Initiation of adenovirus DNA replication in vitro requires a specific DNA sequence

(origin-containing plasmid/deletion mapping/oligonucleotide mutagenesis/conserved sequence)

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ABSTRACT Initiation of adenovirus DNA replication in vitro occurs on a linearized plasmid DNA containing 3,327 base pairs of the adenovirus terminal sequence. Various deletions have been constructed in the plasmid DNA and their template activities examined. Deletions from an internal restriction enzyme cleavage site that retain only 20 base pairs or more of the adenovirus terminal sequence support initiation and limited chain elongation, whereas deletions that leave 14 base pairs or less of the terminal sequence do not. On the other hand, all deletions extending from the very terminus of the adenovirus DNA destroy the template activity. The terminal 20 base pairs of adenovirus DNA contain a sequence A-T-A-A-T-A-T-A-C-C, which is perfectly conserved in the DNAs from different serotypes of human adenovirus. Base changes within the conserved sequence greatly reduce the template activity. These results suggest that the terminal 20 base pairs constitute a functional origin for the initiation of adenovirus DNA replication in vitro.

The adenovirus genome is a linear duplex DNA of \( \sim \)36,000 base pairs (bp), which has a 55-kilodalton (kDa) terminal protein covalently attached to the 5' end of each strand (1, 2). The terminal protein is synthesized as an 80-kDa precursor protein (terminal protein precursor, pTP), which, by virtue of its role in priming DNA synthesis, is attached to the ends of nascent DNA strands (3-8). Initiation of DNA replication takes place at the end of the DNA molecule by formation of a covalent linkage between pTP and dCMP (pTP-dCMP complex), and the dCMP becomes the 5'-terminal nucleotide in the DNA (4, 9-11). Elongation of DNA chains from this primer proceeds by a strand-displacement mechanism (12). The development of a cell-free DNA replication system (13) and subsequent in vitro studies have uncovered many novel features of the replication mechanism. Both the terminal protein precursor and the adenovirus DNA binding protein have been purified in a functional form (14-18). DNA replication is also dependent upon another virus-encoded protein of 140 kDa, which contains DNA polymerase activity (14-17, 19). The terminal protein precursor and the 140-kDa adenovirus DNA polymerase (140-kDa DNA polymerase) form a tight complex and together are involved in the formation of the pTP-dCMP complex in vitro. In addition to these viral proteins, nuclear factors from HeLa cells are required for replication. Recently, Nagata et al. (20) have isolated one such factor—a 47-kDa protein that is required for formation of the pTP-dCMP complex.

In contrast to the abundant information obtained about proteins, our knowledge about the substrate DNA is limited. To date, a functional analysis of the DNA sequences required for initiation of DNA replication has not been carried out in a systematic way. This situation is in sharp contrast to the study of simian virus 40 DNA replication. With this system, a functional origin has been defined by creating deletions and point mutations in the origin sequence and then by analyzing their ability to replicate in monkey cells (21-26).

Previous information about the origin of adenovirus DNA replication has come from DNA sequence determination. DNA sequences within the inverted terminal repetition of human and other adenoviruses have been determined (27-38). The template activity in vitro of DNA-protein complexes derived from different human adenovirus serotypes has also been compared (35).

Previously we demonstrated that a plasmid DNA containing the terminal sequence of adenovirus type 5 DNA functions as a template for the initiation of adenovirus DNA replication when a partially purified enzyme fraction was used (9). This provided a system for mutagenesis of the origin-containing DNA and for investigation of the effect of the mutations. In this paper we show that the terminal 20-bp sequence of adenovirus DNA is sufficient for template activity in vitro and also that base changes within this region affect initiation of DNA synthesis.

MATERIALS AND METHODS

Materials. The growth of HeLa cells and adenovirus type 2 were carried out as described (8). Aphidicolin was provided by A. H. Todd (Imperial Chemical Industries, Macclesfield, England) and 2',3'-dideoxy-GTP was purchased from P-L Biochemicals. [\( \alpha ^{32} \)P]dCTP (410 Ci/mmol; 1 Ci = 3.7 \( \times \)10\(^{10}\) Bq) and [\( \gamma ^{32} \)P]ATP (\( \sim \)3,000 Ci/mmol) were purchased from the Radiochemical Centre.

DNA Synthesis in Vitro. Partially purified enzyme fractions used in this study have been described (9). Because the initiation activity was inactivated by repeated freezing and thawing, the fraction was divided into aliquots of 100 \( \mu \)L, stored frozen at \( \sim \)70°C, and used only once. Formation of pTP-dCMP complex and the elongation of the complex were carried out as described (9).

Enzyme Reactions. Nuclease BAL-31 digestion was carried out at 22°C with 1.5 \( \mu \)g of Pco II-linearized plAAI DNA (9) and 2 units of nuclease BAL-31 (Bethesda Research Laboratories) in a 20-\( \mu \)L reaction mixture containing 60 mM Tris-HCl (pH 8.0), 12 mM MgCl\(_2\), 12 mM CaCl\(_2\), and 0.6 M NaCl. Approximately 100 bp per min per DNA end were removed under these conditions. Exonuclease III digestion followed by nuclease S1 treatment was carried out in the following way. \( \text{Sal I} \)-linearized DNA (1.3 \( \mu \)g) was digested with 8 units of exonuclease III (Bethesda Research Laboratories) in 16 \( \mu \)L of 6.6 mM Tris-HCl, pH 7.4/6.6 mM MgCl\(_2\)/6.6 mM 2-mercaptoethanol/50 mM NaCl. After a few minutes of incubation at 18°C, equal volumes of

Abbreviations: kDa, kilodalton(s); pTP, 80-kDa terminal protein precursor; 140-kDa DNA polymerase, 140-kDa adenovirus DNA polymerase; bp, base pair(s).
RESULTS

Construction of Deletions. Plasmid pLAl DNA, which contains adenovirus terminal DNA sequences, supports the formation of the pTP–dCMP complex provided that the plasmid DNA is linearized in such a way that the adenovirus terminal sequence is located close to the end of the linear DNA. The resulting complex serves as a primer for DNA chain elongation (9). To investigate the DNA sequences responsible for this template activity we have constructed deletions within the pLAl DNA (Fig. 1).

The plasmid contains 3,327 bp of adenovirus type 5 DNA sequence. Within this sequence there are three EcoRI sites at 453, 624, and 2,481 nucleotides from the adenovirus terminus. We first constructed a set of deletions beginning at the EcoRI site at 453 nucleotides and extending toward the terminus of adenovirus DNA. The deletions were made either by nuclease BAL-31 digestion or by exonuclease III, followed by nuclease S1 digestions. A Sal I linker (G-G-T-C-G-A-C-C) was added to the end point of each deletion to facilitate screening the size and sequence of the shortened viral DNA insert. The second set of deletions extends in the opposite direction from the EcoRI site and removes sequences located at the very terminus of adenovirus DNA. In this case, an EcoRI linker (G-G-A-A-T-T-C-C) was added to the end point of each deletion to preserve a dG residue, which could base pair with a potential initiating dC residue (Fig. 1).

The deletions used were derived from those used in previous work (9). Four deletions S-87, S-93, S-107, and S-111, were constructed from the plasmid pLAl DNA by nuclease BAL-31 digestion. After addition of the Sal I linker, the shortened adenovirus terminal sequence was ligated to the Sal I site of pAT DNA. Deletions S-200 and S-201 were derived from Sal-I-linearized S-84 by the successive treatment with exonuclease III and nuclease S1. Deletions S-87, S-107, and S-111 were derived from S-84. This time S-84 was digested with EcoRI and treated with exonuclease III followed by treatment with nuclease S1. End points of the deletions were determined by analyzing the size of the inserts by gel electrophoresis. In the case of S-114, S-200, S-201, S-111, S-87, R-8, R-7, and R-17 DNA sequences from the EcoRI site were determined as described by Maxam and Gilbert (39). Numbers at the top represent nucleotides from the terminus of adenovirus DNA.

![Fig. 1. End points of various deletions used in this study. Deletions S-87, S-93, S-107, and S-111 were derived from plasmid pLAl DNA. S-200 and S-201 were constructed from pLAl DNA by digestion with nuclease BAL-31 and treatment with exonuclease III and nuclease S1.](image-url)
Effect of Base Changes Within the Conserved Sequence. The results obtained by using deletions suggest that the functional origin is contained within the terminal 20 bp. This region has been shown to contain sequences highly conserved among different serotypes of adenovirus DNA. Particularly notable is a 10-nucleotide sequence 5'-A-T-A-A-T-A-C-G-C-3', which is perfectly conserved in all known serotypes of human adenovirus DNA (27-35). It has been suggested that this sequence plays a critical role in the initiation of DNA replication. To determine whether the conserved sequence is in fact required for the initiation of DNA replication, a plasmid DNA has been constructed containing base changes within the conserved sequence.

A procedure to construct these base changes is outlined in Fig. 4A. In brief, two complementary 20-mers were synthesized chemically. They contain the terminal sequence of adenovirus DNA but have base changes at nucleotides 13 and 14 in the middle of the conserved sequence; the wild-type 5'-A-T-A-A-T-A-C-G-C-3' is altered to 5'-A-T-A-A-G-G-T-A-C-G-C-3'. These oligonucleotides were hybridized and after addition of linkers they were cloned into plasmid pAT. A Kpn I site is created as a result of the base changes, which facilitated detection of the mutant plasmid as well as determination of the orientation of the insert. Out of a total of 21 isolates, 8 had the desired orientation. Fig. 4B shows the DNA sequence of a mutant clone, which confirmed the authenticity of the desired mutants. Examination of the template activity of the mutant DNA demonstrated that it was much lower than the corresponding activity of the plasmid S-201 DNA, which contains wild-type sequence. Fig. 4C shows results for two independent isolates of the mutant; upon longer exposure, a faint band was seen with the mutant DNA. In separate experiments, about 30% of the wild-type activity was detected with the mutant DNA. Thus, the base changes greatly reduce its template activity although they do not abolish it completely.

**DISCUSSION**

By assaying the ability of deletion DNAs to support the formation of a covalent complex between the terminal protein precursor and 5' dCMP, a minimal size of adenovirus DNA required for initiation has been defined (Fig. 5). The terminal 20-bp sequence thus defined was also able to support DNA synthesis, as revealed by elongation to the first dG residue. In some experiments a slight decrease in template activity was observed when deletions beginning at the internal site extended beyond 35 nucleotides from the terminus. This might mean that sequences lying between 20 and 35 nucleotides from the terminus function to stimulate initiation, possibly by facilitating unwinding of the DNA. The presence of a short recognition sequence located at the very end of a DNA molecule may be a general feature of the protein priming mechanism for initiation of DNA replication. It has been reported that bacteriophage φ29, whose mode of DNA replication closely resembles that of adenovirus, contains an identical 6-bp sequence on both ends of the DNA (41, 42), although this sequence has not yet been shown to be involved in DNA replication.

The inverted terminal repeat of adenovirus type 5 is 103 bp and can be divided into an A+T-rich half, which occurs at the termini of viral DNA and a G+C-rich half, which occupies the remainder (29). Sequences conserved among different serotypes are found mostly in the A+T-rich half. In particular, the terminal 22-bp sequence is highly conserved among all human

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**Fig. 2.** Template activity of various deletion DNAs. Plasmid DNAs were digested with EcoRI and used in the reaction to form pTP–dCMP complex. Reaction mixtures (20 μL) contained 25 mM Hepes/KOH, pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol, 3 mM ATP, 0.85 μM [α-32P]dCTP (~400 Ci/mmol), 8 μL of enzyme fraction, and 100 ng of DNAs. Dideoxy-ATP and aphidicolin were added to final concentrations of 40 μM and 40 μg/ml, respectively. After incubation for 2 hr at 30°C, the product was analyzed by electrophoresis on a NaDodSO4/10% polyacrylamide gel as described (9), and the gel was autoradiographed. The arrow indicates the position of the 80-kDa pTP protein. Ad–prot, adenovirus DNA–protein complex.
adeno-viruses and is also partially conserved in the simian, avian, and murine adено-viruses (27-38). Therefore, the functional origin defined in this study is conserved among different sero-types of adenovirus.

Within the functional origin of adenovirus DNA replication, there is a 10-bp sequence (A-T-A-A-T-A-T-A-C-C) that is perfectly conserved among all serotypes of human adenovirus, and it has been suggested that this sequence plays an important role in the initiation of DNA replication (27-38). Results presented in this study provide evidence in support of this claim. Base changes in the middle of the conserved sequence greatly reduce the template activity. However, results obtained by using deletions at the terminus suggest that the sequence at the very end is also important for initiation. Whether the nucleotides between the conserved block and the extreme terminus are also required for initiation is a question that requires further investigation. van Bergen et al. (43) have suggested that these base pairs merely provide a spacer between the conserved sequence and the priming site. They propose that any G residue 4-8 bp from the conserved sequence will provide a template for pTP-dCMP complex formation. However, our result with deletion R-8, which contains a G nucleotide 4 bp from the conserved sequence, but does not prime, must be considered in any model.

Conservation of origin sequences among closely related organisms is a general phenomenon. For example, the negative-strand origins of single-stranded DNA phages G4, ST-1, φK, and α3 share high homology and similar secondary structures can be drawn for all four phages (44). Lymphoid phages, λ, φ80, and φ82 contain highly conserved, tandemly repeated sequences at the origin and these sequences serve as binding sites for the O protein, which is required for initiation (45-47). Sequences homologous to the simian virus 40 DNA replication origin are also found in polyoma virus and BK virus (reviewed by Seif et al. in ref. 48). Finally, a consensus sequence was found between five naturally occurring origins from bacterial DNA (49).

All of the experiments described in this report were carried out by using double-stranded DNA. It has been reported by us and others that single-stranded DNA supports the formation of the pTP-dCMP complex (9, 50). Furthermore, a synthetic oligonucleotide, which contained only the first 15 bases of the ter-

![Image](https://example.com/image.png)

**FIG. 4.** Construction and template activity of a plasmid DNA with base changes in the conserved region. (A) Mutant oligonucleotides containing 20 bases corresponding to both strands of adenovirus terminal sequence were synthesized. One microgram of each of the 20-mers was mixed in 0.1 M Tris-HCl (pH 8), heated at 95°C for 2 min, and slowly cooled to room temperature. The DNA was reacted with [γ-32P]ATP and T4 polynucleotide kinase and was chased with unlabelled ATP. One-tenth microgram of hybridized, kinase-treated DNA was mixed with 0.5 μg each of EcoRI and SalI linkers. Twenty units of T4 DNA ligase were added and the reaction mixture was incubated at 14°C overnight. After heating at 65°C for 10 min, the DNA was digested with EcoRI and SalI and separated by electrophoresis on a 12% acrylamide gel. A major band was detected at the position corresponding to the 20-mer with two linkers. The band was eluted from the gel, precipitated with ethanol, and cloned into plasmid pAT DNA that had been digested with EcoRI and SalI. (B) DNA sequence of the mutant plasmid constructed as in A was determined according to Maxam and Gilbert (39). The mutant DNA was linearized with EcoRI and end-labeled with [γ-32P]ATP and T4 polynucleotide kinase after phosphatase treatment. The labeled DNA was digested with BglI and the short fragment was separated by electrophoresis on an 8% polyacrylamide/7 M urea gel. The arrows show positions where the base changes occur. In the wild-type sequence T-A is present at these positions (see text and Fig. 5). (C) Template activity of two independently isolated mutant DNAs was investigated by the formation of the pTP-dCMP complex as described in the legend to Fig. 2. All DNAs were digested with EcoRI prior to incubation with the enzyme fraction. Fifty nanograms of EcoRI-linearized DNA was used for the reaction. The arrow indicates the position of the 80-kDa protein. wt, Wild type.
minal sequence from the 3' strand, supported complex formation when a highly purified protein fraction containing pTP and 140-kDa DNA polymerase was used (40). We have further analyzed this single-stranded DNA requirement by using the highly purified enzyme fraction. As expected, the 20-mer containing the terminal sequence of the 3' strand supported complex formation but, surprisingly, the mutant 20-mer of the 3' strand that was used to construct the point mutants in this report also supported complex formation to approximately the same extent (unpublished data). On the other hand, the mutant 20-mer of the 5' strand did not support the complex formation. These reactions that used synthetic oligonucleotides differed from those that used double-stranded DNA in the following ways. First, a high concentration of oligonucleotides is required to support complex formation. Second, the reaction is inhibited by the high ATP concentration normally used when double-stranded DNA is the template. Thus, it is likely that the reaction utilizing single-stranded DNA is distinct from that using double-stranded DNA, and it is unclear to what extent the reaction with single-stranded DNA reflects the initiation on a double-stranded viral DNA.

Plasmids containing adenovirus DNA provide useful tools for studying adenovirus DNA replication in vivo and in vitro. In addition to the results reported here and previously (9) on the initiation of DNA replication in vitro, the replication of a cloned adenovirus DNA has been studied in vitro. Y. Gluzman and D. Hanahan (personal communication) have cloned an entire genome of adenovirus into a plasmid DNA and have shown that this plasmid gives rise to infectious virus upon transformation to 293 cells, although the efficiency of transformation was lower than that of Pronase-treated adenovirus DNA. In this case, the plasmid DNA also had to be linearized prior to transformation.

In conclusion, we have shown that a functional origin of adenovirus DNA replication is located at the termini of the DNA. Within this region, a sequence that is perfectly conserved among the human adenoviruses is present and plays an important role in the initiation of DNA replication. We have recently learned that similar results were obtained by M. D. Chalberg and D. R. Rawlin (personal communication). Does this terminal sequence represent a recognition sequence for the pTP, 140-kDa DNA polymerase, or nuclear factors from HeLa cells? Further in vitro analysis of the template requirement, including DNase protection experiments, should clarify this point.

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