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Biochemistry

DNA sequences required for the *in vitro* replication of adenovirus DNA

(nuclear factor I/DNA binding protein/deletion mapping)

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ABSTRACT **Initiation of adenovirus (Ad) DNA replication occurs on viral DNA containing a 55-kilodalton (kDa) protein at the 5′ terminus of each viral DNA strand and on plasmid DNAs containing the origin of Ad replication but lacking the 55-kDa terminal protein (TP). Initiation of replication proceeds via the synthesis of a covalent complex between an 80-kDa precursor to the TP (pTP) and the 5′-terminal deoxynucleotide, dCMP. Formation of the covalent pTP-dCMP initiation complex with Ad DNA as the template requires the viral-encoded pTP and DNA polymerase and, in the presence of the Ad DNA binding protein, is dependent upon a 47-kDa host protein, nuclear factor I. Initiation of replication with recombinant plasmid templates requires the aforementioned proteins and an additional host protein, factor pL. Deletion mutants of the Ad DNA replication origin contained within the 6.6-kilobase plasmid pLA1 were used to analyze the nucleotide sequences required for the formation and subsequent elongation of the pTP-dCMP initiation complex. The existence of two domains within the first 50 base pairs of the Ad genome, both of which are required for the efficient use of recombinant DNA molecules as templates in an *in vitro* DNA replication system, was demonstrated. The first domain, consisting of a 10-base-pair "core" sequence located at nucleotide positions 9–18, has been identified tentatively as a binding site for the pTP [Rijinders, A. W. M., van Bergen, B. G. M., van der Vliet, P. C. & Sussenbach, J. S. (1983) *Nucleic Acids Res.* 11, 8777–8789]. The second domain, consisting of a 32-base-pair region spanning nucleotides 17–48, was shown to be essential for the binding of nuclear factor I.**

Studies on the *in vitro* replication of the 36,000-bp adenovirus (Ad) genome [Ad DNA with a 55-kilodalton (kDa) terminal protein (TP) covalently linked to each 5′ end (Ad DNA-TP)] have provided considerable information concerning the protein requirements and mechanism of Ad DNA replication (see refs. 1–5 for reviews). Reconstitution of Ad DNA replication *in vitro* has been accomplished with five highly purified proteins (3, 6), three of which are viral-encoded (2, 3). These viral proteins include the Ad DNA binding protein (Ad DBP), the 80-kDa precursor (pTP) to the TP found at the 5′ ends of each viral DNA strand, and a 140-kDa DNA-dependent DNA polymerase (Ad Pol). The two remaining proteins required in the reconstituted system have been purified from nuclear extracts of uninfected HeLa cells. Nuclear factor I, a 47-kDa protein involved in the initiation and early elongation of Ad DNA synthesis *in vitro* (7), has been shown to be a site-specific DNA binding protein (8). DNase I cleavage-inhibition experiments have localized the nuclear factor I DNA binding site to a region between nucleotides 17 and 48 of the Ad5 genome (8). Nuclear factor II, a type I DNA topoisomerase, is required for the elongation of replicating intermediates to full-length Ad DNA (6). Initiation of Ad DNA replication proceeds via the synthesis of a covalent complex between the 80-kDa pTP and 5′ dCMP, the first nucleotide of the nascent DNA strand (9–11). Elongation of nascent DNA chains, utilizing the 3′ hydroxyl end of the dCMP residue as a primer terminus, can occur in the reconstituted system. In the presence of Ad DNA-TP, ATP, Mg^{2+}, the four dNTPs, the pTP, Ad DBP, and nuclear factor I, the Ad Pol synthesizes DNA chains that are 25–35% the length of Ad DNA (3, 6). Addition of nuclear factor II leads to the synthesis of 36-kb Ad DNA at a rate that approximates *in vivo* Ad DNA synthesis (6).

In vitro replication of plasmid DNA molecules containing the origin of Ad DNA replication but not the 55-kDa TP also has been reported (12–14). Replication of one such recombinant plasmid, pLA1, resembles the *in vitro* replication of Ad DNA-TP in that (*i*) specific Ad DNA sequences are required at the terminus of the linearized plasmid DNA template for replication to occur (12–14); (*ii*) replication of pLA1 DNA requires the pTP, Ad Pol, Ad DBP, and nuclear factor I, as does replication of Ad DNA-TP (14, 15); and (*iii*) replication of linearized pLA1 DNA is initiated by the formation of a covalent pTP-dCMP complex (12–15). Initiation of DNA replication on linearized pLA1 differs from that on Ad DNA-TP in the relative efficiencies with which these DNAs are used as templates (14) and in the requirement for an additional host protein, factor pL (15), for pTP-dCMP complex formation when pLA1 DNA is used as the template.

Construction of plasmid DNAs containing the origin of Ad DNA replication that support formation of the pTP-dCMP initiation complex has provided a means to determine the DNA sequences required for the initiation of Ad DNA replication. Studies using deletion mutant plasmids containing various lengths of the terminal region of the Ad genome and base alterations introduced by site-directed mutagenesis (12, 16) have demonstrated a requirement for the region between nucleotides 9 and 18 of Ad DNA for the *in vitro* initiation of Ad DNA synthesis. Sequence determinations of various serotypes of Ad DNA have revealed that this 10-bp "core" sequence is perfectly conserved among the human Ads and is partially conserved in the DNA of the simian, avian, and murine Ads (17–27). In this communication, we report that an additional region that is located directly to the 3′ side of the 10-bp core sequence and encompasses the nuclear factor I DNA binding site is required for the efficient use of plasmid

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Abbreviations: bp, base pair(s); Ad, adenovirus; kDa, kilodalton(s); TP, 55-kDa terminal protein; Ad DNA-TP, Ad DNA with a TP covalently linked to each 5′ end; Ad DBP, Ad-encoded DNA binding protein; pTP, 80-kDa percursor of the TP; Ad Pol, 140-kDa Ad-encoded DNA polymerase; pTP-Ad Pol, noncovalent complex between pTP and 140-kDa Ad Pol; kb, kilobase(s).

DNA molecules as templates for the initiation and elongation of Ad DNA replication.

MATERIALS AND METHODS

Materials. [α-^{32}P]dNTPs and [*methyl*-^{3}H]dTTP were purchased from Amersham. Nitrocellulose filters (HA, 0.45 μm) were from Millipore.

Nucleic Acids. Plasmid pLA1 (13) was isolated as described (14). All other plasmid DNAs (see Fig. 1) were constructed as described (16) and isolated from *E. coli* strain DH1 by the alkaline NaDodSO$_4$ lysis method (28). Plasmid DNAs were purified by CsCl gradient centrifugation followed by sedimentation through 5–20% sucrose gradients containing 1 M NaCl (29).

Enzymes. The pTP–Ad Pol fraction (30) and Ad DBP (31) were purified as described. One unit of the pTP–Ad Pol fraction catalyzed the incorporation of 1 nmol of [^{3}H]dTMP into an acid-insoluble form in 20 min at 30°C (14). Extracts of uninfected HeLa nuclei (first DEAE-cellulose pass-through fraction) were prepared as described (7). Nuclear factor I and factor pL were purified by the methods of Nagata *et al.* (7) and Guggenheimer *et al.* (15), respectively. *E. coli* DNA polymerase I "large fragment" was purchased from Boehringer Mannheim; restriction endonucleases *Eco*RI and *Sal* I were purchased from New England Biolabs. Conditions for restriction endonuclease digestion of plasmid DNAs were those recommended by the manufacturer.

End-Labeling of DNA. Recessed ends produced by *Eco*RI digestion of plasmid DNAs were labeled at the 3′ end by using [α-^{32}P]dATP, [α-^{32}P]dTTP, and the *E. coli* DNA polymerase I "large fragment" as described (32).

DNA Synthesis. Reaction mixtures (25 or 50 μl) were as described (14) and were incubated for 60 min at 30°C.

Assay for the Formation of the pTP-dCMP Complex. Reaction mixtures (50 μl), processing of samples, electrophoresis, autoradiography, and quantitation of the pTP-dCMP complex were as described (14).

Filter Binding Assays. Retention of labeled DNA fragments on nitrocellulose filter by nuclear factor I was assayed as described (8).

RESULTS

Nuclear Factor I DNA Binding. The site-specific binding of nuclear factor I to a region of DNA close to the terminus of Ad DNA has been reported (3, 8). DNase I cleavage-inhibition experiments have indicated that the nuclear factor I binding site is located between nucleotides 17 and 48 of the Ad5 genome (8). To confirm the boundaries of the nuclear factor I binding domain, filter binding assays were performed with highly purified nuclear factor I and deletion mutant derivatives of plasmid pLA1. pLA1 DNA contains the left-hand terminal 3.3-kb *Bgl* II D fragment of Ad5 DNA. Plasmid DNAs harboring deletions of the Ad5 terminal region were constructed (16) (Fig. 1).

The interaction of nuclear factor I with 3′ ^{32}P-labeled *Eco*RI-digested plasmid DNAs displayed a marked dependence on the ionic strength of the reaction mixture and filtration buffer. At low concentrations of NaCl (50 mM), all of the plasmid DNAs were retained on nitrocellulose filters by nuclear factor I (Fig. 2*A*). Differential binding of nuclear factor I to plasmid DNAs was observed at 100 and 150 mM NaCl. Only those plasmid DNAs (pLAS108, pLAS107, R8, and R17) containing an intact nuclear factor I binding site (as determined by DNase I inhibition) were retained to a significant extent by nuclear factor I under these conditions. Plasmids pLAS114 and S201 were not retained on the nitrocellulose filters by nuclear factor I at 150 mM NaCl. These two plasmid DNAs were not retained on nitrocellulose filters even in the presence of excess nuclear factor I (Fig. 2*B*).

Double digestion of pLA1 DNA with *Eco*RI/*Sal* I generates two DNA fragments of 3.0 and 3.6 kb. The larger of these two fragments contains the cloned Ad5 DNA sequences and the nuclear factor I binding site. Similar digestion of deletion mutant derivatives of pLA1 DNA yields a 3.0-kb fragment containing only vector DNA sequences and a smaller fragment (20–72 bp) containing various extents of the Ad5 terminal region. The specificity of nuclear factor I binding to 3′ ^{32}P-labeled *Eco*RI/*Sal* I-digested plasmid DNAs was examined (Fig. 3). Analysis by agarose and polyacrylamide gel electrophoresis of the bound DNA fragments eluted from nitrocellulose filters showed that, under conditions of high ionic strength (150 mM NaCl), only those DNA fragments that contained an intact nuclear factor I binding domain were bound to nitrocellulose filters by nuclear factor I. Vector DNA sequences and fragments containing only 4 bp (S201) and 19 bp (pLAS 114) of the nuclear factor I binding site were not bound by nuclear factor I under these conditions. At low ionic strength (50 mM NaCl), fragments containing the nuclear factor I binding site, as well as vector DNA sequences, were retained on nitrocellulose filters in the presence of nuclear factor I. The exception to these findings, the smaller *Eco*RI–*Sal* I fragments of plasmids pLAS114 (35 bp) and S201 (20 bp) were not adsorbed to nitrocellulose under conditions of either high or low ionic strength.

Template Activity of Deletion Mutants. *Eco*RI-digested pLA1 DNA and the deletion derivatives were examined for their ability to support formation of the pTP-dCMP initiation complex (Table 1). In reactions containing the purified viral DNA replication proteins and the purified host factor pL, a basal level of pTP-dCMP complex was formed in the presence of DNAs that contained the 5′ terminal 20 bp of the Ad genome (pLA1, pLAS108, pLAS107, pLAS114, and S201).

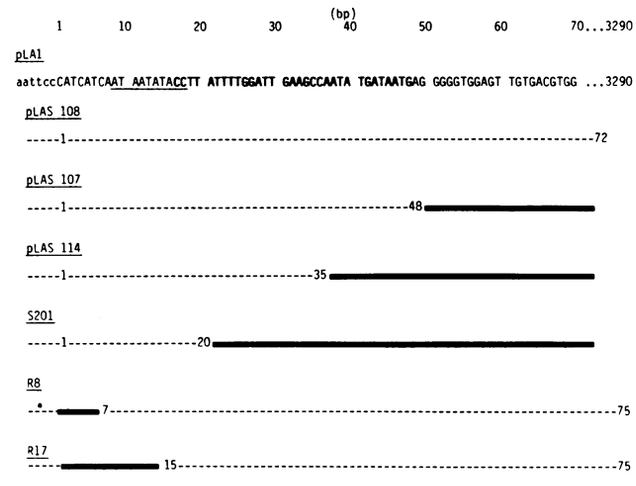

FIG. 1. End points of deletion mutants. The DNA fragments containing deleted sequences extending from the 3′ end towards the *Eco*RI site (pLAS108, pLAS107, pLAS114, and S201) were subcloned into plasmid pAT by the addition of a *Sal* I linker. DNA fragments containing deleted sequences beginning at the 5′ end (R8 and R17) were subcloned by the addition of an *Eco*RI linker. Small letters indicate the *Eco*RI linker DNA sequences at the 5′ end of the plasmid DNA molecules after digestion with *Eco*RI. Capital letters indicate Ad DNA sequences. ------, Sequences present in the various deletion mutants; ——, deleted DNA sequences: underlined sequences (bp 9–18), the 10-bp core sequence (ref. 33; see *Discussion*); bold letters, the nuclear factor I binding site as determined by DNase I cleavage inhibition (8) and nitrocellulose filter binding (this study). Note that, in deletion mutants R8 and R17, the 5′-terminal Ad G·C bp is restored by the addition of the *Eco*RI linker, whereas Ad bp 49 and 50 in deletion mutant pLAS107 are restored by ligation of the *Sal* I linker.

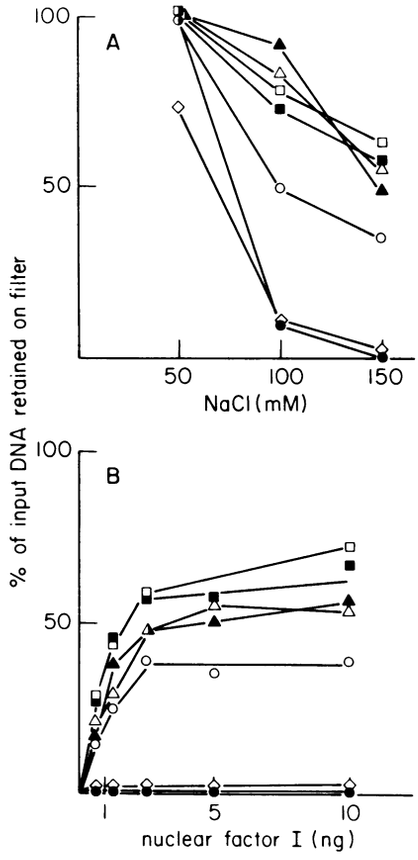

FIG. 2. Retention of plasmid DNAs on nitrocellulose filters by nuclear factor I. Filter binding assays were performed as described (8). (*A*) Binding of plasmid DNAs by nuclear factor I as a function of NaCl concentration. Reaction mixtures (50 μl) containing 0.5 fmol (as molecules) of 3′ ^{32}P-labeled *Eco*RI-digested plasmid DNA (10,000 cpm/fmol), 5 ng of nuclear factor I (glycerol gradient fraction), and the indicated concentration of NaCl were incubated for 20 min at 0°C. The mixtures were filtered, washed five times with 0.4 ml of a mixture containing 25 mM Hepes·NaOH buffer (pH 7.5), 5 mM $MgCl_2$, 4 mM dithiothreitol, and the same concentration of NaCl as that present in the reaction mixture, and dried, and radioactivity was determined by Cerenkov radiation. (*B*) Reaction mixtures (50 μl) containing 0.5 fmol (as molecules) of 3′ ^{32}P-labeled *Eco*RI-digested plasmid DNA, 150 mM NaCl, and the indicated amount of nuclear factor I were incubated, filtered, and washed as described above. Plasmid DNAs and Ad sequences present in these DNAs (in parentheses) were as follows: ○, pLA1 (1–3290); △, pLAS108 (1–72); ▲, pLAS107 (1–48); ●, pLAS114 (1–35); ◇, S201 (1–20); ■, R8 (7–75); □, R17 (15–75) DNAs.

Addition of purified nuclear factor I stimulated formation of the pTP-dCMP initiation complex (5- to 7-fold) with plasmids that contained a minimum of the terminal 48 bp of Ad DNA. Plasmids pLAS114 and S201 showed no increase in efficiency as templates for the initiation reaction upon addition of nuclear factor I. Plasmids R8 and R17 did not support formation of detectable levels of the pTP-dCMP initiation complex in either the absence or presence of nuclear factor I.

Extracts of Ad-infected HeLa nuclei (12, 13), or extracts of uninfected HeLa nuclei supplemented with purified viral DNA replication proteins (14), catalyze the *in vitro* initiation and elongation of nascent DNA chains using linearized pLA1 DNA as the template. Deletion mutant derivatives of pLA1 DNA were examined for their ability to support pTP-primed DNA synthesis (Fig. 4). *Eco*RI-linearized plasmid pLA1 and deletion mutant derivatives pLAS108 and pLAS107 (containing Ad sequences 1–3290, 1–72, and 1–48, respectively) supported DNA synthesis in reaction mixtures containing the purified pTP–Ad Pol fraction and Ad DBP,

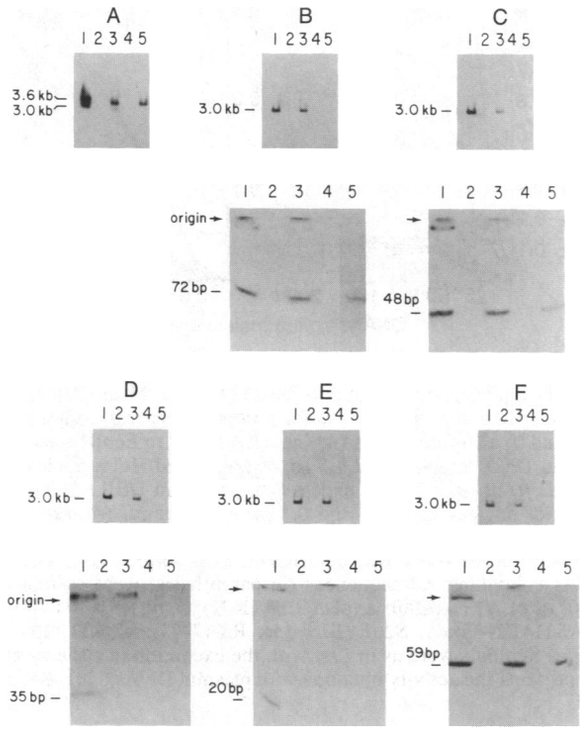

FIG. 3. Specificity of DNA binding by nuclear factor I. Reaction mixtures (50 μl) containing 0.5 fmol (as molecules) of 3′ ^{32}P-labeled *Eco*RI/*Sal* I-digested plasmid DNA and 50 mM (lanes 2 and 3) or 150 mM (lanes 4 and 5) NaCl in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 5 ng of nuclear factor I were incubated, and the reaction products were filtered and washed as described in the legend to Fig. 2. After filtration, DNA fragments of doubly digested pLA1 (*A*), pLAS108 (*B*), pLAS107 (*C*), pLAS114 (*D*), S201 (*E*), and R17 (*F*) DNAs that were retained on the nitrocellulose filters were eluted as described (8) and analyzed on 0.8% agarose (*A–F Upper*) or 17.5% polyacrylamide gels (*B–F Lower*) in 89 mM Tris borate buffer, pH 8.3/2 mM EDTA. Lane 1 (*A–F*) contained *Eco*RI/*Sal* I-digested plasmid DNA that was applied directly to the nitrocellulose filter and eluted as above.

supplemented with either crude extracts of uninfected HeLa nuclei (Fig. 4 *Left*) or purified nuclear factor I and factor pL (Fig. 4 *Right*). *Eco*RI-linearized plasmid DNAs containing deletions of the nuclear factor I binding domain (pLAS114 and S201) and recombinant DNAs containing deletions from

Table 1. pTP-dCMP initiation complex formation with plasmid DNA templates

Template DNA	pTP-dCMP complex formed, % A	B
pLA1	22	100
pLAS108	11	60
pLAS107	10	72
pLAS114	9	13
S201	7	7
R8	<2	<2
R17	<2	<2

Formation of the pTP-dCMP initiation complex was assayed in reaction mixtures (50 μl) containing 6.0 fmol (as molecules) of *Eco*RI-digested plasmid DNA, the pTP–Ad Pol complex (0.014 unit, glycerol gradient fraction), a 20-fold excess (wt/wt) of the Ad DBP to DNA, and factor pL (0.4 μg) in the absence (column A) or presence (column B) of nuclear factor I (7.5 ng, glycerol gradient fraction). In this experiment, 100% was equivalent to 0.1 fmol of pTP-dCMP complex formed in 60 min at 30°C.

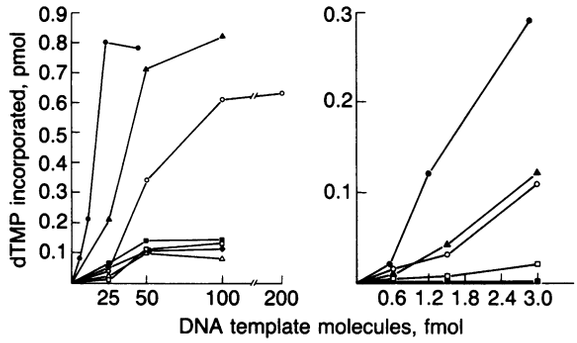

FIG. 4. Template activity of plasmid DNAs. DNA synthesis was measured in reaction mixtures of 50 μl (*Left*) or 25 μl (*Right*) containing 0.018 unit of the pTP–Ad Pol complex (glycerol gradient fraction) and (*i*) a 10-fold excess (wt/wt) of Ad DBP to *Eco*RI-linearized plasmid DNA template and 1.5 μg of uninfected HeLa nuclear extract (*Left*) or (*ii*) a 20-fold excess (wt/wt) of Ad DBP to template, 4.0 ng of nuclear factor I, and 0.2 μg of factor pL (*Right*). After incubation for 60 min at 30°C, reactions were terminated and acid-insoluble radioactivity was determined as described (14). Plasmid DNAs containing Ad sequences (in parenthesis) were as follows. (*Left*) ●, pLA1 (1–3290); ▲, pLAS108 (1–72); ○, pLAS107 (1–48); □, pLAS114 (1–35); △, S201 (1–20); ■, R8 (7–75); ◆, R17 (15–75). (*Right*) Symbols were as in *Left* with the exception that the symbol ■ represents the activity obtained with plasmid DNAs S201, R8, and R17.

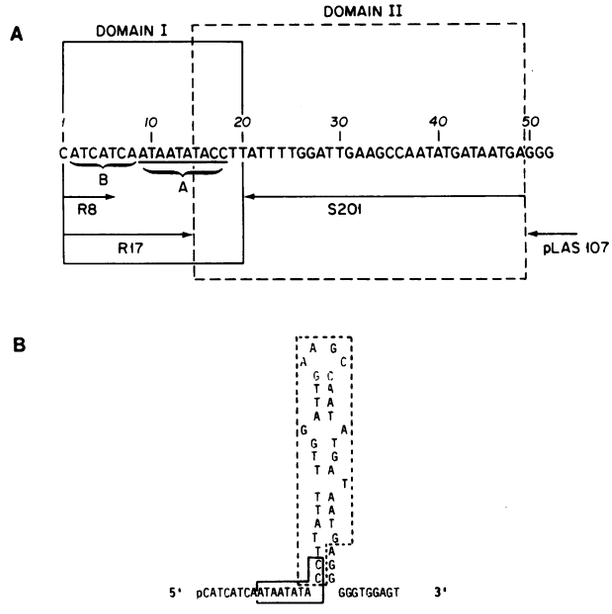

FIG. 5. Schematic representation of the two DNA domains required for Ad DNA replication. (*A*) Domain I can be divided into two areas: A, a 10-bp core sequence (underlined) that the pTP may recognize and bind, and B, a spacer region separating the core sequence and the terminal dG·dC bp. Arrows represent the boundaries of the deletion plasmids used to define both domains. (*B*) Putative secondary structure of the region at the 5′ terminus of *Eco*RI-digested pLA1 DNA. Domains I and II overlap at positions 17 and 18. Boxed areas outline the 10-bp core sequence present in domain I (solid line) and the nuclear factor I binding site in domain II (broken line).

the 5′ *Eco*RI site were inactive as templates for DNA synthesis. DNA synthesis with pLA1, pLAS108, and pLAS107 DNAs, using either the crude extract of uninfected HeLa nuclei or purified host replication factors, commenced after a 10- to 15-min lag and remained linear for 120 min at 30°C. DNA synthesis was not observed when *Eco*RI-digested plasmid DNAs pLAS114, S201, R8, and R17 were used as templates, even after incubation for 120 min at 30°C. Increased concentrations of the Ad DBP or crude extracts from uninfected HeLa nuclei reduced the rate and extent of DNA synthesis with active templates but did not lead to significant dNMP incorporation with inactive DNA templates (data not shown). Therefore, with optimal conditions for the *Eco*RI-digested pLA1 DNA template and under conditions of limiting DNA concentration, DNA synthesis required the 5′-terminal 18 bp of the Ad genome and additional 3′ DNA sequences that comprise the nuclear factor I binding domain.

Alkaline agarose gel electrophoresis of products formed in reactions containing the pTP–Ad Pol fraction, Ad DBP, factor pL, and nuclear factor I demonstrated that full-length DNA was synthesized when either pLA1 (6.6 kb) or pLAS107 (3.0 kb) DNA was used as the template (unpublished results). These results, along with those that demonstrated that equimolar concentrations of *Eco*RI-digested pLA1 and pLAS107 DNAs supported formation of the pTP-dCMP initiation complex to approximately the same extent (Table 1), account for the differences in the dNMP incorporation observed when these DNAs were used as templates (Fig. 4 *Right*).

DISCUSSION

Plasmid pLA1 DNA, which contains the 3.3-kb left end of Ad5 DNA, supports the formation and elongation of the pTP-dCMP initiation complex, provided that the DNA is linearized in such a way as to place the cloned Ad DNA sequences at the terminus of the linear molecule (13, 14, 34). *In vitro* mutagenized derivatives of pLA1 DNA have been constructed (16) and analyzed for their ability to support *in vitro* Ad DNA replication. The results presented in this paper demonstrate that the terminal 50 bp of the Ad genome are required for the efficient use of plasmid DNAs as templates for *in vitro* DNA replication and that these terminal 50 bp are divided into two functionally distinct domains. The two domains are defined by the existence of three classes of plasmid DNAs examined in this study. Class I DNAs are those to which nuclear factor I binds and act as efficient templates for the initiation and elongation reactions (pLA1, pLAS108, and pLAS107); class II DNAs are those to which nuclear factor I does not bind and are poor templates for formation of the pTP-dCMP initiation complex and do not support elongation of the initiation complex (pLAS114, and S201); class III DNAs are those to which nuclear factor I does bind but are inactive for initiation and elongation (R8 and R17).

The maximal limits of domain I (Figs. 1 and 5*A*) are defined by plasmid S201, which contains the terminal 20 bp of the Ad genome. The 5′-terminal dG·dC bp and a 10-bp core sequence (bp 9–18) within domain I are perfectly conserved in the DNA of all human Ads. Plasmid DNAs containing deletions from the 5′ terminus (R8 and R17), deletions from the 3′ end extending into domain I (refs. 16 and 34; unpublished observations), and single-base substitutions within the core sequence are almost completely inactive as templates for the formation of the pTP-dCMP initiation complex. Rijinders *et al.* (33) have presented evidence suggesting that the 10-bp core sequence may provide a binding site for the pTP, thus explaining the absolute requirement for these DNA sequences. The function of the nucleotides between the terminal dG·dC bp and the core sequence is less clear, although it has been proposed that these nucleotides serve as a spacer region, which positions the pTP–Ad Pol complex an appropriate distance (4–8 bp) from the terminus (12). Challberg and Rawlins (34) have found that a plasmid containing a dG·dC to dA·dT transition at position 4 supported the initiation reaction. These results suggest that the exact sequence of the nonconserved terminal nucleotides is not important. Our finding that plasmid R8, which contains a dG·dC bp (do-

nated by the *Eco*RI linker) four bases from the core sequence, is completely inactive as a template for the formation of the pTP-dCMP initiation complex suggests that the required distance between the 5′-terminal dG·dC bp (at which Ad DNA replication is initiated) and the 10-bp core sequence must be greater than four nucleotides.

Domain II, the maximal limits of which are defined from the 5′ end by plasmid R17 to the 3′ end by plasmid pLAS107, is required for nuclear factor I binding and efficient use of the plasmid DNAs as templates for initiation and chain elongation. These results confirm the limits of the nuclear factor I binding site as determined by DNase inhibition patterns (8) and the requirement for nuclear factor I in the replication of Ad DNA-TP and plasmid DNAs containing the origin of Ad DNA replication but lacking the 55-kDa TP (15). It is unclear at present whether nuclear factor I acts both in the initiation and elongation reactions or solely in the initiation reaction. DNA synthesis observed with templates containing domain II may simply be due to the marked increase in the initiation reaction in the presence of nuclear factor I.

The terminal 50-bp of the Ad genome has the potential to form a hairpin structure (Fig. 5*B*) possessing a ΔG of -6.1 kcal/mol (1 cal = 4.19 J) (8). A region of overlap exists between domains I and II at the base of the hairpin structure. Plasmid DNAs containing a dC→dT transition at either position 17 or 18 are still bound by nuclear factor I but are completely inactive as templates for the formation of the pTP-dCMP initiation complex (ref. 34; D. Rawlins, personal communication). This region of overlap, at the 3′ end of the 10-bp core sequence, and the 5′ end of the nuclear factor I binding site may constitute a site of direct protein–protein interaction between the pTP-Ad Pol complex and nuclear factor I.

It has been reported that plasmid DNAs containing the terminal 20 bp of Ad DNA (i.e., deleted within domain II) supported formation of the pTP-dCMP initiation complex and pLA1 DNA replication (16, 34). The apparent discrepancy between these results and the results presented in this study may be accounted for by the use of high template concentrations (4 μg/ml) and a limiting amount of nuclear factor I in previous experiments. Experiments performed with the optimal concentration of crude nuclear extracts of Ad-infected HeLa cells, prepared by the method of Tamanoi and Stillman (13), and with low DNA concentrations (0.4 μg/ml) confirmed the findings presented in this paper (unpublished results). The addition of purified nuclear factor I to nuclear extracts of Ad-infected or uninfected HeLa cells enhanced the initiation reaction on plasmids containing domain II but had no effect on plasmid DNAs deleted in this region. These results suggest that nuclear factor I is a limiting component in crude extracts and that, in order to observe a nuclear factor I (or domain II) dependence, low concentrations of template DNA must be used when extracts are employed as a source of replication proteins.

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