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Adenovirus DNA replication in vitro: duplication of single-stranded DNA containing a panhandle structure

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Adenovirus DNA replicates by displacement of one of the parental strands followed by duplication of the displaced parental single strand (complementary strand synthesis). Displacement synthesis has been performed in a reconstituted system composed of viral and cellular proteins, employing either the viral DNA-terminal protein complex as template or linearized plasmids containing the origin. Previously, evidence was obtained that in vivo complementary strand synthesis requires formation of a panhandle structure originating from hybridization of the inverted terminal repeats. To study the conditions for complementary strand synthesis in vitro, we have constructed an artificial panhandle molecule that contains a double-stranded inverted terminal repetition (ITR) region and a single-stranded loop derived from the left and right terminal *Xma*I fragments of Ad2. Such a molecule appeared to be an efficient template and could initiate by the same protein-priming mechanism as double-stranded DNA, employing the precursor terminal protein. The efficiency of both types of template was comparable. Like for replication of the duplex molecule initiation of panhandle replication was stimulated by nuclear factors I and III, proteins that bind to specific double-stranded regions of the ITR. The Ad DNA-binding protein is essential and the 39 kDa C-terminal domain of this protein that harbors the DNA-binding properties is sufficient for its function. These results support the hypothesis that panhandle formation is required for duplication of the displaced strand.

Introduction

The adenovirus (Ad) genome consists of a linear DNA duplex of about 36 kbp that has a 55 kDa protein (TP) attached to both 5′ termini. The origins of DNA replication are contained within the inverted terminal repeat (ITR) of about 100 bp at the ends of the molecule. Replication initiates by protein priming involving a precursor of the TP (pTP) and proceeds by a strand displacement mechanism, i.e., the 3′ → 5′ strand of the replication fork serves as a template for the Ad DNA polymerase (pol), while the 5′-3′ strand is not replicated simultaneously but displaced. The elongation reaction requires the Ad DNA-binding protein (DBP) that binds strongly to the displaced single-stranded DNA. The displaced strand is subsequently converted into a double-stranded form ('complementary strand synthesis', for reviews see Refs. 1–5).

Initiation of strand displacement synthesis has been studied extensively in vitro. This reaction

Abbreviation: ITR, inverted terminal repeat; Ad, adenovirus; TP, terminal protein; pol, DNA polymerase; NF, nuclear factor; CTF, CCAAT-box binding factor; OTFI, octamer-binding transcription factor.

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requires two viral proteins, pTP and pol, that are present as a pTP-pol complex in infected cells, as well as the core origin sequence encompassing nucleotides 1–18 [6–8]. Upon initiation, a dCMP residue, serving as the first nucleotide of the nascent strand, becomes covalently linked to a serine residue of pTP. The 3′-OH group of dCMP functions as primer for further DNA chain elongation [9–11]. Two cellular proteins, nuclear factors I and III (NFI and NFIII) greatly enhance the rate of initiation by binding specifically to the origin [12–14]. Recognition sequences for NFI and NFIII in the Ad2 origin are closely spaced and their relative position is rather critical [8,15,16]. Contact point analyses indicated that NFI binds at one side of the DNA helix, presumably as a dimer having mainly major groove contacts [16], while NFIII has contacts at all sides of the helix, including minor groove contacts [17].

NFI and NFIII recognition sequences (consensus TGGC/A-N_5-GCCAA and TATGCAA-AT, respectively) have been found in a number of cellular promoter and enhancer sequences and appear to represent parts of these regulatory regions. Several lines of evidence, including direct comparison of proteins and immunological as well as sequence studies [18–21] indicate that NFI and NFIII are very closely related or identical to the transcription factors CTF (CCAAT-box binding factor) and OTFI (octamer-binding transcription factor), respectively. This indicates that adenovirus employs cellular transcription factors for optimal initiation of DNA replication, reinforcing the link between replication and transcription which has been observed for other eukaryotic DNA viruses [22].

Two additional proteins are required for elongation. One is the viral DBP, which binds to the displaced single-stranded DNA. The DBP-single-stranded DNA complex has a regular structure, protecting the DNA against nucleases and presumably facilitating complementary strand synthesis [23]. Moreover, DBP and pol collaborate somehow to enable a high rate of displacement synthesis [24]. Nuclear factor II (NFII) is a cellular protein with topoisomerase activity which is required late during elongation [25].

So far, studies on DNA replication in vitro have been limited to the displacement synthesis reaction, mainly because suitable templates for complementary strand synthesis are not easily prepared. Our knowledge of the complementary synthesis comes, therefore, mainly from in vivo experiments. There are several indications that the complementary ends of the displaced strand hybridize in vivo, forming a panhandle structure with an origin identical to that of the duplex genome. Transfections with Ad DNA of which part of the origin is deleted at one end produce viruses with two intact origins [26]. Repair of the defective origin occurs presumably with the intact end functioning as template after panhandle formation. Cloned Ad DNA fragments, containing ITR sequences at their ends, replicate in 293 cells when they are cotransfected with helper Ad DNA [27–29]. In such a system, repair of a completely deleted ITR takes place if panhandle formation is allowed by the introduction of other inverted DNA sequences [27,28]. Furthermore, circular plasmids containing two ITR sequences replicate to form linear fragments bordered by the ITRs and coupled to the primer protein [27]. A model that explains this result assumes multiple rounds of replication with a panhandle molecule as an essential intermediate. Here we describe the construction of an artificial panhandle molecule consisting of Ad2 left and right terminal sequences. We demonstrate that replication of this partial duplex occurs efficiently in vitro by a pTP-primed initiation reaction similar to that with double-stranded DNA.

Materials and Methods

Construction of panhandle DNA

The plasmid XD-7 [30] contains the left-terminal *Xba*I fragment of 1.3 kb of Ad2 as an *Eco*RI insert in pBR322. The ITR is oriented near the *Hin*dIII site of pBR322. pComaI [31] carries the right-terminal Ad2 *Hin*dIII fragment of 1.0 kb in pUC8 as an *Eco*RI-*Hin*dIII insert. The ITR is located at the *Eco*RI site. The Ad2 DNA has *Xma*I sites at 1006 bp from the left end and 573 bp from the right end. XD-7 and pComaI were digested with *Hin*dIII and *Xma*I, mixed and ligated. HB101 transformants of the ligation products were screened for pUC8 vectors containing an *Eco*RI fragment of 1583 bp (pRL9). In this

fragment, the terminal *Xma*I fragments of AD2 are joined and flanked by *Eco*RI sticky ends. The fragment was cloned in the *Eco*RI site of M13mp9 (mpRL9) and transformed to JM101. Single-stranded mpRL9 DNA was isolated as described [32]. Phage was precipitated with PEG6000, 5.5 h after inoculation of a log-phase culture of JM101.

To generate panhandle molecules, single-stranded mpRL9 DNA (20 μg/ml) was denatured at 90°C and renatured in 6.6 mM Tris-HCl (pH 7.5)/50 mM NaCl/13.6 mM $MgCl_2$. This buffer was adjusted to 50 mM Tris-HCl (pH 7.5)/100 mM NaCl/13.6 mM $MgCl_2$/1 mM DTT; *Eco*RI was added and digestion was allowed at 37°C for several hours. The DNA was then extracted with phenol/chloroform and diethyl ether and precipitated with 2.5 vol. ethanol.

In vitro DNA replication assay

The purification procedures for DBP [33], the complex of pTP and the Ad DNA polymerase (pTP-pol [34]), NFI [35], NFIII [13] and HeLa cytosol [36] were as described. The C-terminal 39 kDa DBP fragment was prepared by digestion with chymotrypsin in 2 M NaCl [37] followed by DNA-cellulose chromatography. Reaction mixtures (15 μl) contained 25 mM Hepes-KOH (pH 7.5), 4 mM $MgCl_2$, 0.4 mM DTT, 0.1 mM aphidicolin, 5 mM creatine phosphate, 5 μg/ml creatine kinase, 2 mM ATP, 17 μM each of dATP, dGTP and dTTP, 2 μM [α-^{32}P]dCTP (3000 Ci/mmol), 2 mU pTP-pol, 1 μg DBP, 1 μl cytosol, 1 μl NFI (HAP fraction) and 1 μl NFIII (phosphocellulose fraction) and 30 ng template DNA or as indicated. Incubation was at 37°C for 60 min. Reaction products were analyzed by electrophoresis on a 1% agarose gel with 89 mM Tris/89 mM boric acid/1 mM EDTA/0.1% SDS as running buffer. Gels were dried and autoradiographed.

Results

Construction of the panhandle molecule

In order to produce a panhandle molecule that mimicks the postulated viral panhandle, we constructed mpRL9. This plasmid consists of the left- and right-terminal *Xma*I fragments of Ad2, joined at their *Xma*I sites, and ligated into the *Eco*RI site of a M13 vector (Fig. 1A). Self-hybridization of the single-stranded DNA leads to a dump-bell structure with a 103 bp double-stranded region containing the ITR. Since these fragments originally contained *Eco*RI linkers [30,31] hybridization restores an *Eco*RI site at the border of the double-stranded region. Despite the single-stranded regions located next to this site, *Eco*RI digestion could be performed (Fig. 1B, Ref. 38), generating the required panhandle molecule.

The panhandle molecule can be used as template for protein-primed replication

The panhandle molecule was tested as a template for in vitro Ad DNA replication using a reconstituted system composed of the viral DBP and pTP-pol complex as well as cellular NFI, NFIII and cytosol fractions (see Materials and Methods). As a control, *Eco*RI digested pRL9 containing the same sequence in double-stranded form was used.

As shown in Fig. 2, a double-stranded DNA fragment of 1583 bp was synthesized which, as a consequence of the protein priming, is covalently coupled to the pTP. The pTP retards the replicated fragment relative to the native dsDNA fragment on SDS-agarose gels, as is demonstrated by pronase digestion of the replication products (lanes 2 and 4). The pronase-digested products comigrate with the 1583 bp *Eco*RI insert of pRL9 (not shown). From these results we conclude that the panhandle molecule serves as a template for pTP-primed Ad DNA replication. Under the conditions employed, no products other than the pTP-coupled 1583 bp fragment were formed with the panhandle template. With the double-stranded templates, labeling of the pUC vector (lanes 3 and 4) can be seen. This is presumably caused by aspecific, repair-like DNA synthesis, as has been observed before [39].

Comparison of replication protein requirements for displacement synthesis and complementary strand synthesis

To study the requirements for optimal DNA replication of the double-stranded template and the panhandle molecule we omitted the purified replication components one at a time from the reconstituted system (Fig. 3A + B). As for the double-stranded template, NFI and NFIII stimu-

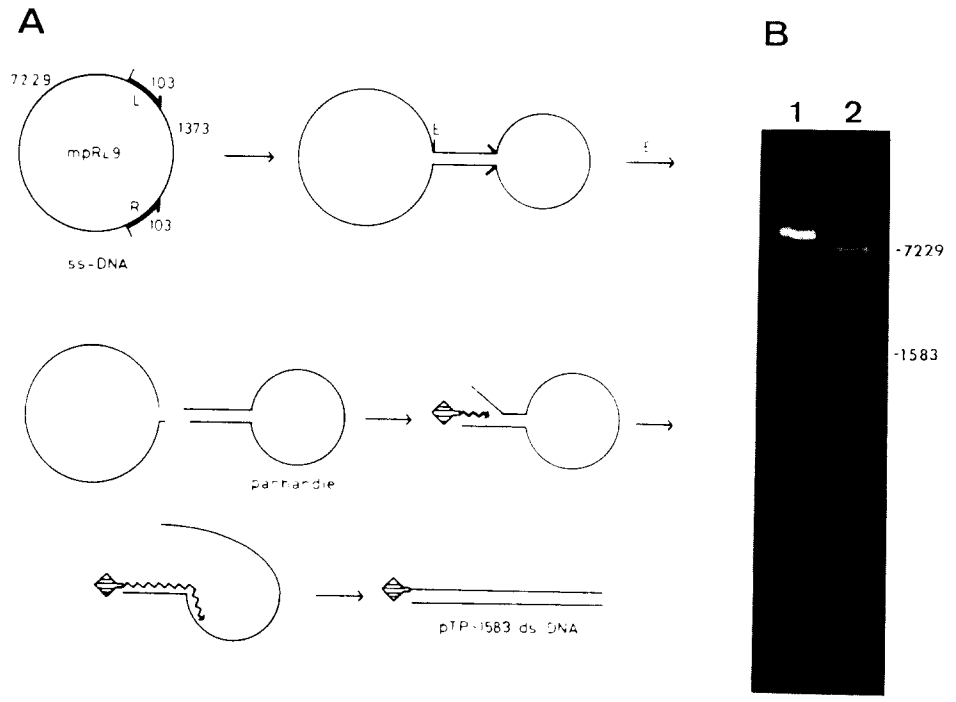

Fig. 1. Construction of the panhandle. (A) Single-stranded mpRL9 containing the left (L) and right (R) Ad2 inverted repeats flanked by an *Eco*RI recognition sequence (E) was hybridized and digested with *Eco*RI to produce the required panhandle molecule. After pTP-primed DNA synthesis, a 1583 bp (1579 + 4 bp from the *Eco*RI site) double-stranded DNA would be formed containing pTP. (B) Ethidium-bromide-stained agarose gel with single-stranded mpRL9 DNA before (lane 1) and after (lane 2) hybridization and *Eco*RI digestion. The length of the fragments is indicated.

lated DNA replication of the panhandle molecule (compare lanes 1, 3 and 4). For both templates, omission of the cytosol reduced the activity of the system (lanes 2). Omission of DBP led to a different response. Both with double-stranded and single-stranded DNA, replication was blocked in the absence of DBP but in addition the panhandle was degraded, a process which was accompanied by aspecific DNA synthesis (Fig. 3B, lane 5). Apparently DBP protects the single-stranded DNA against a nuclease activity present in the reconstituted system, a protective function that has been described before for DBP [40]. The nuclease activity could originate from the DNA polymerase which possesses a $3' \rightarrow 5'$ exonuclease activity on single-stranded DNA [24,41].

To compare the efficiency of replication directly in the same reaction mixture, a double-stranded template of 1006 bp (XD-7 digested with *Eco*RI and *Xma*I) and the panhandle containing template were mixed in equimolar amounts (Fig. 3C). In such a mixture the two templates replicated to the same extent (lane 2). We conclude that, under optimal incubation conditions, the efficiency of both templates is comparable.

Role of DBP in complementary strand synthesis

A profound effect of DBP on displacement synthesis in vitro has been noted before [39]. Since omission of DBP led to breakdown of newly synthesized, panhandle-dependent DNA synthesis, we studied the DBP dependency in more detail by using various DBP concentrations (Fig. 4A). Again, in the absence of DBP the panhandle is degraded and aspecific synthesis occurs (lane 1). A low amount of DBP prevents the degradation of the panhandle but is not optimal for specific replication (lane 2). Most of the label is incorporated in a fragment of 1583 bp which is not coupled to pTP. Apparently, this is caused by a repair activity that starts on the double-stranded region of the panhandle and then complements the single-stranded loop of the molecule. As with double-stranded templates, replication of the pan-

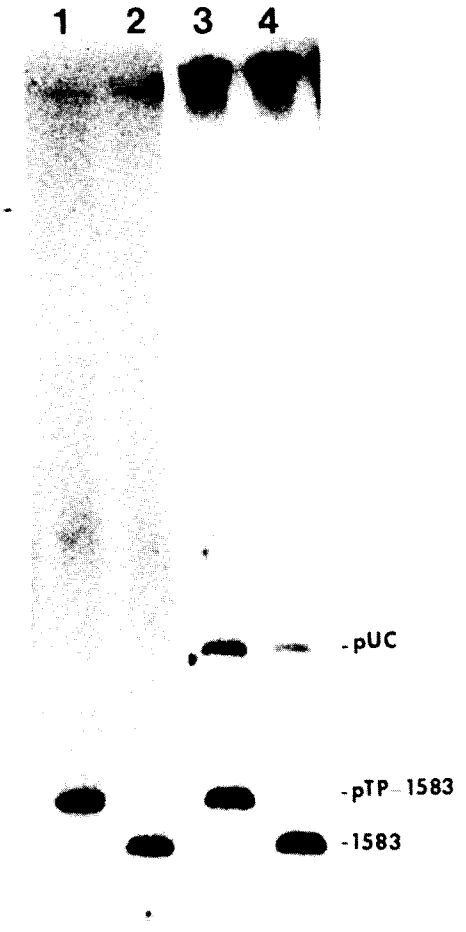

Fig. 2. Replication of the panhandle molecule in vitro. Partially single-stranded panhandle DNA (lanes 1, 2) or double-stranded DNA of the same sequence (lanes 3, 4) was incubated under conditions for Ad DNA replication. Half of the products was incubated with pronase (lanes 2 and 4) and separated on an agarose gel containing SDS, which was subsequently dried and autoradiographed. The length of the DNA fragments is indicated in basepairs. pUC = vector fragment of pRL9.

handle is stimulated further by higher concentrations of DBP in the presence of nuclear factors (lanes 3 and 4). The repair activity becomes fully repressed.

The Ad DBP consists of two domains that can be separated after chymotrypsin digestion. The 39 kDa C-terminal fragment harbors the DNA binding activity and can functionally replace DBP in the replication of ds templates [37,42]. We examined whether the DNA binding domain is also sufficient for replication of the panhandle molecule. For this purpose the replication of the panhandle template was performed with the 39 kDa C-domain replacing DBP (Fig. 4B). With the batches of replication factors used in this experiment, the degradation of single-stranded DNA was diminished and in the absence of DBP, aspecific DNA synthesis occurred with intact templates (lane 1). As for the double-stranded template, a low amount of the 39 kDa C-domain was sufficient to repress this activity (lane 2). Higher concentrations of the DBP fragment led to pTP-primed DNA replication of the panhandle molecule (lanes 3 and 4). So, the 39 kDa C-domain of DBP suffices for its function in specific DNA replication of the panhandle template.

Discussion

Two rounds of DNA synthesis are needed to duplicate the Ad genome. In vitro studies of the first round, the strand-displacement step, have identified novel factors required for Ad DNA replication. Employing an artificial panhandle molecule as template our results indicate that the second round, duplication of the displaced strand, can also be studied in vitro. The reconstituted system that replicates double-stranded fragments via the strand-displacement mechanism, also duplicates the artificial panhandle molecule using pTP-priming and proceeding up to the molecular end of the template. Duplication of the panhandle template is enhanced by NFI and NFIII. Since these proteins do not recognize their binding sequence when present in the single-stranded form, we conclude that initiation indeed takes place at the ITR of the panhandle and not at single-stranded or denatured DNA sequences. In vitro the two types of Ad DNA replication can take place simultaneously with the same efficiency (Fig. 3). Since the nature of the templates is intrinsically different except for the ITR region, this result indicates that the priming reaction is the rate-limiting step of the process. This agrees with calculations made for in vivo replication [43].

The evidence that panhandle formation is an essential intermediate in the replication of adenovirus in infected cells is quite strong.

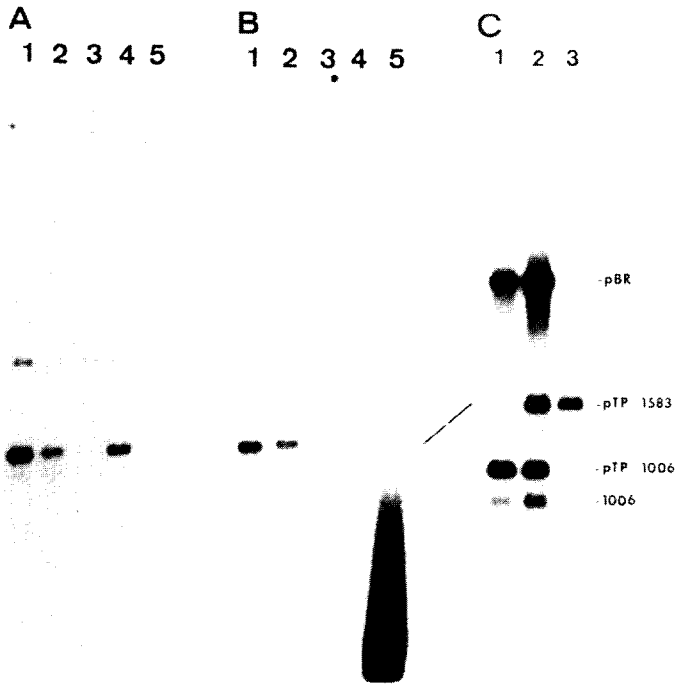

Fig. 3. Factor requirements for replication of the panhandle molecule. DNA synthesis was studied with double-stranded pRL9 DNA (A) as templates or single-stranded mpRL9 panhandle DNA (B) either in the complete system (lanes 1) or deleting cytosol (lanes 2), NFI (lanes 3), NFIII (lanes 4) or DBP (lanes 5). (C) Single- and double-stranded DNA templates, mixed in equivalent amounts. Lane 1, double stranded XD-7 digested with *Eco*RI and *Xma*I (30 ng); lane 3, 30 ng of *Eco*RI digested mpRL9 DNA; lane 2, a mixture of 30 ng of both. The products were analyzed as described in Fig. 2. Replication products are indicated. pBR = vector fragment of XD-7.

Adenovirus genomes lacking part of the left ITR nevertheless produce infectious progeny with two intact ITRs, consistent with panhandle formation or recombination [26]. Similar results were obtained [27,28] employing transfected adenovirus minichromosomes containing cloned ITRs which replicate in the presence of helper adenovirus DNA. In this experimental approach a sequence-independent requirement for inverted sequences was demonstrated, further strengthening the notion that panhandle formation is required. Moreover, the strict conservation of nucleotide sequence in both ITRs is also consistent with a gene correction mechanism employing panhandle formation.

The approach that we followed has its limitations when extrapolating to the situation in vivo. In the first place, any role of the terminal protein already attached to the genome is ignored. It is technically difficult to obtain a single-stranded template containing the terminal protein from viruses, since this requires denaturing conditions which inactivate the terminal protein. Another possibility to obtain such a molecule would be from in vitro displacement synthesis, but here the production of displaced strands is too low and subsequent separation from double-stranded templates is too complicated to make this a feasible approach. It is difficult to predict what the effect of the terminal protein will be. In the case of double-stranded templates, the terminal protein increases the efficiency of the template and also suppresses internal starts, but it does not change the protein requirements for initiation [7,44]. On the other hand, transfected origin containing minichromosomes can initiate correctly, even though they lack the terminal protein [27]. Therefore, we think that the lack of the terminal protein does not essentially affect our results.

Another drawback of the present approach is

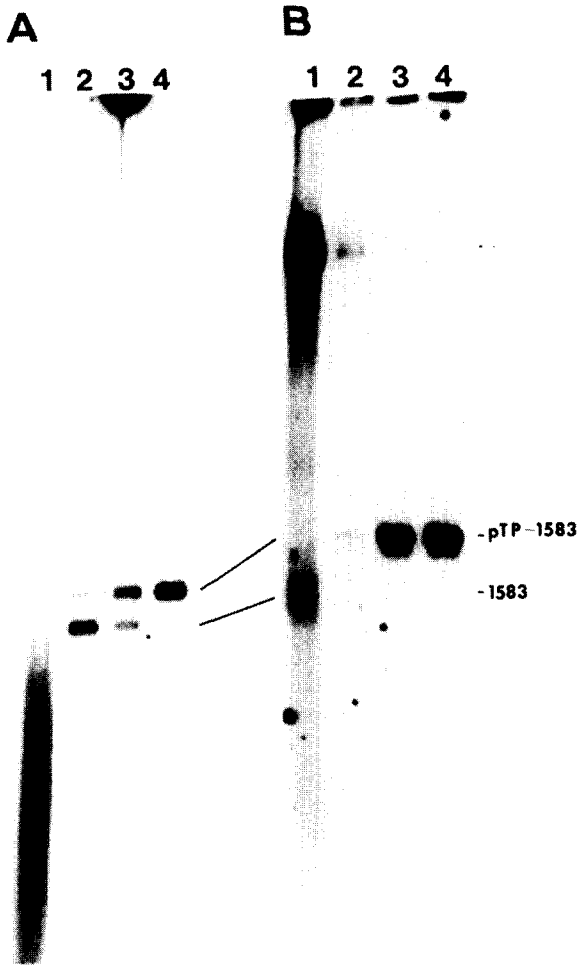

Fig. 4. (A) Effect of DBP concentration on replication of the panhandle molecule. In vitro replication of the panhandle molecule was performed with: lane 1, no DBP; lane 2, 0.5 μgDBP; lane 3, 0.75 μg DBP; lane 4, 1.0 μg DBP (B) Replication of the panhandle molecule in the presence of the 39 kDa C-domain of DBP. In vitro DNA replication with: lane 1, no DBP; lane 2, 0.25 μg DBP C-domain; lane 3, 0.5 μg DBP C-domain; lane 4, 1.0 μg DBP C-domain.

that both processes, displacement synthesis and complementary strand synthesis, might well be coupled in vivo. In such a model a replication complex would remain attached to the displaced strand and could be used in part for duplication of this strand without prior dissociation. We do not know whether such coupling, if it occurs, will influence the protein requirements for initiation of complementary strand synthesis.

Furthermore, we cannot exclude the possibility that in vivo complementary strand synthesis occurs mainly with Ad DNA molecules that are replicated simultaneously from both ends. In such molecules, which have been observed rarely [45], both strands serve as a template at one end of the molecule and are displaced at the other end. Complementary strand synthesis would then take place after encounter of the replication forks and dissociation of the daughter strands.

Ad DBP serves distinct functions in both steps of Ad DNA replication. During strand displacement, DBP is required to bind the single-stranded DNA that comes free. DBP is also necessary for complementary strand synthesis (Fig. 2), not only to protect the single-stranded DNA from nuclease activity but also to form an efficient template for the Ad DNA polymerase (Ref. 24; Fig. 4). These diverse functions of DBP are exerted by the C-terminal DNA binding domain of the protein (Fig. 4B), indicating that the N-terminal domain is dispensable for both types of DNA synthesis.

Interesting, in the case of T4 DNA replication removal of the C-terminal 'A' fragment of T4 gene 32 leads to a loss of lagging strand synthesis in vitro [46]. This effect occurs concomitant with an increase in helix-destabilizing activity and has been attributed to destabilization of the 3'-hydroxy terminus of the RNA primer required for lagging-stranded synthesis. Removal of the N-terminal 'B' region of T4 gene 32 protein leads to loss of cooperativity without drastic effects on lagging-strand synthesis. Clearly, the observed effects depend on the kind of deletion and this may also be true for Ad DBP, explaining why deletion of the N-terminal sequences does not strongly influence its role in displacement synthesis or complementary-strand synthesis. Whether the C-terminal DBP domain is able to perform cooperative binding is still to be determined.

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