terminus, also a deoxycytidine residue.

When ddGTP was replaced with dGTP in the reaction mixture, elongation continued past the first deoxyguanosine residue but only to a limited extent, resulting in DNA-protein complexes of 100–200 nucleotides (data not shown).

DISCUSSION

Extracts from adenovirus-infected HeLa cells are capable of forming a covalent complex between pTP and 5'-dCMP (ref.

5; this paper). Formation of this complex is believed to be a first step in DNA replication. An additional function for the terminal protein was suggested by the observation that its presence on template DNA was obligatory for both complex formation and full-length DNA synthesis (5, 9, 10, 14). We have reinvestigated the template requirement and demonstrated that a specific DNA sequence is required for initiation of DNA replication and that the terminal protein is dispensable. The sequence can be in either double-stranded or single-stranded form, but it is active only when located at the terminus of a DNA molecule. Addition of ≈ 20 dG-dC base pairs to the end of the DNA molecule destroys its template activity. Thus, the activity responsible for pTP-dCMP complex formation recognizes both the sequence and its terminal location on the DNA molecules. The fact that single-stranded DNA can act as template for complex formation suggests that initiation of DNA replication might be a two-step process: (i) the unwinding of duplex DNA at its ends and (ii) complex formation using single-stranded DNA as template. Pronase-treated adenovirus DNA does not function as template when double stranded, presumably because of inter-

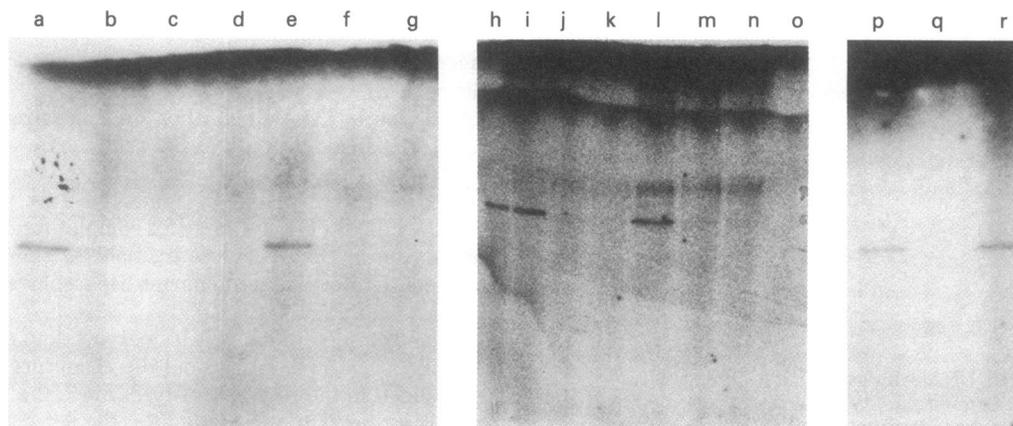


FIG. 4. pTP-dCMP complex formation directed by various template DNAs. Reactions to form pTP-dCMP complex were carried out with 8 μ l of denatured DNA-cellulose fraction 4, aphidicolin, and 80 ng of DNA. Incubation was for 2 hr at 30°C, and these products were separated on 10% polyacrylamide/NaDodSO₄ gels. For lanes a–g and p–r, products were treated with DNase before electrophoresis. Lanes: a and h, Ad-protein complex; b, adenovirus DNA; c, pLA1; d, *8; e, *Eco*RI-linearized pLA1; f, *Pst*I-linearized *8; g and o, no DNA; i, *Eco*RI-linearized pLA1; j, *Sal*I-linearized pLA1; k, *Eco*RI-linearized pBR322; l, heat-denatured *Eco*RI-linearized pLA1; m, heat-denatured *Sal*I-linearized pLA1; n, heat-denatured *Eco*RI-linearized pBR322; p, *Kpn*I-cleaved Ad-protein complex; q, *Kpn*I-cleaved adenovirus DNA; r, piperidine-treated *Kpn*I-cleaved adenovirus DNA.

Fig. 3. Structure of plasmid pLA1 and *8 DNAs. pLA1 DNA contains the *Bgl* II fragment of Ad5 DNA, 0–9.4 map units (▨). The fragment was isolated from viral DNA and the terminal protein was removed by piperidine treatment. The strands were reannealed, an *Eco*RI linker (G-G-A-A-T-T-C-C) was ligated on, and the fragment was cloned into *Eco*RI/*Bam*HI-digested pAT (pBR322 having a deletion around the *Pvu* II site). We have confirmed the presence of the left end of adenovirus DNA by determining the sequence from the *Eco*RI site. *8 DNA contains Ad2 DNA from the left end to 4.83%. This fragment, which has a dG-dC tail on the left end of the viral DNA, was inserted into the region between the *Pst* I and *Eco*RI sites of pBR322. →, Restriction sites on the plasmid DNAs. The sequences that appear at the ends of pLA1 and *8 DNAs after linearization by *Eco*RI and *Pst* I, respectively, are shown below the structures. The sequence at the bottom of the figure is the left-end sequence of Ad2 DNA (19); note that Ad5 DNA has an extra adenosine residue at the ninth position (20).

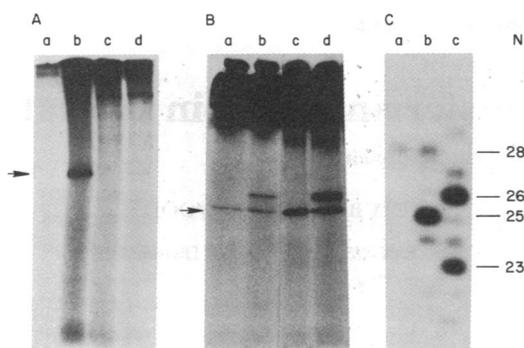


FIG. 5. Identification of initiation sites on Ad-protein complex and on pLA1 DNA. (A) Eight microliters of DNA-cellulose fraction 4 was incubated with 80 ng of pLA1 DNA and aphidicolin in 20 μ l of 0.85 μ M α - 32 P-labeled dATP (lane a), dCTP (lane b), dGTP (lane c), or TTP (lane d) and other components as described in *Materials and Methods* for 2 hr at 30°C and then subjected to electrophoresis on a 10% polyacrylamide/NaDodSO₄ gel. (B) Eighty nanograms of Ad-protein complex (lanes a and b) or pLA1 DNA (lanes c and d) was incubated with 0.85 μ M [α - 32 P]dCTP (lanes a and c) or with 0.85 μ M [α - 32 P]dCTP/10 μ M dATP/10 μ M TTP/40 μ M ddGTP (lanes b and d) for 2 hr at 30°C and then subjected to electrophoresis on a 10% polyacrylamide/NaDodSO₄ gel. \rightarrow , 87,000-dalton protein. (C) Large-scale elongation reactions were carried out by using 1.6 μ g of Ad-protein complex or pLA1 DNA in 400 μ l of 10 μ M dATP/10 μ M dCTP/10 μ M TTP/40 μ M ddGTP. The DNA was labeled with 560 μ Ci of [α - 32 P]dATP (200–400 Ci/mmol), 460 μ Ci of [α - 32 P]dCTP (200–400 Ci/mmol), and 725 μ Ci of [α - 32 P]TTP (200–400 Ci/mmol). After 2 hr of incubation at 30°C, the reaction mixtures were subjected to electrophoresis on a 10% polyacrylamide/NaDodSO₄ gel. The bands corresponding to the elongated products shown in B were cut out, electroeluted, treated with Pronase (1 mg/ml for 1 hr at 37°C), extracted with phenol and chloroform/isoamyl alcohol (24:1), and ethanol precipitated. The precipitate was suspended in H₂O and treated with piperidine (8). The sample was lyophilized three times and then analyzed on a 12% polyacrylamide/7 M urea gel. Lanes: a, marker DNA (*Hpa* II-cut pBR322 DNA filled in with dCTP and [α - 32 P]dGTP by using the Klenow fragment of DNA polymerase I); b and c, DNAs made on the Ad-protein complex and on pLA1 DNA, respectively. N, length of the DNA in nucleotides.

ference by residual amino acids linked to the DNA at its 5' terminus. Piperidine removed these residual amino acids and, after reannealing, double-stranded DNA could function as template for complex formation.

A DNA polymerase activity was present in the denatured DNA-cellulose fraction used for pTP-dCMP complex formation. This permitted elongation of the pTP-dCMP complex, enabling us to determine where exactly initiation occurred on both the Ad-protein complex and linearized pLA1 DNA. Using ddGTP to stop synthesis at the first deoxyguanosine residue and then determining the length of the prematurely terminated DNA chain, we have shown that initiation occurs at the deoxycytidine residue at the extreme 5' end of the Ad-protein complex. However, when pLA1 DNA was used, initiation apparently occurred with about equal efficiency at the first and second deoxycytidine residues in the Ad5 DNA sequence. Initiation at the second deoxycytidine residue is presumably a perturbation of the normal process attributable to the presence of an extra sequence at the end of the *Eco*RI-cleaved pLA1 DNA, since piperidine-treated adenovirus DNA does not give rise to this additional start (unpublished results). Thus, the requirement for the DNA sequence at the end of the template DNA is very strict and a minor deviation affects correct initiation. It is interesting that both deoxycytidine residues used for initiation on pLA1 DNA are 5' nucleotides of the sequence 5'-C-A-T-C-A-3', which is present at the terminus as an overlapping repeat (see Fig. 3). This may mean that the activity to form pTP-dCMP complex recognizes the complementary sequence 3'-G-T-A-G-T-5' on the opposite (template) strand and lays

down deoxycytidine by base pairing at the first deoxyguanosine residue from the 3' end of this sequence. Similar repeats are found at the termini of other adenovirus serotypes and conserved sequences in the inverted terminal repetition of viral DNA can be found in the DNAs of a number of adenovirus serotypes (14, 21). These could also be the recognition sequences for the pTP-dCMP complex-forming activity. This study opens the way to determine whether such DNA sequences are required for initiation of DNA replication by testing the template activity for pTP-dCMP complex formation of cloned origins altered by *in vitro* mutagenesis or of chemically synthesized DNA.

Using crude unfractionated extracts, we found that complex formation occurred with Ad-protein complex but not with linearized pLA1 DNA as template; however, when the activity was purified by denatured DNA-cellulose chromatography or by precipitation with 35% ammonium sulfate (unpublished results), complex formation was detected with both DNAs. This suggests that an inhibitory factor is present in nuclear extracts. The factor may be a nuclease that digests the 5' end of pLA1 DNA; its action would be prevented by terminal protein, a function for terminal protein suggested by Dunsworth-Browne *et al.* (22). Alternatively, this factor might be involved in the initiation and functions to ensure initiation only on the natural viral DNA molecule.

In summary, a function of pTP in adenovirus DNA replication is to form a complex with dCMP, the first nucleotide in the nascent chain. The pTP, possibly with other replication proteins, recognizes a specific sequence on the template DNA and the complex formed then serves as a primer for DNA synthesis.

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