Template requirements for the initiation of adenovirus DNA replication

(site-directed mutagenesis)

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ABSTRACT The first step in the replication of the adenovirus genome is the covalent attachment of the 5'-terminal nucleotide, dCMP, to the virus-encoded terminal protein precursor (pTP). This reaction can be observed in vitro and has been previously shown to be dependent upon either viral DNA or linearized plasmid DNA containing viral terminal sequences. Plasmids containing deletions or point mutations within the viral terminal sequence were constructed by site-directed mutagenesis. In the case of linear double-stranded templates, pTP·dCMP formation required sequences located within the first 18 base pairs of the viral genome. This sequence contains a segment of 10 base pairs that is conserved in all human adenovirus serotypes. Point mutations within the conserved segment greatly reduced the efficiency of initiation, while a point mutation at a nonconserved position within the first 18 base pairs had little effect. Single-stranded DNAs can also support pTP·dCMP formation in vitro. In contrast to the results obtained with duplex templates, experiments with a variety of single-stranded templates, including phage M13-adenovirus recombinants, denatured plasmids, and synthetic oligodeoxyribonucleotides, failed to reveal any requirements for specific nucleotide sequences. With single-stranded templates containing no dG residues, the specific deoxynucleoside triphosphate requirements of the initiation reaction were altered.

The adenovirus genome is a linear duplex DNA molecule of about 35,000 base pairs containing a 55-kilodalton (kDa) protein covalently attached to the 5' end of each DNA strand. The replication of the adenovirus genome has been extensively studied as a model for the mechanisms involved in the replication of eukaryotic chromosomes (see refs. 1–3 for reviews). Replication of adenovirus DNA initiates at either end of the genome, and chain elongation takes place by a strand displacement mechanism in the 5'-to-3' direction. The displaced parental strand also serves as a template, with synthesis beginning at the 3' end of the displaced strand and proceeding in the 5'-to-3' direction.

Several studies on adenovirus DNA replication in vitro have shown that the initiation of viral DNA synthesis takes place by the formation of a covalent complex between an 80-kDa precursor to the terminal protein, pTP, and the 5'-terminal nucleotide of the new chain, dCMP (4–8). The 3'-hydroxyl of the protein-bound dCMP residue then serves to prime subsequent chain elongation. Initiation requires two virus-encoded proteins: the pTP and a 140-kDa DNA polymerase (9–12). The two proteins have been extensively purified from extracts of virus-infected cells and are thought to form a noncovalent bimolecular complex. In addition to the two viral proteins, initiation appears to require at least one factor encoded by the host cell; Nagata et al. (13) have purified a 47-kDa protein from uninfected cells that stimulates the formation of the pTP·dCMP complex. The complete replication of viral DNA in vitro requires a virus-encoded 72-kDa single-stranded-DNA-binding protein and possibly other host-encoded proteins (12, 14, 15) as well as the proteins just mentioned.

In addition to the presence of a covalently bound terminal protein, the genomes of adenoviruses of all serotypes share one other unusual feature: the sequence at one end of an adenovirus genome is identical to that of the other end for about 100–150 nucleotides (16, 17). In the case of human adenovirus type 2 (Ad2) this inverted terminal repetition extends for 102 or 103 base pairs (18, 19). A comparison of the sequence of the inverted terminal repetition of a large number of different serotypes has shown that these sequences share a good deal of homology. The most striking homology is a 10-base-pair sequence (A-T-A-A-T-A-T-A-C-C) that is present near the DNA termini of adenoviruses of every known human serotype and the simian adenovirus SA7 (20, 21). The existence of such a highly conserved sequence near the termini in the face of significant divergence in the rest of the genome suggests that this sequence plays an important role in the initiation of DNA replication.

Tamanai and Stillman (8) have recently reported that linearized plasmid DNA molecules containing sequences from the left end of adenovirus type 5 (Ad5) can support the formation of the pTP·dCMP with a partially purified enzyme fraction from Ad5-infected cells. This observation suggested that it ought to be possible to use in vitro mutagenesis techniques to systematically analyze the viral terminal sequences necessary for this reaction. In this paper we show that the terminal 18 base pairs of the Ad2 genome are sufficient to support the initiation reaction on linear duplex templates in vitro and that single base pair alterations within the 10-base-pair conserved sequence greatly reduce the efficiency of initiation. We also show that single-stranded DNA templates will support the initiation reaction in vitro, but, in contrast to the case of duplex templates, the reaction does not depend upon the presence of a specific nucleotide sequence.

MATERIALS AND METHODS

Materials. The growth of HeLa cells and the preparation of the ts36-complementing fraction from Ad5-infected cells were carried out as described (12). Nuclear extracts from uninfected HeLa cells, prepared as described (12), were applied to a DEAE-cellulose column equilibrated with 50 mM Hepes, pH 7.5/1 mM phenylmethylsulfonyl fluoride/0.1 mM dithiothreitol/10% sucrose and eluted with the same buffer containing 0.1 M NaCl. A partially purified fraction from Ad5-infected HeLa cells containing both viral and host initiation factors was prepared as described by Tamanai and

Abbreviations: pTP, precursor to the terminal protein; Ad2, adenovirus serotype 2; Ad5, adenovirus serotype 5; kDa, kilodalton(s).
RESULTS

Requirements for Initiation on Duplex and Single-Stranded Templates. Initiation of adenovirus DNA replication takes place at the ends of the viral genome and involves the formation of a covalent complex between dCMP and the 80-kDa pTP. An in vitro assay for initiation has been developed in which extracts from adenovirus-infected cells or purified viral replication proteins are incubated with [α-32P]dCTP, Mg2+, ATP, and adenovirus DNA (4-8). The formation of [32P]dCMP-pTP complexes is detected by polyacrylamide gel electrophoresis of the product followed by autoradiography. Recently, Tamanoi and Stillman (8) have utilized this assay to show that DNA of the plasmid pLA1, which contains the left end of Ad5 DNA, will support the initiation reaction, provided that the plasmid DNA is cleaved with a restriction enzyme in such a way that the adenovirus terminal sequence is located near the end of the resulting linear DNA molecule. We have confirmed this result, using an extensively purified preparation of viral replication proteins containing the pTP and the adenovirus polymerase (ts36-complementing activity; see ref. 12). As shown in Fig. 1, pLA1 DNA linearized with EcoRI, which cleaves adjacent to the adenovirus terminal sequences (see Fig. 4), supports formation of pTP-dCMP complexes. In addition to the purified viral proteins, the reaction requires the presence of factors present in uninfected HeLa cells. None of the other three deoxynucleoside triphosphates effectively substitutes for dCTP (data not shown). Neither uncut pLA1 DNA nor pLA1 DNA digested with Sal I (which cleaves more than 3,500 base pairs from the adenovirus terminus; see ref. 8) supported the formation of pTP-dCMP complexes. Similarly, pBR322 cleaved with EcoRI did not support complex formation. Thus, with duplex DNA templates the initiation reaction requires a specific nucleotide sequence and the sequence must be located at or near the end of the linear molecule.

Fig. 2 shows the requirements for the initiation reaction with single-stranded DNA templates. In contrast to the results described above, both pLA1 DNA cleaved with Sal I and pBR322 DNA cleaved with EcoRI, as well as pLA1 DNA cleaved with EcoRI, effectively supported formation of pTP-dCMP complexes if the DNA's were denatured prior to the reaction. In addition, single-stranded circular DNA from M13 phage, either with or without an inserted segment of adenoviral terminal sequences, and the synthetic oligonucleotide (dG-dT)2 were all equally effective as templates in this reaction. As in the case of duplex DNA templates, pTP-dCMP formation was strictly dependent on the presence of a partially purified extract from uninfected cells. We conclude that while the protein requirements for the protein–nucleotide joining reaction are similar for duplex and single-stranded DNA templates, the template sequence requirements are much less stringent for single-stranded DNA.

When duplex adenovirus is used as template, the protein–nucleotide joining reaction is highly specific for dCTP as the initiating nucleotide. Fig. 3 shows data on the nucleotide specificity with single-stranded templates. Reaction mixtures containing a single deoxynucleoside [α-32P]triphosphate (dATP, dGTP, dCTP, or dTTP) were incubated with
M13mp9 DNA containing a cloned strand of adenovirus terminal sequence and analyzed for the formation of a pTP-nucleotide complex in the standard assay. With this template and other G-containing single-stranded templates, the radioactivity incorporated into pTP-nucleotide was at least 20-fold greater in reaction mixtures containing [α-32P]dCTP than in those containing any of the other three deoxyribonucleotide triphosphates. In contrast, with poly(dA) as template, the radioactivity incorporated into pTP-nucleotide was greatest in reaction mixtures containing [α-32P]dTTP. The quantitative interpretation of the experiment with poly(dA) as template is complicated by the possibility of limited chain elongation in the reaction containing dTTP. It seems likely from our results, however, that with a template not containing dG residues, the preferred nucleotide is one which, in principle, can hydrogen bond to the template. When templates containing all four deoxyribonucleotides are used to support pTP-nucleotide formation, protein-nucleotide joining is highly specific for dCTP.

**Construction of Plasmids with Deletions and Point Mutations.** The results in the preceding section demonstrate that initiation of replication on linear duplex templates requires a nucleotide sequence located near the terminus of the adenoviral genome. To define this sequence requirement more precisely, we have constructed plasmids with deletions or point mutations within the viral terminal sequence. The starting plasmid for these constructions was the plasmid XD7, which contains the left terminal XbaI restriction fragment from Ad2 inserted into the EcoRI site of pBR322 (26). Digestion of this plasmid with EcoRI produces a structure that is similar, but not identical, to that found in EcoRI-linearized pLA1 (see Fig. 4). In particular, the viral terminal sequence in EcoRI-digested XD7 is not preceded by the two extra G-C base pairs found in pLA1. We have observed no difference between XD7 and pLA1 as templates for initiation.

Plasmids containing deletions in the viral terminal sequence were constructed by digestion with Bsal 31 nuclease, followed by the insertion of a BamHI linker oligonucleotide (G-G-C-C-A-T-G-G-C-C). The general structure of these plasmids is diagrammed in Fig. 5A. The deletions all begin at a SacII site at nucleotide 338 and extend a variable distance toward the EcoRI site at the terminus of viral DNA. The end points of all of the deletions were determined by DNA sequence analysis. We isolated plasmids containing the first 7, 12, 18, 21, and 31 base pairs of the adenoviral terminal sequence.

Plasmids containing G-C-to-A-T transitions within the viral terminal sequence were constructed by directed mutagenesis with sodium bisulfite (27). The structure of these plasmids is diagrammed in Fig. 5B. The mutant plasmids can all be linearized with EcoRI, exposing a structure identical to that obtained with XD7 and the deletion plasmids. The structure of each of the mutant plasmids was verified by direct sequence analysis. There are four C residues (excluding the...
terminal nucleotide) within the first 18 base pairs of the strand of Ad2 DNA (see Fig. 7); we isolated plasmids containing single C-to-T transitions at each of the positions except position 7.

**In Vitro Initiation Using the Mutant Plasmids.** Plasmids with altered viral terminal sequences were digested with EcoRI and tested for their ability to support initiation in vitro. The results of representative experiments are shown in Fig. 6 and the results of several such experiments are summarized in Fig. 7. All plasmids tested that contain at least the first 18 base pairs of the terminal sequence of the Ad2 genome (pMDC7, pMDC10, dl 31, dl 20, and dl 18) supported pTP-dCMP complex formation to nearly the same extent. In contrast, plasmids containing deletions extending past base pair 18 (dl 12 and dl 7) supported this reaction far less efficiently. These results indicate that the sequences required for the initiation of adenovirus DNA replication are present within the first 18 base pairs of the viral genome. Plasmids with a C-to-T transition at nucleotide 17 or 18 did not support the formation of detectable levels of pTP-dCMP complex. A plasmid containing a point mutation at nucleotide 4 supported pTP-dCMP complex formation nearly as efficiently as the wild-type plasmid. In control experiments, increasing the amount of plasmid DNA in these reaction mixtures did not alter the extent of the reaction. Thus, pTP-dCMP complex formation is dramatically reduced by alterations within the 10-base-pair conserved sequence present at both ends of the adenoviral genome but is affected little, if at all, by sequence alterations outside this region.

**DISCUSSION**

The results presented in this paper provide strong support for the idea that the 10-base-pair sequence (A-T-A-A-T-A-T-A-C-C) present near the termini of the genomes of human adenovirus of all serotypes plays an important role in the initiation of DNA replication (20, 21, 28). In our strain of Ad2, this sequence is located between the 9th and 18th base pairs at each end of the genome. Using plasmids containing deletions, we found that the first 18 base pairs of Ad2 DNA are sufficient to support the formation of a covalent complex between the 80 kDa terminal protein precursor and dCMP, the proposed first step in DNA replication. Moreover, single base pair alterations within the conserved sequence dramatically reduce the extent to which plasmid DNAs support this reaction. Thus, it seems likely that the conserved sequence block is required for an essential step in initiation. There is no information as yet on what this step might be. The simplest hypothesis is that the conserved sequence provides a binding site for one of the proteins required for protein-nucleotide joining. We have recently learned that Tamanoi and Stillman have also constructed plasmid DNAs with alterations in the adenovirus terminal sequence and obtained results very similar to ours (29).

The role of the nucleotides between the terminal G-C base pair and the conserved sequence is less clear. van Bergen et al. (28) have suggested that these nucleotides serve as a spacer, positioning the terminal G-C base pair an appropriate distance away from the conserved sequence. We found that a plasmid with a G-C-to-A-T transition at base pair 4 supported the initiation reaction nearly as well as a plasmid containing the wild-type sequence. Thus, our results are consistent with the view that the exact sequence of the nonconserved terminal nucleotides is not important, but a more detailed mutational analysis of this region will be required before firm conclusions can be drawn about its role in initiation.

It has been previously reported that single-stranded DNA can support pTP-dCMP formation, and that this reaction, like the reaction with duplex DNA as template, occurs in response to a specific nucleotide sequence (8, 30). We have confirmed that single-stranded DNA is an effective template for initiation, but our results indicate that no special sequence on single-stranded templates is required. Every single-stranded DNA that we have tested to date, including synthetic oligonucleotides consisting of a repeating dinucleotide sequence, has been capable of supporting pTP-dCMP formation. We do not know for certain what the relationship of the reaction with single-stranded DNA is to the reaction with duplex DNA. Clearly, a duplex template molecule must begin to unwind in an early step during the course of DNA replication. Thus, an attractive hypothesis is that the reaction with single-stranded DNA reflects a partial reaction of the initiation process, providing some insight into the mechanism by which initiation takes place. Our results are consistent with a simple two-step model for initiation. The first step in initiation is the binding of one or more of the proteins involved in initiation to the conserved subterminal sequence block located between 9 and 18 base pairs from the terminus. As a result of this binding event, the extreme terminal region of the duplex is unwound, exposing a short single-stranded segment. The second step in initiation takes place on the ex-
posed template strand and involves the covalent joining of the initiating nucleotide (dCMP) to pTP. In this model, the high degree of sequence specificity of initiation is determined by the initial binding/unwinding event. The subsequent protein-to-nucleotide joining event requires an exposed single-stranded template but is relatively indifferent to sequence, except for a strong preference for a dG residue at the start site. Even the latter preference is not absolute, since the specificity of the nucleotide covalently joined to pTP can be altered by providing a single-stranded homopolymer template [poly(dA)] without dG residues. This suggests that base pairing between the initiating nucleotide and the template may be an obligatory feature of the protein-nucleotide joining reaction.

Nagata et al. (13) have suggested that a 47-kDa host factor serves to unwind the duplex template prior to pTP-dCMP formation. This suggestion was based on their observation that pTP-dCMP formation with single-stranded templates differed from that with duplex templates in that it required only a purified preparation of pTP and the polymerase, without the addition of any host-encoded factor. In contrast, we find that both single- and double-stranded templates, pTP-dCMP formation is strictly dependent on one or more factors present in uninfected cells. The reason for this discrepancy is unknown. On the basis of our results, however, we suggest that the host factor(s) must have at least one other function in addition to that of unwinding the helix, if in fact it participates in such an activity at all. We have recently found (unpublished results) that purified virus-encoded initiation factors bind to the end of any duplex DNA molecule regardless of sequence. Thus, it is possible that the host factor is at least partly responsible for the specific recognition of the viral origin sequence documented in this paper; this possibility remains to be tested directly.

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