Functional Characterization of Thermolabile DNA-Binding Proteins That Affect Adenovirus DNA Replication

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Received 1 October 1985/Accepted 5 December 1985

The human adenovirus type 2 (Ad2) mutant Ad2*ts*111 has previously been shown to contain two mutations which result in a complex phenotype. Ad2*ts*111 contains a single base change in the early region 1B (E1B) 19,000-molecular-weight (19K) coding region which yields a *cyt deg* phenotype and another defect which maps to the E2A 72K DNA-binding protein (DBP) coding region that causes a temperature-sensitive DNA replication phenotype. Here we report that the defect in the Ad2*ts*111 DBP is due to a single G→T transversion that results in a substitution of valine for glycine at amino acid 280. A temperature-independent revertant, Ad2*ts*111R10, was isolated, which reverts back to glycine at amino acid 280 yet retains the *cyt* and *deg* phenotypes caused by the 19K mutation. We physically separated the two mutations of Ad2*ts*111 by constructing a recombinant virus, Ad2*ts*111A, which contained a wild-type Ad2 E1B 19K gene and the gly→val mutation in the 72K gene. Ad2*ts*111A was *cyt*⁺ *deg*⁺, yet it was still defective for DNA replication at the nonpermissive temperature. The Ad2*ts*111 DBP mutation is located only two amino acids away from the site of the mutation in Ad2⁺ND1*ts*23, a previously sequenced DBP mutant. Biochemical studies of purified Ad2⁺ND1*ts*23 DBP showed that this protein was defective for elongation but not initiation of replication in a cell-free replication system consisting of purified Ad polymerase, terminal protein precursor, and nuclear factor I. Ad2⁺ND1*ts*23 DBP bound less tightly to single-strand DNA than did Ad2 DBP, as shown by salt gradient elution of purified DBPs from denatured DNA cellulose columns. This decreased binding to DNA was probably due to local conformational changes in the protein at a site that is critical for DNA binding rather than to global changes in protein structure, since both the Ad2⁺ND1*ts*23 and Ad2 DBPs showed identical cleavage patterns by the protease thermolysin at various temperatures.

The adenovirus DNA-binding protein (DBP) is a multifunctional phosphoprotein with an apparent molecular weight of 72,000 (72K protein) that is encoded by early region 2A (E2A) (62.6 to 67.0 map units) of the virus genome (28). A number of DBP mutants and second-site revertants have been used to show that the protein plays a functional role in (i) viral DNA replication (13, 44, 47, 48), (ii) control of viral gene expression at both transcriptional (33) and post-transcriptional (5) levels, and (iii) enhanced growth of adenovirus in monkey cells (21).

The first function to be assigned to the DBP was its role in DNA replication. Temperature-sensitive DBP mutants which exhibit reduced viral DNA synthesis at the nonpermissive temperature have been isolated and extensively characterized *in vivo* (for a review, see reference 41). In addition, the development of a system that is capable of replicating adenovirus DNA *in vitro* has facilitated biochemical studies of the role of the DBP in viral DNA synthesis (9). The results of combined *in vivo* and *in vitro* studies have led to the conclusion that the DBP is required for elongation and efficient initiation of adenovirus DNA synthesis (see reference 42 for a review).

When human KB cells are infected by adenovirus type 5 (Ad5) *ts*125, a known DBP mutant, and subsequently incubated at the nonpermissive temperature, increased expression of a number of virus early gene products occurs (8). Studies to determine the mechanism of control of gene expression by the DBP have shown that the DBP affects the stability of E1 mRNAs (5) and the level of transcription of region E4 (33) in virus-infected cells. Furthermore, Handa et al. (14) have demonstrated that transcriptional inhibition by

DBP can be reproduced *in vitro*. They have shown that transcription from linearized plasmids that contain the E4 promoter is specifically inhibited by the addition of purified DBP to a whole-cell transcription extract.

Another function of the DBP is revealed by a series of host range (*hr*) mutants with defects that map within the 5' half of the DBP coding region, thereby allowing adenovirus to grow efficiently in monkey cells (1, 7, 21). In fact, all nine independently isolated *hr* mutants that grow well in monkey cells have the identical defect, a substitution of tyrosine for histidine at amino acid 130 (2, 7, 24). The block to adenovirus growth in monkey cells is due to reduced expression of a number of late gene products, especially the mRNA encoding late protein IV (fiber), which exhibits altered splicing patterns in adenovirus-infected CV1 monkey cells (3). This block can be overcome either by coinfection with simian virus 40 (37), by nondefective hybrid viruses which contain the carboxy terminus of the simian virus 40 T antigen integrated into the adenovirus genome (10), or by the previously mentioned host range mutants.

Physical and genetic evidence suggests the existence of two independent functional domains within the DBP. The two domains can be separated by mild chymotrypsin digestion, which results in a highly phosphorylated 27K amino-terminal fragment and a nonphosphorylated 44K carboxy-terminal fragment that retains DNA-binding activity (20). Other studies have determined that the 44K chymotryptic fragment is nearly as active as the wild-type protein in *in vitro* elongation assays, thereby defining the DBP domain that is required for replication activity (4, 13). All DBP mutants that are defective for replication have alterations that map within this fragment.

Ad2⁺ND1*ts*23 was isolated by J. F. Williams (personal

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communication) as a temperature-sensitive, replication-defective virus after mutagenesis of Ad2⁺ND1, a nondefective adenovirus-simian virus 40 hybrid which grows well in monkey cells. The DBP gene of Ad2⁺ND1ts23 has been completely sequenced, revealing a single C→T transition which results in the substitution of phenylalanine for leucine at amino acid 282 (25). The replication defect of this virus has not been characterized previously in vitro, but previous studies have suggested that Ad2⁺ND1ts23 is defective for initiation of replication at the nonpermissive temperature in vivo (47).

Ad2ts111 is a nitrous acid-induced mutant that was originally isolated by Martin et al. (30) and has subsequently been shown to contain two independent mutations (11, 44). Ad2ts111 has a temperature-sensitive DNA replication defect as well as a temperature-independent defect which results in a complex phenotype, including an increased cytopathic effect (*cyt*), as well as the induction of cellular DNA degradation (*deg*). It has been shown previously that the *cyt* and *deg* phenotypes are the result of a single base substitution in the E1B 19K tumor antigen coding region (46), while the temperature-sensitive replication phenotype is due to a defective DBP (44).

We extended studies of the replication defect in Ad2ts111 by mapping the DBP mutation at the nucleotide level and isolating a revertant of the DBP mutation. The DBP mutation affecting DNA replication was separated from the E1B 19K mutation by construction of a recombinant virus which contains the Ad2ts111 DBP mutation and a wild-type Ad2 19K protein coding region. Since the Ad2ts111 DBP mutation maps only two amino acids away from the Ad2⁺ND1ts23 mutation, we also biochemically characterized the replication defect of the Ad2⁺ND1ts23 DBP, together with its ability to bind to single-strand DNA. These studies on the structure and function of the DBP should complement other studies to determine the three-dimensional structure of this protein (46).

MATERIALS AND METHODS

Cells and viruses. HeLa cells were propagated both in monolayers and in suspension cultures; 293 cells were grown in monolayers. Ad2ts111 was originally obtained from J. Sussenbach (State University of Utrecht, The Netherlands). Ad2⁺ND1ts23 was obtained from J. F. Williams (Carnegie-Mellon University, Pittsburgh). Ad2 was grown in HeLa suspension cultures at 37°C, while Ad2ts111 and Ad2⁺ND1ts23 were grown at 32.5°C. Plaque assays were performed at both the permissive (32.5°C) and nonpermissive (38.5°C) temperatures. Ad2ts111R10 was isolated by passaging Ad2ts111 twice in HeLa cells at 38.5°C followed by plaque purification on 293 cells. Individual plaques were grown in HeLa cells at 38.5°C and screened for the desired phenotypes by examination of *SacI*-digested Hirt supernatant DNAs. Ad2ts111A was constructed by using the calcium phosphate precipitation method to transfect gel-purified Ad2ts111 *SalI* A fragment and Ad2 *EcoRI* A fragment (1 µg of each plus 8 µg of carrier DNA) into 293 cells at 32.5°C. Recombinant viruses were harvested, passaged once on 293 cells at 32.5°C, and plaque purified. Purified plaques were screened for temperature sensitivity by performing plaque assays on 293 cells at 32.5 and 38.5°C. Plate lysates of temperature-sensitive viruses were further screened by examination of *SacI*-digested Hirt supernatants.

Cloning and sequencing of E2A gene. The following plasmids were constructed for the purpose of sequencing por-

tions of the DBP coding region of Ad2ts111. Plasmid pGP2 contains the Ad2ts111 *SphI* F fragment (63.6 to 70.0 map units) cloned into the *SphI* site of pBR322. The *HindIII*-*BglII* fragment of this plasmid (which contains sequences from 63.6 to 64.2 map units of Ad2) was ligated to the *HindIII*-*BamHI* fragment of mp9. The *BglII*-*PstI* fragment (64.2 to 64.9 map units) of pGP2 was ligated to the *BamHI*-*PstI* fragment of mp18. The pGP2 *PstI*-*XhoI* fragment (64.9 to 66.6 map units) was ligated to the *PstI*-*SalI* mp18 fragment. Analogous constructs were also made with Ad2 and Ad2ts111R10 DBP gene sequences. The *SphI*-*BglII*, *BglII*-*PstI*, and part of the *PstI*-*XhoI* fragments were then sequenced by the Sanger et al. (39) dideoxy termination procedure.

Isolation of viral DNA. Viral DNA was isolated by the procedure of Hirt (16). Hirt supernatants were then extracted with phenol and chloroform-isoamyl alcohol (24:1 [vol/vol]), followed by ethanol precipitation and digestion with 20 µg of RNase A per ml.

Protein purification. DBP was purified by the procedure of Schechter et al. (40). Adenovirus terminal protein precursor (pTP) and adenovirus polymerase (Adpol) were purified as described previously (43). The native DNA cellulose fraction was used for in vitro replication assays. Nuclear factor I was purified, essentially as described by Nagata et al. (31), by using a nitrocellulose filter-binding assay described by J. Diffley and B. W. Stillman (submitted for publication). The denatured DNA cellulose fraction was used for in vitro replication assays.

Thermolysin digestion of DBP. Purified DBP was digested by the protease thermolysin (Sigma Chemical Co.) for 30 min in a 20-µl reaction mixture containing 1.5 ng of thermolysin, 1.5 µg of DBP, 10 mM Tris (pH 8.0), 2 mM CaCl₂, 0.1 mM EDTA, 1.4 mM 2-mercaptoethanol, 5% glycerol, and 200 mM KCl. Reactions were terminated by adding EDTA to a final concentration of 10 mM. The products of the reaction were then subjected to electrophoresis through a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and stained with Coomassie blue (26).

Replication assays. In vitro 80K initiation assays and 88K partial elongation assays contained 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.5), 5 mM MgCl₂, 2 mM dithiothreitol, 3 mM ATP, 60 ng of Ad-terminal protein complex, 70 ng of pTP-Adpol, 0.4 µg of nuclear factor I, 0.85 µM α³²P-dCTP, 40 µg of aphidicolin per ml, and 1.25 µg of DBP that had been preincubated for 30 min at the reaction temperature (either 30 or 39.5°C). In addition, each 80K assay contained 40 µM di-dATP, while each 88K assay contained in addition 10 µM dATP, 10 µM dTTP, and 40 µM di-dGTP. Reaction mixtures (20 µl) were incubated at either 30 or 39.5°C for 1 h, and then they were stopped by addition of EDTA to 25 mM and sodium pyrophosphate to 90 mM. Products were precipitated by trichloroacetic acid, washed with acetone, and subjected to electrophoresis through a 10% SDS-polyacrylamide gel, followed by autoradiography on Kodak XAR film.

Elongation assays contained 25 mM HEPES (pH 7.5), 5 mM MgCl₂, 4 mM dithiothreitol, 3 mM ATP, 40 µM dATP, 40 µM dGTP, 40 µM dTTP, 10 µM dCTP, 200 µg of bovine serum albumin per ml, 60 ng of *SphI*-digested Ad-protein complex as template, 70 ng of pTP-Adpol, 0.4 µg of nuclear factor I, 0.1 µM α³²P-dCTP, and 1.25 µg of DBP that was preincubated for 30 min at the reaction temperature. Reaction mixtures (20 µl) were incubated for 1 h at either 30 or 39.5°C. The products were then incubated for 1 h in the

FIG. 1. Nucleotide sequence analysis of Ad2, Ad2ts111, and Ad2ts111R10 DBP coding region. (A) Sequencing gels of the same portion of the DBP gene (nucleotides 23,206 to 23,257) from Ad2, Ad2ts111, and Ad2ts111R10. Circled letters indicate the base that is altered in Ad2ts111 (nucleotide 23,241). (B) Nucleotide sequence of the region surrounding the sites of the Ad2ts111 and Ad2⁺ND1ts23 mutations and the deduced amino acid sequence of the DBP. The nucleotide substitutions found in these mutants and the corresponding amino acid substitutions are indicated by arrows.

presence of 0.12% SDS and 0.37 mg of pronase per ml, followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1), spin dialysis, gel electrophoresis through a 0.8% agarose gel, and autoradiography.

RESULTS

Identification of Ad2ts111 DBP mutation and characterization of a revertant. Previous genetic and biochemical studies have shown that the temperature-sensitive replication defect of Ad2ts111 was due to the presence of a defective DBP in this virus (44). Analysis of Ad2-Ad5 restriction site polymorphisms in Ad2cyt106, a temperature-independent recombinant between Ad2ts111 and Ad5 that contains a hybrid Ad2-Ad5 DBP, had indicated that the mutation in the Ad2ts111 DBP must lie to the left of the *Xho*I site (at 66.6 map units), which lies within the amino terminus of the DBP coding region (49). Subsequent marker rescue experiments have mapped the mutation between the *Bgl*II site at 64.2 and the *Sma*I site at 66.6 map units (11). We therefore set out to sequence part of the DBP coding region between the carboxy terminus (at 62.6 map units) and the *Xho*I site at 66.6 map units. This was accomplished by ligating portions of the Ad2ts111 DBP gene into the appropriate M13 vectors and nucleotide sequencing by the Sanger et al. (39) dideoxy termination procedure (see Materials and Methods for details). We sequenced more than 600 base pairs between the *Sph*I site at 63.6 map units and the *Xho*I site at 66.6 map units and found complete agreement with the previously published Ad2 DBP sequence, except for a single G→T transversion at nucleotide 23,241 in Ad2ts111 that was not

found in the wild-type gene (Fig. 1A). This mutation would result in the substitution of a valine for glycine at amino acid 280.

To show that this mutation was indeed the cause of the temperature-sensitive replication phenotype, we selected revertants and plaque purified them after two passages of Ad2ts111 on HeLa cells at 38.5°C as described in Materials and Methods. One temperature-independent virus, Ad2ts111R10, was isolated, which grew well at the nonpermissive temperature yet retained the *cyt* and *deg* phenotypes. Extrachromosomal DNA prepared by a modification of the method of Hirt from HeLa cells that had been infected with Ad2, Ad2ts111, or Ad2ts111R10 showed the reduced levels of viral DNA and the degradation of cellular DNA at 39.5°C that are typical of Ad2ts111 infection (Fig. 2, Ad2ts111 lane 2A). The revertant, on the other hand, retained the *deg* phenotype, as evidenced by the smear of ethidium bromide-stained DNA (Fig. 2, Ad2ts111R10 lane 2B), yet produced wild-type levels of viral DNA at the restrictive temperature (Fig. 2, Ad2ts111R10 lane 2A). That this was in fact an Ad2ts111 revertant was supported by the presence in Ad2ts111R10 of the Ad2ts111 *Sac*I restriction pattern (Fig. 2; compare lanes 2A of Ad2, Ad2ts111, and Ad2ts111R10). Ad2 and Ad2ts111 differ in their *Sac*I restriction patterns because the Ad2ts111 19K mutation results in loss of a *Sac*I site at 4.9 map units and fusion of the *Sac*I D and F fragments in Ad2ts111 (49).

Phenotypic reversion back to wild type can occur by a number of mechanisms. One possible mechanism is simple reversion back to wild-type sequence at the original mutation site. We therefore sequenced the *Bgl*II-*Pst*I fragment

FIG. 2. Characterization of Ad2ts111 temperature-independent revertant. Ad2ts111R10 was isolated as described in Materials and Methods. HeLa cells were infected with either Ad2, Ad2ts111, or Ad2ts111R10 at a multiplicity of infection of 5 PFU per cell. Cells were harvested after 60 h of incubation at 32.5°C (lanes 1) or after 48 h of incubation at 39.5°C (lanes 2), and Hirt supernatant DNAs were isolated. (A) Hirt supernatant DNA was digested by *SacI*, and the products were subjected to electrophoresis through a 1% agarose gel containing 0.5 μ g of ethidium bromide per ml. *SacI* fragments are indicated to the left of the panel. The fragment indicated by the asterisk is the fused *SacI* D + F fragment caused by the loss of the *SacI* site at 4.9 map units in Ad2ts111 and its derivatives. (B) Undigested Hirt supernatant DNAs showing more clearly the degradation of cellular DNA in Ad2ts111- and Ad2ts111R10-infected HeLa cells.

(64.2 to 64.9 map units) of Ad2ts111R10 to determine whether this virus was indeed a primary-site revertant. Fig. 1A shows that Ad2ts111R10 contains a T→G transversion at the Ad2ts111 mutation site, resulting in a return back to wild-type Ad2 sequence.

Ad2ts111 replication defect is separable from the 19K mutation. Since Ad2ts111 contains mutations in two different genes, it was necessary to physically separate the two mutations to show that the Ad2ts111 temperature-sensitive replication defect was independent of the Ad2ts111 19K mutation. This was done by cotransfecting 293 cells with the *EcoRI* A fragment (0 to 59.4 map units) from Ad2 (which contains a wild-type 19K gene) and the *SalI* A fragment (46.6 to 100 map units) from Ad2ts111 (which contains the mutant DBP gene). This procedure requires that recombination between the two fragments occurs in vivo to produce intact viral genomes. Temperature-sensitive viruses generated by recombination of the two fragments were plaque purified, and one virus, which we named Ad2ts111A to indicate that it is a derivative of Ad2ts111, was further characterized. Extrachromosomal DNA was isolated by the procedure of Hirt (16) from cells infected with Ad2, Ad2ts111, or

Ad2ts111A. Viral DNA synthesis in the recombinant Ad2ts111A was dramatically lower at the nonpermissive temperature relative to Ad2 (Fig. 3; compare lanes 2A of Ad2ts111 and Ad2ts111A). The *SacI* restriction pattern and the lack of degradation of cellular DNA demonstrated that this was in fact a recombinant and eliminated the possibility that this was merely recovery of intact Ad2ts111 or Ad2 during the transfection procedure.

Ad2⁺ND1ts23 DBP binds less tightly to single-strand DNA.

When the site of the DBP mutation in Ad2ts111 was compared with that of known DBP mutants, it was found to lie only two amino acids away from the site of the mutation in Ad2⁺ND1ts23 (see Fig. 1 and 9). Ad2⁺ND1ts23 is a nondefective virus isolated by J. F. Williams (personal communication) after mutagenesis of the Ad2-simian virus 40 hybrid Ad2⁺ND1. The temperature-sensitive replication of Ad2⁺ND1ts23 has been investigated to some extent in vivo, identifying the DBP as the cause of the temperature-sensitive phenotype (47), but there has been very little biochemical characterization of the Ad2⁺ND1ts23 DBP. We therefore set out to characterize the Ad2⁺ND1ts23 mutant protein in more detail. The Ad2⁺ND1ts23 DBP was purified by the procedure described by Schechter et al. (40). In this procedure, the final step of purification required binding the DBP to denatured DNA cellulose in 0.1 M NaCl, washing the column with 0.5 M NaCl, and, finally, eluting the DBP with 2 M NaCl. When this procedure was performed to purify the Ad2⁺ND1ts23 DBP, the mutant DBP bound to the

FIG. 3. Separation of Ad2ts111 replication defect from the Ad2ts111 E1B 19K mutation. HeLa cells were infected with Ad2, Ad2ts111, or Ad2ts111A at a multiplicity of infection of 5 PFU per cell, followed by incubation for 60 h at 32.5°C (lanes 1), or 48 h at 39.5°C (lanes 2). *SacI*-digested (A) or undigested (B) Hirt supernatant was then subjected to electrophoresis through a 1% agarose gel containing 0.5 μ g of ethidium bromide per ml. The *SacI* fragments and the *SacI* D + F fusion fragment (*) are indicated to the left.

column in 0.1 M NaCl but unexpectedly eluted from the column in the 0.5 M wash. The protein in the final pooled fractions was >90% DBP, with some slight contamination by other proteins owing to the early elution (Fig. 4, lane 1). To show that the DBP was binding less tightly to DNA, we rechromatographed the purified Ad2 and Ad2⁺ND1*ts*23 DBPs on separate denatured DNA cellulose columns at 4°C and eluted them with a 0.25 M/1 M NaCl gradient. Figure 5 shows that the Ad2 protein bound more tightly to the column, with peak fractions eluting from the column at ~600 mM NaCl, while the Ad2⁺ND1*ts*23 DBP was eluted at ≥250 mM NaCl, showing that even at 4°C the Ad2⁺ND1*ts*23 DBP bound DNA less tightly than did wild-type protein. The poor DNA binding of the Ad2⁺ND1*ts*23 DBP was a result of a single amino acid substitution, since the sequence of the remainder of the protein was identical to that of the wild-type DBP (25).

Thermolysin digestion of Ad2 and Ad2⁺ND1*ts*23 DBPs. The mutation in the Ad2⁺ND1*ts*23 DBP could result in reduced binding of DNA by either (i) causing global denaturation, thereby reducing the stability of the protein or (ii) directly altering a specific site that is responsible for DNA recognition without affecting other regions of the protein. One method to demonstrate this difference is to determine the sensitivity of the mutant and wild-type proteins to digestion by the protease thermolysin at various temperatures. Thermolabile proteins that denature at high temperatures should be more sensitive to thermolysin digestion than the corresponding wild-type proteins or mutant proteins that have only local conformational changes. Hecht et al. (15) used this assay to show that mutations in lambda repressor

fall into two categories: (1) those that affect the DNA-binding site, thereby directly lowering the affinity of the repressor for substrate, or (2) those that affect the global conformation of the protein, resulting in a less stable structure. Mutants in the first category are just as stable as the wild-type protein. Mutants that fall into the second category, however, show increased sensitivity to thermolysin digestion relative to the wild-type protein as the digestion temperature is increased. This test of protein stability was applied to the Ad2 and Ad2⁺ND1*ts*23 DBPs (Fig. 6), and the digestion patterns of both proteins were virtually identical at temperatures ranging from 20 to 68°C, suggesting that overall protein structure in the mutant was not dramatically altered as the temperature was increased.

In vitro replication assays with Ad2⁺ND1*ts*23 DBP. Previous in vitro replication studies with either nuclear extracts prepared from cells infected by Ad2*ts*111 or purified Ad5*ts*125 DBP have shown that those proteins are temperature sensitive for elongation but not for initiation of viral DNA replication (13, 19, 44). Initiation of adenovirus replication is defined as covalent attachment of dCMP to the 80K pTP, which is subsequently used as the primer for DNA synthesis (29, 45). Limited elongation can be assayed by adding dATP, dCTP, dTTP, and di-dGTP to the replication reaction. Elongation will proceed up to the first deoxycytidine residue in the template strand at nucleotide 26, at which point termination of the nascent chain will occur owing to incorporation of di-dGMP into the nascent strand (29). The product of this reaction, a pTP-26-mer complex, migrates as an 88K band in SDS-polyacrylamide gels. An in vitro replication system consisting of purified Ad polymerase, pTP, and nuclear factor I, plus either the Ad2 or Ad2⁺ND1*ts*23 DBP was used to assay formation of the 80K initiation complex and the 88K partial elongation product (Fig. 7). In the initiation assay, there was slightly more incorporation of α-³²P-dCMP into the 80K band at the higher temperature in reaction mixtures that contained the Ad2 DBP, while the level of incorporation in reaction mixtures with Ad2⁺ND1*ts*23 DBP was relatively unchanged. In contrast, partial elongation reaction mixtures which contained Ad2⁺ND1*ts*23 DBP at 39.5°C showed decreased incorporation of ³²P-dCMP into the 88K pTP-26-mer band relative to the level observed with the wild-type DBP. These results (Table 1) were quantitated by cutting the appropriate bands out of the gel and by scintillation counting. The results of the elongation assay showed a nearly twofold increase in formation of the 88K pTP-26-mer product in reactions with the Ad2 DBP at 39.5°C relative to 30°C, while incorporation with Ad2⁺ND1*ts*23 DBP showed a fourfold decrease. This net eightfold difference between the activities of the two proteins confirmed that DBP stimulated elongation of adenovirus replication, even at this early stage of the elongation process. The lower set of bands in Fig. 7 were the initiation and partial elongation products of a 60K cleavage product of the pTP.

Another assay for the elongation of replication is the preferential replication of the terminal origin containing fragments of restriction enzyme-digested Ad DNA-protein complex (17). In this assay, Ad-terminal protein complex is digested by *Sph*I before being used as the template for the in vitro reaction. Fig. 8 shows that ³²P-dCMP was specifically incorporated into the terminal *Sph*I D and E fragments and that this incorporation was defective in reactions containing the Ad2⁺ND1*ts*23 DBP at the nonpermissive temperature, while the wild-type DBP was not defective at the nonpermissive temperature.

FIG. 4. Purified adenovirus DBPs. The DBP was isolated by the procedure of Schechter et al. (40) from HeLa cells that had been infected with either Ad2 or Ad2⁺ND1*ts*23. Protein (1 µg) from the Ad2⁺ND1*ts*23 (lane 1) and Ad2 (lane 2) final pooled fractions was subjected to electrophoresis through a 10% SDS-polyacrylamide gel and stained with Coomassie blue. The contaminating protein in the Ad2⁺ND1*ts*23 lane was due to the early elution of the Ad2⁺ND1*ts*23 protein from the DNA cellulose column. Molecular weight markers (lane M) were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500).

FIG. 5. NaCl gradient elution of purified DBPs. Purified Ad2 (100 μ g) (A) or Ad2*ND1ts23 (100 μ g) (B) DBP was applied to a denatured DNA cellulose column in a buffer containing 100 mM NaCl. The protein (●) was then eluted from the column with a 0.25 M/1 M NaCl gradient. The protein content of the fractions was assayed by the method of Bradford (6). The concentration of NaCl (○) was determined by conductivity.

DISCUSSION

The adenovirus DBP is probably the best characterized of the adenovirus proteins, yet much remains to be known about the mechanism of action of this versatile protein. Genetic studies with DBP mutants have attributed a number of functions to this protein (41), while nucleotide sequencing studies of those DBP mutants have provided clues as to which region of the DBP is important for each of its functions. Further detailed characterization of the protein has been made possible by the development of *in vitro* replication and transcription systems to determine the exact role of the protein in these processes.

The DBP is one of five proteins required for the replication of full-length adenovirus DNA *in vitro*. Three of these proteins are encoded within the viral genome: DBP, Ad polymerase, and the Ad pTP. In addition, two host cell proteins are required for the synthesis of full-length adenovirus DNA. One of these host proteins, nuclear factor I, binds to a specific site within the adenovirus origin of replication and stimulates the initiation of viral DNA synthesis (31). In the presence of the pTP-Adpol complex, DBP, nuclear factor I, and Ad terminal protein template, viral DNA synthesis *in vitro* produces molecules that are only about 30% of the length of the viral genome. Addition of the

FIG. 6. Thermostability of purified DBPs. Purified Ad2 (1.5 μ g) or Ad2*ND1ts23 (1.5 μ g) DBP was incubated with 1.5 ng of thermolysin (Sigma) for 30 min at the temperature indicated above each lane. Lanes indicated by a minus sign contained undigested DBP. The reactions were terminated, and the products were subjected to electrophoresis through a 10% SDS-polyacrylamide gel, followed by staining with Coomassie blue. Molecular weight markers are the same as in Fig. 4.

FIG. 7. Initiation and partial elongation assays with purified proteins. Ad2 and Ad2⁺ND1^{ts23} DBPs were preincubated for 30 min at the reaction temperature. Each reaction contained 25 mM HEPES (pH 7.5), 5 mM MgCl₂, 2 mM dithiothreitol, 3 mM ATP, 40 μg of aphidicolin per ml, 60 ng of Ad-terminal protein complex, 70 ng of pTP-Adpol, 0.4 μg of nuclear factor I, 0.85 μM α³²P-dCTP, and 1.25 μg of DBP. In addition, each 80K initiation assay contained 40 μM di-dATP, while each 88K assay contained, in addition, 10 μM dATP, 10 μM dTTP, and 40 μM di-dGTP. After incubation for 1 h at either 30 or 39.5°C as indicated, reactions were terminated by addition of EDTA to 25 mM and sodium pyrophosphate to 90 mM. The products were isolated and subjected to 10% SDS-polyacrylamide gel electrophoresis and autoradiography. Molecular weight markers were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000).

other host-encoded protein, nuclear factor II, allows DNA synthesis to continue, resulting in full-length virus DNA (32). Nuclear factor II has been found to have DNA topoisomerase I activity, and topoisomerase I isolated from calf thymus can substitute for nuclear factor II in adenovirus elongation reactions *in vitro* (32).

The requirement of the Ad DBP in viral DNA replication is well established at this point, mainly owing to the avail-

FIG. 8. Temperature-sensitive elongation with Ad2⁺ND1^{ts23} DBP. Purified Ad2 DBP or Ad2⁺ND1^{ts23} DBP was first preincubated for 30 min at the reaction temperature. Reaction mixtures (20 μl) contained 60 ng of *Sph*I-digested Ad-protein complex, 70 ng of pTP-Adpol, 0.4 μg of nuclear factor I, 1.25 μg of DBP, 0.1 μM α³²P-dCTP, 4 μg of bovine serum albumin, 25 mM HEPES (pH 7.5), 5 mM MgCl₂, 4 mM dithiothreitol, 3 mM ATP, 40 μM dATP, 40 μM dGTP, 40 μM dTTP, and 10 μM dCTP. Reactions were incubated at 30 (lanes 1) or 39.5°C (lanes 2) for 1 h. The reaction products were then incubated for 1 h at 37°C after addition of SDS to 0.12% and pronase to 0.37 mg/ml. The products were extracted once by phenol-chloroform-isoamyl alcohol (25:24:1) and subjected to spin dialysis and electrophoresis through a 0.8% agarose gel followed by autoradiography. The location of *Sph*I fragments A through E are indicated to the left. Fragments D and E are the left and right terminal fragments, respectively. The smaller *Sph*I fragments (F to I) are not visible on this autoradiogram, since little ³²P-dCMP was incorporated into those fragments.

TABLE 1. Incorporation of dCMP into 80K and 88K products in *in vitro* replication assays with purified proteins

Virus DBP and temp (°C)	fmol of dCMP incorporated ^a		
	80K ^b	80K ^c	88K ^c
Ad2			
30	0.4	0.2	0.9
39.5	0.6 (1.5)	0.3 (1.3)	1.7 (1.9)
Ad2 ⁺ ND1 ^{ts23}			
30	0.3	0.2	0.4
39.5	0.3 (1.0)	0.4 (2.0)	0.1 (0.25)

^a Numbers in parentheses indicate ratios between the amounts incorporated at 39.5 and 30°C for each pair of values.

^b Data derived from panels labeled 80K at left of Fig. 7.

^c Data derived from panels labeled 88K at right of Fig. 7.

ability of a cell-free replication system and the large number of available DBP mutants. The DBP is required during the elongation of DNA synthesis, since extracts prepared from cells infected with temperature-sensitive viruses are defective for elongation at the nonpermissive temperature *in vitro*, and this defect can be complemented by the addition of wild-type DBP (13, 19, 44). The role of the DBP in the initiation stage of replication is more complex, however. Although initiation of adenovirus replication, defined as covalent attachment of dCMP to pTP, can occur to some extent in the absence of DBP, the addition of purified DBP to a reaction that contains purified pTP-Adpol, nuclear factor I, and Ad terminal protein template stimulates the formation of the pTP-dCMP complex threefold (31). This indicates that the DBP is not absolutely required for initiation yet still has some role in the initiation reaction. It is not known whether

FIG. 9. Map of adenovirus mutants and intragenic second-site revertants. The sites of known DBP mutations are indicated above the arrow, and the locations of second-site DBP revertants are indicated below the arrow. At the top of the figure is a scale of the 529 amino acids that constitute the DBP. The vertical zig-zag line indicates the proposed chymotrypsin cleavage site. The bottom of the figure shows a summary of the functions that have been assigned to the physical domains of the DBP (*hr*, host range). Compiled from references 2, 7, 22, 24, and 25.

it is a direct role, such a stabilization of single-strand DNA regions at the origin of replication, an indirect role involving interactions with Adpol and nuclear factor I, or even a combination of the first two possibilities. Our results with the Ad2⁺ND1*ts*23 DBP, however, suggest that the first possibility is unlikely, since the Ad2⁺ND1*ts*23 DBP was defective for binding to DNA, yet initiation of replication was virtually unaffected at the nonpermissive temperature with this protein. This result is most consistent with direct protein-protein interactions between the DBP and either Adpol or nuclear factor I at the origin of adenovirus replication.

Mutations that affect the DNA replication function of the DBP are all confined in the 3' half of the gene, and the positions of those mutations suggest where the DNA-binding site of the protein may be located (Fig. 9). In addition, biochemical analyses of defective DBPs presented in this report and elsewhere (44) indicate that the region around amino acids 280 and 282 is probably important for DNA binding. The mutation that alters amino acid 413, and which is common to Ad5*ts*125, Ad5*ts*107, and Ad2*ts*400, results in a DBP that is rapidly dephosphorylated and degraded at the nonpermissive temperature *in vivo* (27, 48). In contrast, the mutation at amino acid 280, which is present in Ad2*ts*111, results in a DBP that is normally phosphorylated and is stable at the nonpermissive temperature yet is still thermolabile *in vivo* and in cell-free replication assays (44). In this report, we showed that the Ad2⁺ND1*ts*23 DBP was also thermolabile for elongation but not for initiation of replication *in vitro* and that the Ad2⁺ND1*ts*23 DBP bound less tightly to single-strand DNA than did the wild-type Ad2 DBP. The Ad2⁺ND1*ts*23 DBP also appeared to be as stable as the wild-type protein, since it exhibited the same digestion pattern as the wild-type Ad2 DBP when incubated with the protease thermolysin at various temperatures. Taken together, these observations suggest that the region around amino acids 280 to 282 is part of a site that directly affects the binding of DBP to single-strand DNA without causing global changes in the protein structure. It is not known whether amino acid 413 is also a part of the binding site, but this should be determined shortly by analysis of the recently crystallized carboxy-terminal fragment of the Ad2 DBP (46),

which should help to determine the three-dimensional structure of this protein.

Although we have not sequenced the entire DBP gene of Ad2*ts*111, we feel confident that the mutation that we have identified is responsible for the temperature-sensitive phenotype, based on the isolation of a temperature-independent revertant which reverts back to wild-type sequence at the original mutation site and previously reported marker rescue experiments which localized the temperature-sensitive replication defect of Ad2*ts*111 to between 64.2 and 66.6 map units within the DBP coding region (11). It may be significant that the glycine residue that is affected by the Ad2*ts*111 DBP mutation is conserved between the four human adenovirus serotypes that have been sequenced thus far in this region (23–25, 36).

It is likely that important functions of the DBP during the normal viral growth cycle involve interactions with cellular proteins. Analyses of DBP mutants that exhibit host range effects with regard to virus assembly (35), autoregulation of DBP levels (34), and possibly splicing of mRNA (3) suggest that the DBP interacts with host proteins that are involved in these processes. It has also been suggested that the DBP may interact with the Ad polymerase (12), since the Ad DBP, but not the *E. coli* single-strand DBP, can stimulate DNA synthesis by the Adpol by using poly(dT) as the template and oligo(rA) or oligo(dA) as the primer, whereas the Ad DBP does not stimulate DNA synthesis by the HeLa DNA polymerase α with the same template and primer. This is reminiscent of the specific interactions that have been reported to occur between procaryotic DNA polymerases and single-strand DBPs (18, 38). The elucidation of the host proteins and the DBP domains that are important for these interactions should contribute greatly to the understanding of the role of this multifunctional protein in the viral growth cycle.

ACKNOWLEDGMENTS

We thank Barbara Faha for excellent technical assistance and John Diffley and Ron Guggenheimer for valuable discussions.

This work was supported by Public Health Service grant AI20460 from the National Institutes of Health and a Rita Allen Foundation

grant to B.S. G.P. is a predoctoral fellow partially supported by training grant GMO7964 from the National Institutes of Health.

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