Temperature-sensitive Mutants of Adenovirus Single-stranded DNA-binding Protein

INABILITY TO SUPPORT DNA REPLICATION IS ASSOCIATED WITH AN ALTERED DNA-BINDING ACTIVITY OF THE PROTEIN*

(Received for publication, January 8, 1991)

Masayoshi Tsuji, Peter C. van der Vliet†, and Geoffrey R. Kitchingman§
From the Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101 and the †Laboratory for Physiological Chemistry, University of Utrecht, Utrecht, The Netherlands

The adenovirus single-stranded DNA-binding protein (DBP) is an essential factor in viral DNA replication. Three temperature-sensitive (ts) adenoviruses (Ad2−ND1ts23, Ad2ts111A, and Ad5ts125) are known to have single amino acid substitutions in their DBPs that result in defective DNA replication at the nonpermissive temperature. To elucidate the mechanism(s) involved in the ts phenotype, we purified the three mutant DBPs and studied their DNA-binding properties and their ability to support DNA replication in an in vitro system. The results confirm that the three ts DBPs were incapable of supporting DNA replication at the nonpermissive temperature (40 °C). The defect was found at both the initiation and elongation steps of DNA replication. The 2-fold stimulation of pTP-dCMP formation by the DBP was lost by prior heating of the ts DBPs. The pronounced effect of the DBP on the early elongation process was severely diminished, but not abolished, by prior heating to 40 °C. The functional change at 40 °C was irreversible, as the ts DBPs preincubated at 40 °C were no longer active when assayed at 30 °C. Upon heating to 40 °C, all three ts DBPs lost their ability to bind to oligonucleotides, although they still retained some binding activity for large single-stranded DNAs such as M13 DNA. Thus, the inability of these three ts DBPs to support DNA replication is attributable to their altered DNA-binding properties.

* This work was supported by National Institutes of Health Grant AI-17654, and by the American Lebanese and Syrian Associated Charities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed.

1 The abbreviations used are: Ad, adenovirus; ss, single-stranded; ds, double-stranded; DBP, single-stranded DNA-binding protein; ts, temperature-sensitive; DNA-prot, adenovirus DNA with 55-kDa terminal protein covalently linked to each 5' end; pTP, 80-kDa precursor to terminal protein; Adpol, adenovirus DNA polymerase; V pTP and V pol, crude extracts prepared from HeLa cells infected with recombinant vaccinia viruses expressing Ad pTP and pol, respectively; NF, HeLa cell nuclear factors involved in adenovirus DNA replication; 2-ME, 2-mercaptoethanol; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

2 The protein performs multiple roles in various aspects of viral infection, but its major function is support of viral DNA replication.

Adenovirus DNA replication has been studied extensively since the first report on the development of an in vitro DNA replication system (6). At least six proteins have been found to be involved in the synthesis of full-length genomic DNA (reviewed in Refs. 7-9). Three of these are virally encoded proteins that are essential for DNA replication; the 140-kDa adenovirus DNA polymerase (Adpol), the 80-kDa precursor to the terminal protein (pTP), and the DBP. The pTP is essential for the protein-primed DNA replication mechanism (10), in which the β-hydroxyl group of a serine residue (11) in the pTP serves as a primer (12) to which the first nucleotide, dCMP, is added by Adpol. The Adpol and pTP are tightly associated and are usually isolated from infected cells as a stoichiometric complex (13, 14). The DBP is thought to play two roles in viral DNA synthesis: as a general ssDNA-binding protein that protects the displaced strand from nuclease attack (15) while separating it from the replicating template strand, and as an auxiliary factor for Adpol, enhancing processivity of the polymerase and facilitating DNA chain elongation (16, 17). It has been inferred that the DBP specifically interacts with the Adpol in the presence or even absence of template DNA (17), although the existence of a stable Adpol-DBP complex has never been demonstrated.

In addition to viral proteins, at least three host cell nuclear factors (NFI, NFII, and NFIII) have been shown to be involved in viral DNA replication (18-20). NFI and NFIII bind to specific DNA sequences at the origin of DNA replication and stimulate the initiation process (21). NFII, a type I topoisomerase, is believed to be required for removing topological constraint, thereby permitting replication of genome-length DNA (19).

Genetic evidence for the requirement of DBF in DNA replication has accumulated from studies on conditionally lethal adenoviruses that have a mutation in the DBP (22-26). Ad5ts125 (27), which is identical to Ad5ts107 (28), is the prototype DBP mutant used in many studies, and has a Pro-to-Ser substitution at amino acid 413 of its DBP (28). Only a limited number of studies have used Ad2−ND1ts23 or Ad2ts111A (29, 30). These two viruses have single amino acid substitutions in their DBPs that are located in close proximity: Ad2ts111A has a Gly-to-Val change at amino acid 280 (30), while Ad2−ND1ts23 has a Leu-to-Val change at 282

16178
Temperature-sensitive Adenovirus DNA-binding Proteins

(31). Interestingly, the region surrounding these two mutations has been proposed (32) to form a putative metal-binding domain known as a “zinc finger” (33). All three of the ts DBP mutants have been shown to be fully functional at the permissive temperature but defective at the nonpermissive temperature for supporting DNA replication in vivo (22-24) and in vitro (16, 25, 26, 30).

Efforts to find a change in the DNA-binding properties of these ts DBP mutants linked to their inability to support DNA replication at the nonpermissive temperature have produced inconclusive results. The first such study (34) demonstrated that the Ad5ts125 DBP was eluted from an ssDNA-cellulose column at a lower temperature (20 °C) than was expected to elute wild-type DBP (40 °C), indicating that the mutant protein is defective in DNA-binding at elevated temperatures. This thermal elution, however, was carried out in the presence of 250 mM NaCl, a salt concentration inhibitory for DNA replication in vitro. A subsequent study (35) showed that the Ad5ts107 DBP was capable of binding to ssDNA, even at the nonpermissive temperature, if the binding was examined at salt concentrations comparable to that used for in vitro replication (20 mM NaCl). Similar results were recently obtained for the Ad2ts111A DBP (36), reinforcing the hypothesis that the defect for ts DBPs in DNA replication does not result from impaired DNA-binding activity, but may be due to an altered DBP-Adpol interaction. A puzzling finding has been reported for the Ad2*ND1ts23, Ad2ts111A and Ad5ts125) to examine a possible linkage between their ability to support DNA replication and their DNA-binding properties at the permissive and nonpermissive temperatures. Various assay systems were used to study slightly different aspects of DNA binding. Our results demonstrate that the DNA-binding properties of all three mutant DBP's change when they are heated to the nonpermissive temperature. The changes observed are quite obvious in some assays but equivocal in others. We believe that the defect of the ts DBPs in DNA replication can be ascribed to an alteration in their DNA-binding activity.

MATERIALS AND METHODS

Materials—Radioactive labels used were: [32P]orthophosphate (carrier free), [α-32P]ATP(3,000 Ci/mmol), [α-32P]dCTP (5,000 Ci/mmol) and [γ-32P]ATP (5,000 Ci/mmol) from ICMP Biomedicals; [H] thymidine (50 Ci/mmol) from Schwarz/Mann; and t-[4,4,5-3H]leucine (147 Ci/mmol) from Du Pont-New England Nuclear. Restriction enzymes and T4 polynucleotide kinase were purchased from New England Biolabs, creatine phosphokinase and ssDNA-cellulose (dehydrated and autoradiographed at -80 °C using Kodak XAR film with [γ-32P]ATP (1-4 × 10^6 cpm/ng) was prepared from phage M13mp18 grown on Escherichia coli JM101 in TG1 medium (0.5 g of NaCl, 8.0 g of KCl, 11.0 g of NH4Cl, 0.4 g of MgCl2-6H2O, 0.22 g of Na2SO4, 0.8 g of sodium pyruvate, and 12.1 g of Tris base in 1 liter of water) (38) containing [3H]orthophosphate at 0.1 mCi/ml. [H]-labeled M13 DNA (3.4 × 10^6 cpm/ng) was made in an identical manner except that the infected E. coli were grown in M9 medium containing [3H]thymidine (0.1 mCi/ml) and 250 μg/ml of deoxyguanosine. Pol(DT) and oligo(A)7 were obtained from Pharmacia LKB Biotechnology Inc.

**Purification of DBP**—The DBPs were prepared from 2-liter suspension cultures of KB cells (4 × 10^7 cells/ml) infected with each virus at a multiplicity of 10, except for Ad2*ND1ts23 which was used at a multiplicity of 3. Cells infected with wild-type virus were incubated at 37 °C and harvested at 50-h postinfection. Cells infected with ts viruses were incubated at 32.5 °C for 72-96 h. Purification of DBP was carried out according to the method of Schechter et al. (40) with two modifications: (a) DBP was eluted from an ssDNA-cellulose column with a linear rather than a step NaCl gradient; and (b) no apparent polymeric sequence structure, and (b) no apparent bias in nucleotide content.

**DNA Replication Assay**—Adenovirus specific DNA replication was assayed in vitro by detecting the preferential incorporation of [3H]thymidine into the nascent strand of DNA templates incorporating in vivo synthesized DNA.

A nuclear extract containing host cell factors was prepared from HeLa cells, and cytoplasmic extracts (designated as V pol and pTP, respectively) were prepared as described by Stunnenberg et al. (37).

A nuclear extract containing host cell factors was prepared from uninfected HeLa cells (6) and passed through a DEAEC-cellulose column as described previously (43). Reactions were set up on ice by mixing 15 μl of a solution containing the indicated amounts of DBP with 10 μl of a solution containing other replication components such that the final reaction (25 μl) contained 35 μg of Xhol digested Ad DNA-prot, 0.5 μl of V pol, 0.5 μl of V pTP, 0.15 μl of uninfected HeLa cell nuclear extract, 0.8 μg of calf thymus DNA, 0.1 μg of [3H]thymidine, 4 μCi of [3H]thymidine, 0.002 μg of [3H]thymidine, 0.002 μg of [3H]thymidine, and 0.002 μg of [3H]thymidine.

**Initiation and Partial Elongation Assays**—Formation of initiation complexes (pTP-dCMP) was assayed by incubating the specified
amounts of DBP with 50 ng of DNA-prot, 1 μl of V pol, 1 μl of V pTP, 0.3 μl of uninfected HeLa cell nuclear extract, 0.8 μg of creatine kinase, 40 mM HEPES/KOH, pH 7.5, 7.5 mM MgCl₂, 1 mM DTT, 3 mM ATP, 7.5 mM phosphocreatine, 100 μM aconitase, 40 μM dideoxy-ATP, and 0.5 μM [α-32P]dCTP (200 Ci/mmol) in a total volume of 40 μl. After incubation at 40 °C for 30 min, 2-ME, 10% glycerol, and 0.02% bromphenol blue. The pH was neutralized by adding 1–2 μl of 1 M Tris base. Products were separated by SDSPAGE and visualized by autoradiography.

Synthetic Template Assay—DBP has been shown to specifically stimulate the synthesis of poly(dA) by Adppl with an oligo(dA): oligo(dT) template (45). This stimulation was measured with 1 μg of DBP, 0.4 μl of V pol, 165 ng of poly(dT), 165 ng of oligo(dA), 50 mM Tris-HCl, pH 8.0, 8 mM MgCl₂, 4 mM DTT, 200 μg/ml BSA, 3 mM ATP, 7.5 mM phosphocreatine, 100 μM aconitase, 40 μM dideoxy-ATP, and 0.5 μM [α-32P]dCTP (200 Ci/mmol) in a final volume of 40 μl. DBPs were used without any heat treatment or after heating at 30 or 40 °C for 30 min. Reactions were carried out at 30 or 40 °C for 30 min, and terminated by the addition of 950 μl of 200 mM sodium pyrophosphate and 50 mM EDTA followed by SDS-PAGE (44) and visualized by autoradiography.

Impaired DNA Replication in Vitro—Purified DBPs were tested in vitro for their ability to support adenovirus DNA replication. Although our assay system contained crude cell extracts, the system was free from possible contamination with DBP because Adppl and pTP were not prepared from adenovirus-infected cells, but rather from cells infected with recombinant vaccinia viruses expressing Adppl and pTP (37). The template DNA-prot was digested with XhoI prior to the reaction, so that successful replication should result in preferential incorporation of [32P]dCMP into the terminal fragments (XhoI B and C) which contain the DNA replication origins. As shown in Fig. 3, specific DNA synthesis required functional DBP. Wild-type DBP was active at both 30 and 40 °C, while DBP mutants were inactive at 40 °C but had full activity at 30 °C. These results indicate that DBP from either adenovirus can support adenovirus DNA replication in vitro.

DBPs—The DBPs of wild-type adenovirus (Ad5d301), Ad2'ND1ts23, Ad2ts111A, and Ad5ts125 were purified to 90–95% homogeneity, based on densitometer analysis of 72-kDa bands on SDS-PAGE (Fig. 1). All preparations contained a few extra bands in the 38–46-kDa region, some of which are presumably degradation products of the 72-kDa DBP at 47 °C. During purification, each DBP had a slightly different elution peak in ssDNA-cellulose chromatography: the wild-type DBP eluted at 0.60 M NaCl; Ad2'ND1ts23 at 0.47 M; Ad2ts111A at 0.56 M; and Ad5ts125 at 0.55 M. We subsequently realized that comparisons based on NaCl concentrations were inaccurate because the elution peak for the same DBP varied significantly across column runs depending on the age of the column. To correct for this problem, we carried out an analytical column binding assay in which [32P]-labeled mutant DBPs were chromatographed together with wild-type [32P]DBP (Fig. 2). The results indicated that both Ad2ts111A and Ad5ts125 DBPs have a wild-type DNA-binding affinity, while the Ad2'ND1ts23 DBP has a slightly decreased affinity.

Adenovirus DNA Replication in Vitro—Purified DBPs were tested in vitro for their ability to support adenovirus DNA replication. Although our assay system contained crude cell extracts, the system was free from possible contamination with DBP because Adppl and pTP were not prepared from adenovirus-infected cells, but rather from cells infected with recombinant vaccinia viruses expressing Adppl and pTP (37). The template DNA-prot was digested with XhoI prior to the reaction, so that successful replication should result in preferential incorporation of [32P]dCMP into the terminal fragments (XhoI B and C) which contain the DNA replication origins. As shown in Fig. 3, specific DNA synthesis required functional DBP. Wild-type DBP was active at both 30 and 40 °C, while DBP mutants were inactive at 40 °C but had full activity at 30 °C. These results indicate that DBP from either adenovirus can support adenovirus DNA replication in vitro.
Temperature-sensitive Adenovirus DNA-binding Proteins

Fig. 3. Temperature-sensitive DNA replication in vitro. Wild-type and three ts DBPs (1.5 µg) were tested for their ability to support adenovirus DNA replication. Reactions were carried out either at 30 °C for 120 min or at 40 °C for 60 min. The DBPs were preincubated for 30 min at the reaction temperatures. The template DNA-prot was digested with XhoI prior to the reaction, so that successful replication should result in the incorporation of [32P]dCMP into the origin containing end fragments (B and C). Additional slower-migrating bands (B-R1 and C-R1) are replicative intermediates in which one of the parental strands is partially displaced by the new DNA but still connected to the template strand. Also labeled are faster-migrating single-stranded B and C fragments (ssB and ssC) that represent released, replicated strands.

Fig. 2. Elution profiles of wild-type and mutant DBPs in ssDNA-cellulose chromatography. [32P]-labeled extracts prepared from ts adenovirus infected cells were mixed with purified wild-type [3H]DBP, and loaded on ssDNA-cellulose columns. After extensive washing with buffer A containing 0.1 M NaCl gradient in buffer A, and the elution profiles of the wild-type [3H]DBP (C) and the mutant [32P] DBPs (bars) were differentially determined. The position of the [3H]-labeled wild-type DBP was determined by liquid scintillation counting. Autoradiographs used for quantification of the [32P]-labeled mutant DBPs are shown above the elution profiles.

40 °C, whereas the three mutant DBPs were functional at 30 °C (the permissive temperature) but not at 40 °C (the nonpermissive temperature), confirming their ts phenotype. The functional change at 40 °C was an irreversible process: once the ts DBPs were heated to 40 °C, they were no longer functional even if chilled on ice for an extended period of time and then assayed at 30 °C (data not shown). This characteristic enabled us to study the heat-inactivation kinetics of the DBPs. At 40 °C, Ad5ts125 DBP was inactivated most rapidly, followed by Ad2'N123 and Ad2ts111A (Figs. 4 and 5). Little inactivation was observed with wild-type DBP. A mixture of wild-type DBP and each ts mutant, after being heated at 40 °C, had replicative activity comparable to that of the wild-type DBP alone, indicating that the inactivated ts DBPs were not inhibitory to active DBP. It should be noted that when Ad5ts125 DBP lost its ability to support specific DNA synthesis, a smear of small [32P]-labeled DNAs concomitantly appeared at the bottom of gel (Fig. 4). We do not know the origin of this smear. Similar smear bands also appeared at 40 °C, where the wild-type DBP was still functional. The inactivation kinetics of the wild-type and ts DBPs were studied by the in vitro DNA replication assay. The DBPs (1.5 µg) in TEM buffer containing 200 µg/ml BSA and 10% glycerol, were incubated at 40 °C for the indicated periods of time, and their ability to support DNA replication was measured at 30 °C for 90 min.

Fig. 4. Inactivation of the ts DBPs at 40 °C measured by the in vitro DNA replication assay. The DBPs (1.5 µg) in TEM buffer containing 200 µg/ml BSA and 10% glycerol, were incubated at 40 °C for the indicated periods of time, and their ability to support DNA replication was measured at 30 °C for 90 min.

Fig. 5. Heat-inactivation kinetics of the wild-type and ts DBPs. The autoradiograms shown in Fig. 4 were analyzed by a scanning densitometer to quantify the intensity of bands B-R1, C-R1, B and C. A total intensity of these four bands at each time point was expressed as percentage relative to that at time 0 (unheated DBP).
with the heated Ad2ts111A and Ad2"ND1ts23 DBPs, but they were seen only on an overexposed autoradiogram.

Initiation and Partial Elongation Assays—The in vitro assay described above can be modified to further investigate early events in adenovirus DNA replication. The initiation step is assayed by measuring the formation of the 80-kDa pTP-dCMP complex. An early elongation step is studied by substituting dGTP with dideoxy-GTP, which terminates DNA synthesis at the 26th nucleotide (pTP-26-mer, 88-kDa). The results of the initiation and partial elongation assays are shown in Fig. 6. Although the DBP was not essential for initiation, both wild-type and ts DBPs stimulated the formation of pTP-dCMP approximately 2-fold. This stimulatory activity was not seen when the ts DBPs were heated at 40 °C prior to the assay (Fig. 6 and Table I). In contrast to the minor effect on the initiation reaction, the DBPs had a pronounced stimulatory effect on the early elongation process. Stimulatory activity of the ts DBPs for elongation was severely diminished, but not abolished, by prior heating to 40 °C (Fig. 6 and Table I). The effect of heating was greater for Ad5ts125 DBP than for the other two ts DBPs, and the 88-kDa band produced with the heated Ad5ts125 DBP was not only fainter but also more diffuse than the other 88-kDa bands, indicating a possible heterogeneity in the initiation or termination sites.

Synthetic Template Assay—The elongation process was studied more closely using a synthetic template assay. This assay consists of only three components: Adpol, DBP, and oligo(dA):poly(dT) as a template. Neither pTP nor any of the

| DBP          | Heating at 40 °C | Initiation* | Partial elongation* |
|--------------|------------------|-------------|---------------------|
| None         |                  |             |                     |
| wt           | -                | 758         | 945                 |
| +            | 1548             | 2.04        | 1528                |
| Ad2ts111A    | -                | 1438        | 839                 |
| +            | 1586             | 2.09        | 1522 (1.00)         |
| Ad2"ND1ts23  | -                | 615         | 775                 |
| +            | 829              | 0.81        | 1082 (0.39)         |
| Ad5ts125     | -                | 1921        | 706                 |
| +            | 1376             | 2.53        | 1074 (0.36)         |
|              |                  | 735         | 932                 |

Table I

Quantification of initiation and partial elongation products

*The amounts of initiation product (80 kDa) and partial elongation product (88 kDa) in Fig. 6 were measured by a scanning densitometer, and expressed by arbitrary intensity units. Numbers in parentheses indicate ratios between the amounts of partial elongation products (88 kDa) formed with the heated and unheated DBPs.
Temperature-sensitive Adenovirus DNA-binding Proteins

Fig. 7. Stimulation of poly(dA) synthesis by DBP in the synthetic template assay. The wild-type and ts DBPs (1 μg) were incubated at 0, 30, or 40 °C for 30 min in 20 μl of a solution consisting of 50 mM Tris-HCl (pH 8.0), 20 mM NaCl, 8 mM MgCl₂, 4 mM DTT, 3 mM ATP, and 200 μg/ml BSA. They were combined with 30 μl of the reaction mixtures containing poly(dT):oligo(dA), V pol, and [³²P]dATP (250 cpm/pmol) to yield the desired concentrations. The reactions were carried out at 30 or 40 °C for 30 min, and incorporation of [³²P]dAMP into acid insoluble material was determined by liquid scintillation counting.

Fig. 8. Elution profiles of the ts DBPs heated at 40 °C in ssDNA-cellulose chromatography at 4 °C. Partially purified ts [³²P]DBPs were mixed with purified wild-type [³²H]DBP, and incubated 40 °C for 30 min. Samples were loaded onto an ss-DNA-cellulose column and chromatographed at 4 °C. Bound proteins were eluted with a linear 0.02–0.85 M NaCl gradient in buffer A, and the elution profiles of the wild-type and mutant DBPs were differentially determined as described in the legend to Fig. 2.

Fig. 9. Filter-binding assay with unheated and heated DBPs. The wild-type and ts DBPs were used either without any heat-treatment or after incubation at 40 °C for 30 min. A constant amount (20 ng) of [³²H]-labeled M13 DNA (344 cpm/ng) was mixed with increasing amounts of unheated or heated DBPs in 20 μl of TEM buffer containing 20 mM NaCl, 200 μg/ml RSA, and 10% glycerol. After overnight incubation at 4 °C, the mixtures were filtered through nitrocellulose filters. Radioactivity retained on filters was determined by liquid scintillation counting and expressed as a percentage relative to the input counts.

of individual DBP molecules to bind to ssDNA may be better examined using oligonucleotides; because only a limited number of DBP molecules can bind to an oligonucleotide, any cooperativity can be minimized. Since the oligonucleotide-binding capacity of the DBP has not been characterized, we first analyzed the DNA size requirement for binding. Synthetic oligonucleotides (8–84 nucleotides) were [³²P]-labeled and mixed with increasing amounts of wild-type DBP. The DBP-DNA complexes were separated from the free DNAs by agarose gel electrophoresis (Fig. 10). The patterns of the shift of DNA bands indicated that stable DBP-DNA complexes were formed with the 30-mer, 40-mer, and 84-mer DNAs. Complete shift of these oligonucleotides was achieved at a DBP/DNA (w/w) ratio between 30 and 40, which is consistent with the expected saturation point assuming that one DBP molecule occupies 9–11 nucleotides (50). The DBP-24-mer complex appeared to be marginally stable. Smeared bands were observed with a 16-mer and excess amounts of DBP, indicating unstable binding. An octamer had no shift even at a DBP/DNA ratio of 300. Binding to oligonucleotides under 24 bases could be demonstrated by UV cross-linking (data not shown).

Using the 84-mer as a DNA probe, we next examined the ts DBPs for their oligonucleotide-binding activity. The wild-type and mutant DBPs, unheated or heated to 40 °C for 30
Temperature-sensitive Adenovirus DNA-binding Proteins

Fig. 10. Binding of DBP to various sizes of oligonucleotides. Various amounts of wild-type DBP were mixed with 2 ng of $^{32}$P-labeled oligonucleotides (1-4 x 10$^6$ cpm/ng) to yield the indicated DBP/DNA (w/w) ratios, and the DBP-DNA complexes were separated at 4 °C by electrophoresis through a 1% agarose gel.

Fig. 11. Gel mobility shift assay showing oligonucleotide-binding activity of the ts DBPs. The DBPs (100 μg/ml, either unheated or heated at 40 °C for 30 min) were diluted appropriately and mixed with 5 ng of $^{32}$P-84-mer DNA (16,000 cpm/ng) to give the indicated DBP/DNA ratios, and then electrophoresed at 4 °C through a 1% agarose gel.

Virtually identical results were obtained when a 30-mer was used instead of the 84-mer as a probe DNA (results not shown). We consistently observed that the three ts DBPs, when heated to 40 °C, formed one or two faint band(s) shifting between the free DNA and the DBP-DNA complex (Fig. 11). These bands are probably due to contamination with other ssDNA-binding proteins in the DBP preparations. Evidence for this came from several lines of experimentation. First, these bands were not stained with an anti-DBP monoclonal antibody (B6) in a Western blot analysis (Fig. 12). Second, when mixtures of the heated ts DBPs and $^{32}$P-84-mer were UV cross-linked and analyzed by SDS-PAGE, a major band of approximately 75-kDa was detected (data not shown). The unheated ts DBPs and $^{32}$P-84-mer, in contrast, formed a major band of about 100 kDa, which is close to the estimated molecular weight of the DBP-84-mer DNA complex. SDS-PAGE analysis showed that the DBPs themselves were not degraded after heating to 40 °C. Further, when the UV cross-linked samples were immunoprecipitated with B6 anti-DBP antibody, the 100-kDa band was quantitatively precipitated, but the 75-kDa band was not. All of these results indicate the presence of some contaminants with ssDNA-binding activity in the DBP preparations. This is not surprising because such contaminants cannot be completely removed by ssDNA-cellulose chromatography.

Gel Mobility Shift Assay with M13 DNA—The results of the oligonucleotide-binding assay are seemingly contradictory to the findings obtained in the column binding and filter-binding assays. The discrepancy might be due to differences in either the assay methods or the DNAs used for each assay. To distinguish between these possibilities, we carried out another gel mobility shift assay with $^{32}$P-labeled M13 DNA. The results are shown in Fig. 13. Unlike oligonucleotides, the mobility of M13 DNA was progressively shifted as the number
of DBP molecules bound to the DNA increased. All three ts DBPs had significantly different patterns of shift before and after heating at 40 °C. However, the heated ts DBPs were still capable of shifting M13 DNA, and therefore appeared to retain some DNA-binding activity.

**DISCUSSION**

We examined three ts mutant adenovirus DBPs for their ability to support DNA replication and to bind to ssDNA. All three ts DBPs were unequivocally defective in adenovirus DNA replication at the nonpermissive temperature. The DBPs were further tested by several in vitro assays to determine whether the defect was in the initiation or elongation phase of DNA synthesis. In agreement with previous findings (16, 26, 30, 36), our results in the partial elongation and synthetic template assays showed that the three ts DBPs had a severe defect in DNA chain elongation at the nonpermissive temperature. Furthermore, we were able to detect a defect in the initiation step, a finding that may have been overlooked previously (16, 26, 30) because the DBP is not an essential factor but only a weak stimulator for formation of the pTP-dCMP initiation complex. Also, previous studies tested the ts DBPs at the permissive and nonpermissive temperatures. Since the rate of initiation complex formation differs depending on temperature, it is difficult to directly compare the activities of ts DBPs at the two temperatures. We found that the functional change of the mutant proteins at the nonpermissive temperature was irreversible. Taking advantage of this characteristic, we carried out the initiation assay at a constant temperature (30 °C) using the unheated and heated ts DBPs. The unheated ts DBPs and wild-type protein stimulated pTP-dCMP synthesis 2-fold, whereas the heated ones were not stimulatory.

Results from various assays seemed inconsistent regarding the DNA-binding properties of DBP. The column binding assays using ssDNA-cellulose or ssDNA-agarose failed to show an unequivocal change in the relative DNA-binding affinity of the ts DBPs at the permissive and nonpermissive temperatures. The filter binding assay using M13 DNA showed that the three ts DBPs heated at 40 °C were still capable of retaining as much as 70% of the input DNA on the filters, although the amount of protein needed was significantly larger than that required by unheated proteins. Similar results were also obtained in the gel shift assay using M13 DNA. On the other hand, the gel shift assay using an 84-mer oligonucleotide clearly indicated that the ts DBPs lost their DNA-binding capacity by heating at 40 °C. These seemingly contradictory results may simply reflect the difference in the length of DNA used in each assay. Because of the cooperativity between DBP molecules (50, 52), the overall binding affinity of the protein for an ssDNA molecule apparently increases as the number of DBPs bound to DNA increases. If one assumes that approximately 10 bases are covered by one DBP molecule (50), an 84-mer and M13 DNA could bind eight and 720 DBPs, respectively. Therefore, binding to oligonucleotides is far more sensitive to changes in the affinity of individual DBP molecules than is binding to large DNA molecules. Another explanation for the observed differences is that the ts DBPs may change the mode of DNA binding in such a way that a certain length of DNA is needed for binding. Electron microscopic observations that support such a possibility have recently been obtained.2

The seemingly conflicting results described above may actually explain the discrepancy between the conclusions of this and previous studies (35, 36). Based on the change observed in oligonucleotide-binding activity, we suggest that the defect of ts DBPs in DNA replication results from their altered DNA-binding properties. Krevolin and Horwitz (35), using a filter-binding assay with denatured adenovirus DNA, showed that the ability of the Ad5ts107 DBP to bind to ssDNA did not significantly change between the permissive and nonpermissive temperatures and suggested that the inability of this mutant DBP to support DNA replication might reflect an alteration in DNA-Adpol interaction. The results of our filter-binding assay were similar to their results in that the ts DBPs had some DNA-binding activity at the nonpermissive temperature, even though we observed that the binding capacities of ts DBPs were significantly deteriorated after incubation at 40 °C.

The Ad2°ND1ts23 DBP was originally described as having a drastically decreased DNA-binding affinity, yet was fully functional in DNA replication at the permissive temperature (30, 36). In those studies, the mutant protein eluted from an ssDNA-cellulose column at 4 °C with a significantly lower concentration of NaCl (0.2 M) than that needed for wild-type DBP (0.6 M). We did not observe such a large difference despite repeating the column assay three times. However, we did observe that the NaCl concentration needed to elute Ad2°ND1ts23 DBP varied over a considerable range (0.52–0.47 M NaCl), depending on the age of the column, although the relative difference with respect to the internal control was nearly constant in every run (0.10–0.14 M lower than 3H-wild-type DBP). Thus, the large difference reported in the previous studies appears to include the variation between separate column runs, in addition to the real difference between the wild-type and mutant DBPs. We believe that at the permissive temperature, the ability of Ad2°ND1ts23 DBP to bind to ssDNA is strong enough to function normally in DNA replication. It is possible, however, that the column binding assay is measuring primarily electrostatic interactions between the DBP and ssDNA. The actual binding affinity is presumably composed of a combination of electrostatic force and hydrophobic interaction such as aromatic amino acid side chains stacking between the bases of DNA (55, 56). Therefore, if the

---

1. M. Tsuji and G. R. Kitchingman, unpublished observations.

---

**FIG. 13. Gel mobility shift assay with M13 DNA.** Various amounts of DBPs (either unheated or heated at 40 °C for 30 min) were mixed with 20 ng of 32P-labeled M13 DNA (4,800 cpm/ng) to give the indicated DBP/DNA ratios, and then electrophoresed at 4 °C through a 0.5% agarose gel.
Temperature-sensitive Adenovirus DNA-binding Proteins

relative contribution of electrostatic forces to an overall DNA-binding affinity is greater for Ad2+ND1ts23 than for wild-type DBP, it is possible that the mutant protein binds to ssDNA very poorly at high salt conditions, yet binds as tightly as wild-type DBP under the low salt condition in the DNA replication assay. In fact, the binding capacity of Ad2+ND1ts23 DBP appeared to be indistinguishable from that of wild-type DBP under low salt conditions, as seen in the filter-binding and gel shift assays.

Except for the slight difference in the elution profile in ssDNA-cellulose chromatography, Ad2+ND1ts23 and Ad2ts111A DBPs were virtually identical in all characteristics studied. This would be expected because of the close proximity of the two mutations. Rather unexpectedly, the Ad5ts125 DBP also had very similar characteristics despite the fact that this mutation is located more than a hundred amino acids from the other two. However, Ad5ts125 DBP was inactivated at 40°C more rapidly than the other two mutants. Moreover, the Ad5ts125 DBP produced at the nonpermissive temperature has been found to be degraded rapidly in vivo (24, 54), whereas the Ad2ts111A DBP appears to be stable (29, 30). Thus, the mechanism of protein alteration by the Ad5ts125 mutation may somehow differ from that of the other two mutations. Several studies have indicated that the Ad5ts125 mutation affects not only the DNA replication function but also many other functions performed by DBP, such as transcriptional regulation (57), mRNA stability (58), virus assembly (59) and transformation (51). Whether the Ad2+ND1ts23 and Ad2ts111A mutations also affect these other DBP functions is currently under investigation.

It is well established that the DBP consists of two functionally and structurally distinct domains. Mild chymotrypsin treatment separates the protein into 26-kDa amino-terminal and 44-kDa carboxyl-terminal fragments (47). The carboxyl-terminal fragment is still capable of binding to ssDNA (47) and is still fully functional in DNA replication (16). These findings are consistent with the fact that the three ts DBPs have mutations within their carboxyl-terminal domain. Although DBPs from Ad2 and Ad5 differ by nine amino acids (31), these differences are in the amino-terminal domain; the carboxyl-terminal domains are identical. The DBPs of these two serotypes can be used interchangeably in the in vitro DNA replication assays using components prepared from reciprocal Ad serotypes.

How does the altered DNA binding at the nonpermissive temperature give rise to the defect in DNA replication? As mentioned above, all three ts DBPs appear to be defective in both the initiation and elongation phases of DNA replication. The role of the DBP in the initiation of replication is complex. Although not essential for the initiation process, the DBP stimulates the formation of pTP-DcMP initiation complex in the presence of NFI. Part of the reason for this has been explained by recent studies (3, 53) showing that the DBP enhances the affinity of NFI for its binding site at the origin of replication. This enhanced NFI binding appeared to be an indirect effect accompanied by the binding of DBP to DNA (3, 53), although the possibility of direct DBP-NFI interaction cannot be excluded. Cooperation with NFI, however, is not the only role of DBP in initiation, since many studies have shown the stimulation of initiation by DBP in a system consisting of purified components free from NFI (3, 18, 54). Thus, the DBP may have other effects, such as enhancement of the binding of Adpol-pTP to the end of adenovirus DNA and assistance in unwinding the terminal regions of the template DNA prior to initiation.

In contrast to its unknown role in initiation, the function of the DBP in DNA chain elongation has been relatively well defined. The DBP acts as a facilitator of Adpol and makes the enzyme highly processive (45). Stimulation of Adpol by the DBP can result from either a direct interaction between the two proteins or an indirect effect, wherein the DBP changes the topology of template DNA to one more favorable for the polymerase. Although there is circumstantial evidence for a direct Adpol-DBP interaction (45), attempts to isolate the Adpol-DBP complex have not been successful. In the elongation process of DNA synthesis, and perhaps also in the initiation process, the DBP has to bind to single-stranded regions of template DNA, although it is obvious that the binding to DNA per se is not sufficient to promote DNA synthesis. Other, functionally equivalent DBPs such as E. coli single-stranded DNA-binding protein, cannot substitute for the Ad DBP (17, 18, 45).

Based on the results of the DNA-binding assays, we propose that at the nonpermissive temperature, the three ts DBPs either no longer bind to the template DNA, or bind to the template DNA in an aberrant fashion. The alternations are experimentally distinguishable; in the former case, the defective ts DBPs should be readily complemented by wild-type DBP; in the latter, the binding of altered ts DBPs will generate an aberrant template DNA which may be inactive even in the presence of wild-type DBP (assuming that the binding of altered ts DBPs is tight enough to inhibit the binding of wild-type DBP). We have obtained preliminary results supporting the latter possibility using the synthetic template assay, which showed that DNA synthesis with wild-type DBP was inhibited by the addition of the Ad5ts125 DBP preincubated at 40°C for 30 min in a dose-dependent fashion. However, no such inhibition was observed in the in vitro replication assay using the authentic adenovirus DNA-protein complex (Fig. 5). Therefore, further work in this area is clearly required. In addition, it should be emphasized that this explanation does not necessarily imply that the defect in ts DBPs is confined to DNA-binding properties. Whether the ts DBPs have aberrations in possible interactions with Adpol and NFI remains an open question.

In summary, we have shown that the inability of the three mutant DBPs to support DNA replication at the nonpermissive temperature can be ascribed to their altered DNA-binding properties. Further work will be required to determine the nature of the changes to the DBP that result in these altered binding properties.

Acknowledgments—We thank Jack Sublett and Pat Stow for assistance with cell culturing, and Dr. Rakesh Goorha for supplying the 84-mer. Christy Wright provided editorial assistance.

REFERENCES
1. van der Vliet, P. C., and Levine, A. J. (1973) Nature 246, 170–174
2. Fowkes, D. M., Lord, S. T., Linne, T., Pettersson, U., and Philipson, L. (1979) J. Mol. Biol. 132, 163–180
3. Stuiver, M. H., and van der Vliet, P. C. (1990) J. Virol. 64, 379–386
4. Clegern, V. G., and Kissig, D. F. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 8947–8951
5. Seiberg, M., Aloni, Y., and Levine, A. J. (1989) J. Virol. 63, 1134–1141
6. Challeberg, M. D., and Kelly, T. J., Jr. (1979) Proc Natl. Acad. Sci. U.S.A. 76, 655–659
7. Challeberg, M. D., and Kelly, T. J., Jr. (1979) Annu. Rev. Biochem. 58, 671–717
8. Frield, B. R., Lichy, J. H., Field, J., Gronostajski, R. M., Guggenheimer, R. A., Krevalin, M. D., Nagsta, K., Hurwitz, J., and Horwitz, M. S. (1983) Curr. Top. Microbiol. Immunol. 110, 221–255
9. Hay, R. T., and Russell, W. C. (1989) Biochem. J. 258, 3–16
Temperature-sensitive Adenovirus DNA-binding Proteins

10. Rekosh, D. M. K., Russell, W. C., Bellet, A. J. D., and Robinson, A. J. (1977) Cell 11, 283-295
11. Desiderio, S. V., and Kelly, T. J., Jr. (1981) J. Mol. Biol. 145, 319-337
12. Challberg, M. D., Desiderio, S. V., and Kelly, T. J., Jr. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5105-5109
13. Enomoto, T., Lichy, J. H., Ikeda, J.-E., and Hurwitz, J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6779-6783
14. Lichy, J. H., Field, J., Horwitz, M. S., and Hurwitz, J. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5225-5229
15. Leegwater, P. A. J., Rombouts, R. F. A., and van der Vliet, P. C. (1988) Biochem. Biophys. Acta 951, 403-410
16. Friefeld, B. R., Krevolin, M. D., and Horwitz, M. S. (1983) Virology 124, 380-389
17. Lindenbaum, J. O., Field, J., and Hurwitz, J. (1986) J. Biol. Chem. 261, 10218-10227
18. Nagata, K., Guggenheimer, R. A., Enomoto, T., Lichy, J. H., and Hurwitz, J. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6435-6442
19. Nagata, K., Guggenheimer, R. A., and Hurwitz, J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4266-4270
20. Pruijn, G. J. M., van Driel, W., and van der Vliet, P. C. (1986) Nature 322, 656-659
21. van der Vliet, P. C., Claessens, J., de Vries, E., Leegwater, P. A. J., Pruijn, G. J. M., van Driel, W., and van Miltenburg, R. T. (1988) Cancer Cells 6, 61-70
22. van der Vliet, P. C., and Sussenbach, J. S. (1975) Virology 67, 415-426
23. van der Vliet, P. C., Zandberg, J., and Janz, H. S. (1977) Virology 80, 98-110
24. Ginsberg, H. S., Lundholm, U., and Linne, T. (1977) J. Virol. 23, 142-151
25. Kaplan, L. M., Ariga, H., Hurwitz, J., and Horwitz, M. S. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5534-5538
26. van Bergen, B. G. M., and van der Vliet, P. C. (1983) J. Virol. 45, 642-648
27. Ensinger, M. J., and Ginsberg, H. S. (1972) J. Virol. 10, 328-339
28. Kruijer, W., Nicolas, J. C., van Scheik, F. M. A., and Sussenbach, J. S. (1983) Virology 124, 425-433
29. Stillman, B. W., White, E., and Grodzicker, T. (1984) J. Virol. 50, 598-605
30. Pritch, G., and Stillman, B. W. (1986) J. Virol. 57, 883-892
31. Kruijer, W., van Schaik, F. M. A., and Sussenbach, J. S. (1982) Nucleic Acids Res. 10, 4493-4500
32. Vos, H. L., van der Lee, F. M., Reemst, A. M. C. B., van Loon, A. E., and Sussenbach, J. S. (1988) Virology 163, 1-10
33. Berg, J. M. (1990) J. Biol. Chem. 265, 6513-6516
34. van der Vliet, P. C., Levine, A. J., Ensinger, M. J., and Ginsberg, H. S. (1975) J. Virol. 15, 348-354
35. Krevolin, M. D., and Horwitz, M. S. (1987) Virology 156, 167-170
36. Meyers, M. L., Keating, K. M., Roberts, W. J., Williams, K. R., Chase, J. W., and Horwitz, M. S. (1990) J. Biol. Chem. 265, 5875-5882
37. Stunnenberg, H. G., Lange, H., Philipson, L., van Miltenberg, T. R., and van der Vliet, P. C. (1988) Nucleic Acids Res. 16, 2341-2444
38. Rivera, M. J., Smits, M. A., Quint, W., Schoenmakers, J. G. G., and Konings, R. H. (1979) Nucleic Acids Res. 5, 2895-2912
39. Tamanoi, F., and Stillman, B. W. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2221-2225
40. Schechter, N. M., Davies, W., and Anderson, C. W. (1980) Biochemistry 19, 2802-2810
41. Dunsworth-Browne, M., Schell, R. E., and Berk, A. J. (1980) Nucleic Acids Res. 8, 545-554
42. Green, M., and Wold, W. S. M. (1979) Methods Enzymol. 58, 425-435
43. Ikeda, J.-E., Enomoto, T., and Hurwitz, J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 884-888
44. Maizel, J. V., Jr. (1971) Methods Virol. 5, 170-246
45. Field, J., Gronostajski, R. M., and Hurwitz, J. (1984) J. Biol. Chem. 259, 9487-9495
46. Neale, G. A. M., and Kitchingman, G. R. (1989) J. Biol. Chem. 264, 3153-3159
47. Klein, H., Maltzman, W., and Levine, A. J. (1979) J. Biol. Chem. 254, 11051-11060
48. Reich, N. C., Sarnow, P., Duprey, E., and Levine, A. J. (1983) Virology 128, 490-484
49. Mannatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 382-389, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
50. van Amerongen, H., van Grondelle, R., and van der Vliet, P. C. (1987) Biochemistry 26, 4646-4652
51. Carter, T. H., Nicolau, P.-C., Young, C. S. H., and Fisher, P. B. (1982) Virology 117, 619-621
52. Kuil, M. E., van Amerongen, H., van der Vliet, P. C., and van Grondelle, R. (1988) Biochemistry 27, 9795-9800
53. Cleet, P. H., and Hay, R. T. (1989) EMBO J. 8, 1841-1848
54. Kuil, M. E., and Hurwitz, J. (1988) J. Biol. Chem. 263, 9809-9817
55. Brayer, G. D., and McPherson, A. (1983) J. Mol. Biol. 169, 565-596
56. de Jong, E. A. M., van Duynhoven, J. P. M., Harmen, B. J. M., Teijster, G. L., Konings, R. N. H., and Hilbers, C. W. (1987) J. Mol. Biol. 206, 135-152
57. Nevins, J. R., and Winkler, J. J. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1893-1897
58. Babich, A., and Nevins, J. R. (1981) Cell 26, 371-379
59. Nicolas, J. C., Sarnow, P., Girard, M., and Levine, A. J. (1983) Virology 126, 225-239