The adenovirus single-stranded DNA (ssDNA)-binding protein (DBP) is necessary for the elongation step in viral DNA replication. In an attempt to characterize the putative ssDNA-binding domain of the DBP, we purified and characterized the Ad2ts111A DBP, which contains a glycine-to-valine substitution at amino acid 280. This mutation is adjacent to that in the previously studied Ad2*ND1ts23. Ad2*ND1ts23 exhibits a temperature-sensitive defect in DNA replication, and its DBP has previously been shown to bind ssDNA with reduced affinity. Ad2ts111A DBP, like Ad2*ND1ts23, does not support adenovirus DNA replication in vitro at elevated temperatures. However, the Ad2ts111A DBP binds ssDNA more tightly than does Ad2*ND1ts23 and is not temperature sensitive in this function.

To determine the nucleic acid-binding properties of DBP, we applied spectrofluorometric techniques, which had not been used previously to study adenovirus DBP. Using the homopolymer, poly(1,N'-ethenoadenylic acid (poly[rcA]), we have determined that the binding site size is approximately 16 nucleotides. In 20 mM NaCl, the Ad2wt, Ad2ts111A, and Ad2*ND1ts23 DBP proteins all bound stoichiometrically to poly[rcA] with overall apparent affinities above 10^6 M^-1. Based on titrations carried out at higher salt concentrations, however, the stability of these complexes did appear to increase in the order Ad2*ND1ts23 < Ad2ts111A < Ad2wt. By these techniques, we have confirmed also that the DBP of another temperature-sensitive mutant, H5ts107, like the Ad2ts111A DBP, retains its ability to bind ssDNA even at a restrictive temperature utilizing the salt concentration compatible with adenovirus DNA replication in vitro. The H5ts107 DBP, which contains an amino acid substitution at position 413, is defective for in vitro replication at nonpermissive temperature but is not temperature sensitive for binding to ssDNA.

In summary, our results indicate that the replication defects of the Ad2ts111A are similar to those of H5ts107 and cannot be attributed to defective, nonspecific ssDNA binding by the DBP. It appears that ssDNA binding by itself is not sufficient to account for the role of DBP in adenovirus DNA replication.

The adenovirus (Ad)1 single-stranded (ss) DNA-binding protein (DBP) is a multi-functional protein that is required for 1) viral DNA replication (van der Vliet et al., 1975; Horwitz, 1978; Friefeld et al., 1983), 2) regulation of viral gene expression at both transcriptional (Carter and Blanton, 1978; Nevino and Winkler, 1980) and post-transcriptional levels (Babich and Nevis, 1981), 3) determination of host range for viral infectivity (Klessig and Grodzicker, 1979), and 4) viral assembly (Nicolas et al., 1983). DBP was isolated initially from adenovirus-infected cells based upon its ability to bind to ssDNA (van der Vliet and Levine, 1973). Studies of the roles of DBP in viral DNA synthesis have been facilitated by the development of in vitro DNA replication systems (Horwitz, 1978; Challberg and Kelly, 1979; Friefeld et al., 1983), which have allowed a more detailed analysis of the individual protein requirements at each of the different stages of the replicative reaction. In vitro analysis of temperature-sensitive DBP mutants has provided genetic and biochemical evidence that DBP is essential for elongation of nascent adenovirus DNA but not for initiation of replication (Kaplan et al., 1979; Friefeld et al., 1983).

Although DBP migrates on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) with a mobility that corresponds to a 72,000 dalton protein, it is a 529-amino acid protein which has a molecular mass of 59,049 daltons (Kruijer and Winkler, 1980; Friefeld et al., 1983; Krevolin and Horwitz, 1987). Limited proteolysis with a variety of enzymes has indicated that the DBP is composed of two relatively discrete structural
domains; chymotryptic digestion indicates that the amino-terminal and carboxyl-terminal domains consist of approximately 173- and 356-amino acid residues, respectively (Tsernoglou et al., 1985). Chymotryptic digestion produces nester 44- and 34-kDa fragments, representing the carboxyl-terminal domain; each fragment is capable of binding DNA and is active in the in vitro complementation of adenosivirus DNA replication (Ariga et al., 1980).

The isolation of conditional mutants and second site revertants, mapping within the carboxyl-terminal region has been important in establishing the relationships between the structure and function of DBP. All mutations in DBP which affect viral DNA replication have been localized to the carboxyl region. The localization of these mutations to discrete clusters within this domain has suggested sites which may be involved in the DNA replication process. H5ts125 and H6ts107 are independently isolated replication-defective mutants which have identical serine-for-proline substitutions at amino acid residue 113 of the DBP (Kruijer et al., 1983). The H5ts125 DBP has been reported to exhibit decreased binding to ssDNA-cellulose, but this altered phenotype was elicited at a relatively high salt concentration (0.25 M) (van der Vliet et al., 1975). Analysis of H5ts107 has suggested that the capacity to bind DNA is retained at nonpermissive temperatures at the salt concentration (20 mM) used during the in vitro synthesis reaction (Krevolin and Horwitz, 1987). However, the complementing activity of the H5ts107 and H5ts125 DBPs for in vitro replication is unequivocally defective at elevated temperatures. This result suggested that the replication defect in H5ts107 is not the result of impaired DNA binding by the mutant DBP.

Recently, studies describing mutations located toward the NH2-end of the carboxyl domain have been reported. Purification and biochemical characterization of the Ad2+ND1ts23 DBP (substitution of phenylalanine for leucine at amino acid 282 (Kruijer et al., 1982)) suggested a linkage in this mutant between its defective DNA replication activity and its reduced affinity to bind single-stranded DNA even at permissive temperature (Frellich and Stillman, 1986). The defective DNA binding of the Ad2+ND1ts23 DBP has resulted in speculation that the mutation is localized to the binding site. Another DBP mutant, Ad2ts111A, maps only 2 amino acid residues away from that in Ad2+ND1ts23 and contains a valine substitution for glycine at position 280, as demonstrated by nuclease sequencing (Frellich and Stillman, 1986).

In this report, the purification of Ad2ts111A DBP as well as its effect on replication activity and binding to single-stranded DNA are presented. Although the alteration in Ad2ts111A is located very close to that in Ad2+ND1ts23 and neither will support adenosivirus DNA replication at elevated temperatures in several in vitro assays, each mutant DBP has distinct properties in the nucleic acid-binding studies. In addition, we describe the application of fluorescence spectroscopy techniques, similar to those previously used to study protein-nucleic acid interactions involving the Escherichia coli SSb and T4 gene 32 protein (gp32), for determining the ssDNA-binding properties of DBP and its mutant forms.

MATERIALS AND METHODS

Cells and Viruses—Wild-type Ad2 infected HeLa S3 cell cultures were incubated at 37 °C and harvested for virus purification at 48-72 h post infection; cells infected with temperature-sensitive mutants of adenosivirus were kept at 32-33 °C (permissive temperature) for 5-6 days (Horwitz, 1978). Ad2, H5ts107, and Ad2ts111A viruses were purified as previously described (Horwitz, 1978; Maizel et al., 1968; Green and Pina, 1984). Ad2ts111A and Ad2+ND1ts23 were generously provided by B. Stillman (Cold Spring Harbor Laboratory).

Purification of DBP—DBPs were prepared from 8-liter suspensions of infected HeLa cells (5 × 106 cells/ml). For the preparation of temperature-sensitive DBPs, infected cells were incubated for 6 days at 32 °C prior to harvesting; for Ad2 wild-type DBP, infected cells were incubated for 40-48 h at 37 °C. Ad2 wild-type and H5ts107 DBP were purified by a previously described method (Seebelter et al., 1980), which was modified as detailed below. Because it was observed that a significant quantity of wild-type DBP eluted from the ssDNA-cellulose column when the column was washed with buffer containing 0.1 M NaCl, a 10-column volume (150 ml) 0.2-0.5 M NaCl gradient was used instead. Subsequently, the bulk of the DBP was eluted with 2.0 M NaCl and 0.01 M Tris-HCl, pH 8.0, in 3 ml fractions. Fractions containing the DBP were identified by SDS-PAGE (Laemmli, 1970); protein bands were visualized by staining with Coomassie Blue R-250.

The method used for the initial purification of Ad2ts111A DBP was modified to account for the unknown characteristics of this protein, particularly with respect to its elution from ssDNA-cellulose. The solubilized ammonium sulfate fraction containing the DBP, dialyzed against 0.05 M NaCl and 0.01 M Tris-HCl, pH 8.0, was loaded onto a phosphocellulose column (7.5 ml) equilibrated against the same buffer. The protein was eluted with a 20-column volume (150 ml), 0.05-0.90 M linear NaCl gradient, collected in 3 ml fractions. The Ad2ts111A DBP eluted between 0.30-0.35 M NaCl. The fractions containing maximal amounts of DBP were pooled, and the salt concentration was reduced to 0.1 M by dialysis. The DBP pool was loaded onto a 4-column volume (15 ml) sephadex column (9/300) equilibrated with 0.1 M NaCl and 0.01 M Tris-HCl, pH 8.0, and eluted with a 20-column volume (300 ml), 0.1-1.0 M linear NaCl gradient, collected in 6 ml fractions. Fractions containing DBP were identified by SDS-PAGE.

Purified DBP preparations were concentrated by vacuum dialysis against 2 M NaCl and 0.01 M Tris-HCl on ice using a Schleicher & Schuell membrane. Concentrated DBP was subsequently dialyzed against TMEG (0.05 M Tris-HCl, pH 7.5, 1 mM 2-mercaptoethanol, 1 mM NaEDTA, and 10% glycerol) containing either 0.5 M NaCl or no added NaCl. Concentrated DBP was stored at −70 °C, which prevents most of the rapid degradation found at 4 °C, even in 2 M NaCl.

Protein concentrations were determined by amino acid analysis on a Beckman 6300 analyzer.

In Vitro Assay for Adenovirus-specific DNA Replication—The in vitro "end fragment" assay for origin-specific adenovirus DNA replication has been described previously (Horwitz and Ariga, 1981). The terminal fragments of adenovirus DNA-pro (adenovirus DNA with the 5'-covalently-linked 55-kDa terminal protein (TP)) contain the binding sites for nuclear factors I and III. Preferential labeling of these fragments is a measure of specific adenovirus DNA synthesis. Reactions utilized adenovirus DNA-pro prepared from Ad35 virions (Sharp et al., 1976), which is fully functional under the specified assay conditions when compared with Ad2 DNA-pro (Valderrama-Leon et al., 1979). The adenovirus DNA-pro (0.1-0.5 µg) was incubated with the restriction endonuclease Smal (5 units) for 2 h at 37 °C prior to addition of the replication reaction mixtures. Each reaction mixture (50 µl) contained 25 mM HEPES, pH 7.5, 5 mM MgCl2, 0.5 mM dithiothreitol, 3.75 mM ATP, 0.05 mM each of dATP,-dGTP, and dCTP, 1.5 mM (α-32P)TP, DBAPE-purified fraction of nuclear extract from uninfected HeLa cells, adenovirus pol/TP extracts (pTP is the 80 kDa precursor to the 55-kDa TP), as well as DBP as specified for individual reactions. Demonstration of the temperature-sensitive properties of mutant DBPs required preincubation of DBP at the specified reaction temperature for 30-60 min before initiation of the reaction by addition of the other components. After initiation of reaction, the reaction mixture was incubated for 40-60 min at 30 °C and was then stopped by addition of 0.2% SDS. Pronase (4 µg) was added, and the mixture was incubated at 37 °C for 45 min to remove pTP covalently bound to the labeled DNA end fragments. After precipitation of the DNA in ethanol and subsequent solubilization in 40 µl of TBE dye (0.09 M Tris-borate buffer, pH 8.5, 2.5 mM EDTA, 0.05% bromphenol blue, 0.025% xylene cyanol) (Maniatis et al., 1982), the labeling of the terminal Smal DNA fragments (B and G) was analyzed by electrophoresis on a 0.7% agarose gel in Lowing buffer (36 mM Tris, 30 mM NaH2PO4, 1 mM EDTA, pH 7.8) (Maniatis et al., 1982), followed by washing, drying, autoradiography of the gel, and autoradiography of the gel with the aid of film.
8 &mgr;d [&gamma;32P]dATP (approximately 2000 cpm/pmol), and the indicated amounts of DBP. DBP was preincubated at the designated reaction temperature for 30 min with the reaction components, excluding the template-primer and adenovirus pol. The poly(dT) (0.35 &mgr;d) and oligo(dA) (0.33 &mgr;d) were incubated at 65 °C for 10 min, then added to the reaction mixture, and allowed to incubate at the reaction temperature for 15 min. The 60-min reaction was initiated by addition of the adenovirus pol. Reactions were terminated by the addition of 0.2 ml of chilled sodium pyrophosphate (0.2 M), 150 &mgr;g each of bovine &gamma;-globulin and yeast tRNA, and 0.5 ml of 20% trichloroacetic acid. The acid-insoluble incorporation of radioactive label was measured by vacuum-drying or liquid scintillation counting.

Fluorescence Spectroscopy of DBP-Nucleic Acid Interaction—Titration were monitored by observing the increase of the fluorescence of poly(1,N7)-ethenoadenine acid (poly(rA)) as the result of binding by DBP. These experiments were performed by adding aliquots of protein to a 0.69 solution of nucleic acid in a starting sample volume of 2 ml. In some experiments, the “salt-back-titration” procedure was performed, in which case saturation of a fixed amount of poly(rA) by the addition of DBP under tight binding conditions was followed by adding a concentrated NaCl solution to dissociate the DBP-nucleic acid complex. Dissociation of the complex was monitored by measuring the resulting decrease in poly(rA) fluorescence. Tris-HCl (10 mM), pH 8.0, Na2EDTA (0.1 mM) was used as the buffer in all experiments, with the salt concentration as specified for individual experiments. Poly(rA) was purchased from Pharmacia LKB Biotechnology Inc. Fluorescence of poly(rA) was monitored using the SLM 8000 and SLM 8000C (SLM Urbana, IL) spectrofluorometers with an excitation wavelength of 309 nm and an emission wavelength of 406 nm. The SLM 8000C was controlled and data acquisition processed by a user-defined language executed on an IBM PC XT. In each fluorescence titration, the protein concentration was kept below 20 &mgr;d/ml in order to prevent aggregation (van der Vliet et al., 1978). For the calculation of binding site size, the experimental data were analyzed by a nonlinear least-squares technique (Johnson and Frasier, 1985) implemented on a MicroVax II assuming an e25, of 3.7 &times; 104 M⁻¹/mol of phosphate for the poly(rA) (Karpel et al., 1987; Ledneva et al., 1978).

Binding parameters were estimated by visually comparing the experimental binding isotherms with theoretical curves that were generated by the use of equation 15 in McGhee and von Hippel, 1974. Typically, a constant value of the cooperativity parameter, &omega, was assumed and the intrinsic affinity &Ki was allowed to vary until a best fit was obtained. With this constant value of &Ki, the cooperativity parameter was then varied until this parameter was also optimized. The overall apparent affinity, &Kapp, was then calculated as the product of the &Ki and &e.

Regardless of which parameter was being varied, only data points in the range from 0 to 50% maximal fluorescence enhancement were considered in determining the “best fit” as recommended by Kowalczykowski et al. (1981) and Newport et al. (1981).

RESULTS

Purification of Ad2ts111A DBP—When the Ad2”ND1ts23 DBP was purified without modifying the procedure described by Schecter et al. (1980), it eluted from the ssDNA-cellulose column in the 0.5 M NaCl wash and was contaminated by other proteins (Prelich and Stillman, 1986). Because of the possibility that the Ad2ts111A DBP might also elute with the 0.5 M wash and not be sufficiently purified, the DBP was loaded onto the ssDNA-cellulose column in 0.1 M NaCl and resolved by elution with a 20-column volume, 0.1–1.0 M NaCl linear gradient. As shown in Fig. 1, the elution of Ad2ts111A DBP peaked at approximately 0.6 M NaCl and was well separated from contaminants which eluted at lower salt concentrations. By densitometry of SDS-PAGE, DBP fractions eluting at greater than 0.50 M NaCl were judged to be greater than 99% pure with respect to protein.

Using conditions similar to those described above for the purification of Ad2ts111A DBP, we observed that Ad2 wild-type and H5ts107 DBPs eluted from ssDNA cellulose at NaCl concentrations similar to Ad2ts111A. However, when we purified Ad2”ND1ts23 DBP by the same protocol, peak elution occurred at 0.2 M NaCl, and the protein had almost totally eluted by 0.5 M NaCl (data not shown), confirming the results of Prelich and Stillman (1986). These results suggested that Ad2ts111A DBP binds with a higher affinity to single-stranded DNA than does Ad2”ND1ts23 DBP, even though the mutations in these two DBPs are located only 2 amino acid residues apart.

Activity of Ad2ts111A DBP in adenovirus-specific DNA replication in vitro—The end fragment assay which measures adenovirus DNA synthesis proceeding from specific initiation sequences at both ends of the molecule was used to study the activity of Ad2ts111A DBP. The results are presented in Fig. 2. As expected, the reaction was dependent on exogenous DBP (lanes 1 and 5). Purified Ad2ts111A DBP was active in the elongation assay at 30 °C (lane 3) but was inactive at 38.5 °C (lane 7). In contrast, Ad2 wild-type DBP was active at both temperatures (lanes 2 and 6). Purified H5ts107 DBP, like Ad2ts111A DBP, was inactive at the nonpermissive tem-
perture (lane 8). The temperature-sensitive phenotypes of the H5ts107 and Ad2ts111A DBPs were manifest only if the DBP was preincubated for 30–60 min prior to initiation of the reaction and were not reversed when incubation at nonpermissive temperature was followed by equilibration and incubation of the reactions at permissive temperature. Intrageneric complementation between Ad2ts111A DBP (amino acid 282) and H5ts107 DBP (amino acid residue 413) was attempted at nonpermissive temperature, but the defect in adenovirus DNA replication was not corrected. In contrast, at permissive temperature, identical mixtures of Ad2ts111A and H5ts107 DBPs were able to replicate adenovirus DNA effectively (data not shown). Neither Ad2ts111A DBP nor H5ts107 DBP was inhibitory to Ad2wt DBP function at elevated temperature.

Stimulation of DNA Synthesis on Poly(dT)-Oligo(dA) by Temperature-sensitive DBPs Is Temperature Sensitive—A recently developed assay has proven useful in studying the isolated role of DBP in the elongation of DNA by the adenovirus polymerase (Field et al., 1984). This assay, which utilizes poly(dT)-oligo(dA) as a primed template, requires only the adenovirus polymerase and the DBP as protein components; the reaction does not require the pTP or any of the nuclear factors from uninfected cells. Using this template, the DBP greatly stimulates DNA synthesis activity of its cognate adenovirus polymerase, suggesting that DNA synthesis on adenovirus polymerase (Field et al., 1984). This assay, which utilizes poly(dT)-oligo(dA) requires a specific, functional interaction between adenovirus DBP and adenovirus pol.

We have utilized this assay to further characterize the activities of the Ad2ts111A and H5ts107 DBPs. Representative results are shown in Table I. Although each of the temperature-sensitive DBPs was able to stimulate the activity of the adenovirus polymerase at 30 °C, neither Ad2ts111A or H5ts107 DBP was active at 38.5 °C. Similarly, the Ad2′ND1ts23 DBP, which was active in this assay at 30 °C, was inactive at 38.5 °C (data not shown).

Partial Proteolysis of Ad2ts111A DBP—Partial proteolysis of the wild-type DBP by chymotrypsin generates relatively stable, nested 44- and 34-kDa polypeptides corresponding to the carboxyl-terminus of the protein. The primary amino-terminal peptide (27 kDa) is degraded under these conditions and does not appear as a band on the gel. Other proteases generate similar sized fragments, suggesting a bi-domain tertiary structure for wild-type DBP. Partial chymotryptic proteolysis of Ad2ts111A DBP was performed at permissive and nonpermissive temperatures to determine whether the altered activity of the mutant protein might be the result of conformational changes affecting the global two-domain structure of the DBP. As shown in Fig. 3, similar peptide fragments are produced at both temperatures from the wild-type (lanes 3 and 5) and Ad2ts111A (lanes 4 and 6) DBPs indicating that major conformational changes resulting in the accessibility of other potential chymotryptic sites for Ad2ts111A DBP do not occur at nonpermissive temperatures. However, by increasing the protease/DBP ratio (data not shown) or by extending the reaction time, we were able to examine the intrinsic stability of the 34-kDa carboxy-terminal domains of the various DBPs. When the proteolytic reaction was extended to 60 min at 30 °C, the 34-kDa proteolytic fragments from Ad2ts111A DBP (Fig. 3, lane 8) and from wild-type DBP (Fig. 3, lane 7) were approximately equally stable. However, at 37 °C, the major chymotryptic fragment (34 kDa) of Ad2ts111A DBP is almost completely degraded (lane 10), while that of the wild-type DBP (lane 9) remains at a level similar to that observed at the lower temperature (lane 7). Similarly, the 34-kDa proteolytic fragment of the H5ts107 DBP was observed to be more susceptible to proteolysis than the corresponding wild-type fragment at nonpermissive temperature (data not shown). The increased susceptibilities of the 34-kDa proteolytic fragments of the Ad2ts111A and H5ts107 DBPs to further proteolysis at 37 °C indicate that at nonpermissive temperature the carboxyl-terminal domains of these temperature-sensitive DBPs are either destabilized or exist in different, more protease-sensitive configurations than the corresponding region of the wild-type DBP.

Fluorescence Studies—Fluorescence spectroscopy has been used to study the binding of other ssDNA-binding proteins to nucleic acids. Such studies can be used to define the binding site size (n), expressed in terms of nucleotides occluded/DBP molecule, the apparent binding constant (Kapp) which is equal to the product of the intrinsic binding constant Kbind and the cooperativity parameter α. The binding of protein to nucleic acid can be monitored either by measuring the quenching of the intrinsic fluorescence of the protein or by measuring the enhancement of fluorescence of a fluorescent nucleic acid. In experiments in which the quenching of the inherent fluorescence of the wild-type Ad DBP was utilized as a measure of adenovirus DBP-poly(dT) binding, we found that the protein was unusually photosensitive. The extensive bleaching of control protein samples in the absence of nucleic acid upon repeated excitation at 282 nm made the titration curves erratic and difficult to reproduce reliably (data not shown). Therefore, we utilized the enhancement of fluorescence of poly(rA) induced by DBP binding to investigate the interaction of DBP with nucleic acid. The use of poly(rA) permitted excitation at a longer wavelength where considerably less photo-bleaching occurred. Our initial experiments indicated that when poly(rA) was saturated with wild-type DBP, the fluo-
rescence emission of poly(rA) was increased by approximately 200% (data not shown). This increase can probably be attributed to the ability of DBP to unstack the bases of the polynucleotide and suggests that DBP imposes an extended conformation upon the bound nucleic acid. Titrations were performed with each DBP (wild-type, Ad2ts111A, and H5ts107) at various temperatures and NaCl concentrations. The binding curves obtained for a representative set of titrations for Ad2ts111A DBP at 25 °C are presented in Fig. 4A. From titrations carried out at low salt concentration, where binding was essentially stoichiometric (i.e. virtually all added protein is bound), values for the site size and minimal estimates of binding constants were calculated. These values, as determined in 20 mM NaCl, for Ad2wt DBP and H5ts107 DBP, as well as Ad2ts111A DBP, are compiled in Table II. A site size of 15.6 nucleotides was obtained for Ad2wt DBP and similar values were calculated for both Ad2ts111A DBP and for H5ts107 DBP. It should be noted that the values obtained represent minimum estimates of site size because the titration curves observed even at 20 mM NaCl did not indicate ideal stoichiometric binding. Under conditions of tight binding, at 20 mM NaCl and 25 °C, we calculated the lower limits for the $K_{app}$ to be $4 \times 10^6$ M$^{-1}$ (Table II) for Ad2 wild-type DBP, as well as for Ad2ts111A DBP and H5ts107 DBP. As indicated in Table II, all three proteins bind with a low but nonetheless significant degree of cooperativity. The cooperativity parameter of 35 for wild-type DBP is in excellent agreement with the value of 20-30 that was recently determined for the DBP-poly(rA) complex based on circular dichroism spectroscopy (Kuil et al., 1989). Titrations at 20 mM NaCl were performed for another important reason; the in vitro replication assays used to characterize the DBP are typically performed in the presence of 20 mM NaCl. Activity in these assays is sensitive to increased salt concentration, with virtually no DNA synthesis above 40 mM NaCl.

The titrations for each DBP at higher NaCl concentrations, at which binding was weaker, were utilized to more accurately determine the nuclear acid-binding affinities of the various DBPs (Kowalczykowski et al., 1986). The values for $K_{app}$ of the respective DBPs in 100 mM NaCl at 25 °C are compiled in Table II and indicate that increasing the [NaCl] from 20 to 100 mM results in at least a 4-40-fold decrease in $K_{app}$.

Sets of titrations at varied NaCl concentrations were performed to determine the effects of NaCl concentration on the nuclear acid-binding of the various DBPs. The results of a characteristic series of titrations with Ad2ts111A DBP are displayed in Fig. 4A. At increasing concentrations of NaCl, the titration curves are progressively shifted to the right indicating that Ad2ts111A binds poly(rA) less tightly at higher NaCl concentrations. The relationship between NaCl concentration and $K_{app}$ for Ad2ts111A DBP is displayed in Fig. 4B in the form of a log-log plot. The slope of this plot, as well as similar plots for wild-type and H5ts107 DBPs, is reported in Table II. The increase in salt sensitivity at 25 °C on going from the Ad2wt to either the Ad2ts111A or H5ts107 DBP proteins is not significant.

An alternative method for studying the dependence of binding upon salt concentration is the salt back-titration procedure in which the dissociation of the protein-poly(rA) complex is measured as a function of salt concentration. As in the titrations reported above at low salt, tight-binding conditions, a fixed quantity of poly(rA) is added to a cuvette and is titrated by addition of aliquots of DBP until the decrease in fluorescence reaches the plateau indicating saturation. Then, aliquots of concentrated NaCl (5 M) are added stepwise, and the decreases in fluorescence due to dissociation of the protein-nucleic acid complex are monitored until the fluorescence reaches the minimum value, indicating that the polynucleotide exists only in its free form. The curves for such a series of salt titrations comparing wild-type, Ad2ts111A, and Ad2*ND1ts23 DBPs are presented in Fig. 5. Ad2*ND1ts23 DBP was observed to dissociate from single-stranded polynucleotide at a lower salt concentration than Ad2ts111A DBP, a finding which is qualitatively consistent with results obtained by ssDNA-cellulose chromatography.

Fluorescence spectroscopy was also utilized to characterize the effects of elevated temperature upon the abilities of Ad2ts111A, H5ts107, and wild-type DBPs to bind to single-stranded nucleic acid in 20 mM NaCl. In Fig. 6, binding curves obtained at 25 and 42 °C for each DBP are compared. The $K_{app}$ values for the various DBPs at 25 °C and 20 mM NaCl are compiled in Table II. The results obtained for Ad2ts111A DBP and H5ts107 DBP are comparable to that observed for the wild-type DBP; these data demonstrate that the ability of Ad2ts111A DBP, as well as H5ts107 DBP, to bind nucleic acid in 20 mM NaCl is not significantly changed on increasing temperature-sensitive DBP; this result is consistent with our observation that temperature has little effect on the $K_{app}$ values of these mutant DBPs.

![Fig. 4. A, titration curves of the binding of Ad2ts111A DBP to poly(rA). At each salt concentration (from 20 to 300 mM), 0.69 µM poly(rA) was titrated with Ad2ts111A DBP at 25 °C, and binding parameters were obtained from curves as described under "Materials and Methods." The theoretical binding curves (solid lines above) were generated using a site size of 16 nucleotides and the following values of the overall apparent affinity, $K_{app}$, and the cooperativity parameter, $\omega$: 20 mM NaCl, $K_{app} = 7.0 \times 10^6$ M$^{-1}$, $\omega = 7$; 50 mM NaCl, $K_{app} = 5.2 \times 10^3$ M$^{-1}$, $\omega = 13$; 100 mM NaCl, $K_{app} = 1.9 \times 10^5$ M$^{-1}$, $\omega = 9$; 300 mM NaCl, $K_{app} = 2.3 \times 10^4$ M$^{-1}$, $\omega = 45$. B, effect of NaCl concentration on $K_{app}$ for binding of Ad2ts111A DBP to poly(rA) derived from data presented in titration curves of A.](http://www.jbc.org/content/5879/1/5879/F1.large.jpg)
to H5ts107 DBP and to Ad'NDlts23 DBP. The DBP mutation in by construction of a recombinant virus AdBts111A. The DBP mutation in temperature (in 20°C, 37°C, 58°C, 100°C, 25°C, 5°C) No No No No No

| Site of mutation | Ad2wt | Ad2tst111A | H5ts107 | Ad2 + NDlts23 |
|------------------|-------|-----------|---------|--------------|
| Site size        |       |           |         |              |
| ΔIg (log K)      |       |           |         |              |
| 15.6 ± 1.0       | 4.2 x 10^9 | 15.8 ± 1.5 | 15.5 ± 1.0 | 15.5 ± 1.0 |
| K_m (20 mM NaCl, 25 °C) |       |           |         |              |
| ω                | 35-70 | 7-15      | 10-40   | 282^-9      |
| K_m (100 mM NaCl, 25 °C) |       |           |         |              |
| ω                | 17    | 9         | 75      | 282^-9      |
| ΔIg (log K)      | -1.02 | -2.07     | -2.34   |              |
| 15.6 ± 1.0       | 4.2 x 10^9 | 15.8 ± 1.5 | 15.5 ± 1.0 | 15.5 ± 1.0 |
| K_m (20 mM NaCl, 42 °C) |       |           |         |              |
| ω                | 85    | 10        | 35      | 282^-9      |
| Effect of temperature (in 20 mM NaCl) |       |           |         |              |
| ω                | No    | No        | No      | No          |

These data are the average of two or three determinations. The cooperativity factor ω is expressed as a range of values calculated from these titrations. The values of ω presented in the remainder of the table were determined from single titrations. However, these values of ω would also be expected to vary 2-4-fold.

The DNA synthesis assay utilizing a polv(dT)-oligo(dA) template-primer for adenovirus pol has allowed for the further characterization of the role of DBP in DNA replication. Unlike the end fragment assay, in which elongation proceeds from an obligate adenovirus-specific initiation event and for which at least five proteins are required, the synthesis of polv(dA) from the polv(dT)-oligo(dA) template-primer by the adenovirus polymerase is dependent only on the presence of DBP (Field et al., 1984). Studies using this assay suggest that the DBP functions not only by binding single-stranded DNA but also by directly and specifically interacting with and stimulating the cognate adenovirus polymerase. DBP does not stimulate the activity of HeLa DNA polymerase α. Moreover, the E. coli ssDNA-binding protein (SSB) alone does not stimulate the adenovirus pol in this reaction; but, saturating levels of SSB do increase the degree of stimulation produced by low levels of DBP (Lindenbaum et al., 1986). We have extended the functional characterization of the three temperature-sensitive DBPs by analyzing their abilities to stimulate elongation by adenovirus pol of a poly(dT)-oligo(dA) template-primer. Ad2ts111A DBP, H5ts107 DBP, and Ad'NDlts23 DBP each are active in this assay at permissive temperature but inactive at nonpermissive temperature.

The H5ts107 DBP is considerably less stable than the wild-type DBP at nonpermissive temperature in vivo (van der Vliet et al., 1975). Our limited proteolysis studies of Ad2ts111A and H5ts107 DBP indicate that the global two-domain structure of wild-type DBP is conserved in both Ad2ts111A DBP and H5ts107 DBP, and that under the specified reaction conditions, the intact Ad2ts111A and H5ts107 DBP molecules are not significantly less stable than the wild-type protein. However, when proteolysis is extended, it is seen that the carboxyl-terminal domain of both Ad2ts111A and H5ts107 DRP is more susceptible to proteolysis at nonpermissive temperature than is the corresponding domain of the wild-type DBP. These data suggest that at nonpermissive temperature the carboxyl-terminal domain may exist in a slightly different configuration relative to the wild-type DBP. They do not suggest that the replication defects observed in vitro for H5ts107 and Ad2ts111A DBPs are attributable to increased degradation of the molecules but that they may be attributable to the alterations in configuration.

We have determined single-stranded nucleic acid-binding parameters for the Ad2ts111A, H5ts107, and Ad'NDlts23 DBPs, as well as wild-type DBP, by applying the technique of fluorescence spectroscopy. The values obtained for the site size of the wild-type DBP (n = 16) is just slightly larger than the values of 10-15 nucleotides recently reported by Kuil et al. (1989) based upon circular dichroism and ultraviolet ab-
AdBwt, Kepp = 7.0

temperature, the Ad2tslllA and H5ts107 DBPs differ by less reliably calculated. The KBpp for the wild-type DBP was

DBPs were 3.0 * 10^7 NaCl, binding was less tight, and binding constants could be sorption measurements. The site sizes calculated for the Ad2tslllA DBP and the H5ts107 DBP did not differ significantly from that calculated for the wild-type protein.

Binding of DBP to poly(rA) at 20 mM NaCl was nearly stoichiometric, and as a result, only minimum estimates of the apparent binding constant Kapp could be obtained at this salt concentration. The values obtained for the Kapp values of the wild-type, H5ts107, Ad2tslllA, and Ad2ND1ts23 DBP proteins were all above 10^6 M^-1 in 20 mM NaCl. In 100 mM NaCl, binding was less tight, and binding constants could be more reliably calculated. The Kapp for the wild-type DBP was 1.0 * 10^6 M^-1, whereas those for Ad2tslllA and H5ts107 DBPs were 3.0 * 10^5 M^-1 and 2.1 * 10^5 M^-1, respectively, in 100 mM NaCl. Thus, in terms of binding affinity at permissive temperature, the Ad2tslllA and H5ts107 DBPs differ by less than a factor of five from the wild-type in 100 mM NaCl.

When Ad2tslllA DBP was preincubated at elevated temperature, the shape of the binding curve was not altered, indicating that the affinity of binding was not decreased. However, the absolute increase in poly(rA) fluorescence at saturation was only 150% as compared with about 250% for the wild-type DBP titrations (data not shown), suggesting that the temperature-sensitive DBP had imposed a slightly different, probably less extended, conformation upon the poly(rA) at elevated temperature.

A series of titration curves was generated at varying salt concentrations for each of the DBPs to compare the salt dependence of the Kapp values for poly(rA). The slopes of the resulting log Kapp versus log [NaCl] curves ranged from ~1.02 to ~2.34. These experiments demonstrate that binding of the DBP is significantly less dependent upon salt concentration than is the binding of gp32, for which the corresponding slope is equal to ~7 (Newport et al., 1981). Thus, a 10-fold increase in salt concentration would be expected to decrease the affinity of gp32 for ssDNA by seven orders of magnitude as opposed to only about 1 to 2 orders of magnitude in the cases of the DBP proteins examined.

From the results presented above, it is apparent that the temperature-sensitive replication defects of Ad2tslllA and H5ts107 DBPs cannot be readily attributed to their nucleic acid-binding affinities, which do not differ significantly from those of the wild-type protein at 42 °C under conditions necessary for adenovirus replication in vitro. This result confirms and extends previous studies on H5ts107 utilizing different experimental techniques for measuring DNA-binding (Krevolin and Horwitz, 1997). The Ad2ND1ts23 DBP, with its phenylalanine for leucine substitution at amino acid 282, had been reported to be defective for DNA binding because it eluted from a ssDNA-cellulose column at lower NaCl concentration than the wild-type (Prelich and Stillman, 1986), and we have reproduced this result. The results of our spectrofluorometry experiments investigating the dissociation of the DBP-poly(rA) complexes as a function of salt concentration confirm a second method that the binding of Ad2ND1ts23 DBP to single-stranded polynucleotide is weaker than that of Ad2tslllA DBP, as well as the wild-type DBP.

In summary, the focus of the work reported here has been the delineation of structure function relationships of the DBP for single-stranded nucleic acid binding and complementation of DNA replication. We have shown that the H5ts107 and Ad2tslllA DBP mutant proteins, which are defective in replication assays in vitro, are not temperature sensitive for binding under the same conditions. These results reinforce the postulate that the ability of DBP to bind single-stranded DNA is by itself not sufficient for DNA replication. The results of the limited proteolysis studies probably signal small but significant changes in the conformation of the COOH-terminal domains of Ad2tslllA and H5ts107 DBP at nonpermissive temperature. Therefore, it appears that the amino acid substitution in Ad2tslllA, as well as that in H5ts107, affects the protein's activity by decreasing the thermal stability of the protein structure rather than by directly disrupting the nucleic acid binding. The conformational changes that are induced in the Ad2ts107 and H5ts107 proteins at restrictive temperatures are not sufficient to substantially lower the affinity of these proteins for single-stranded nucleic acids. However, they may disrupt a presumed DBP:DNA polymerase interaction or perhaps alter the topology of the bound nucleic acid. In fact, the decreased extent of enhancement of the poly(rA) fluorescence caused by binding of the Ad2tslllA DBP at nonpermissive temperature suggests that the
Ad2ts111A DBP may impose a slightly different structure upon the bound nucleic acid than does the wild-type protein. The spectrofluorometric data reported here represent an initial step in the quantitative investigation of the physicochemical properties of DBP and its binding of single-stranded nucleic acid. Unfortunately, other productive techniques, such as NMR and calorimetry, that have been utilized for the study of T4 gp32 and E. coli SSB would require larger amounts of DBP than are currently available. Cloning and overproduction of wild-type DBP and mutants, as has been accomplished with SSB and gp32, would appear to be an important step in the further characterization of this protein.

Acknowledgments—We wish to acknowledge the secretarial assistance of Gina De Filippi. M. L. M. greatly appreciates Michael Brenowitz for his assistance in analyzing the fluorescence data and for valuable discussions.

REFERENCES

Arita, H., Klein, H., Levine, A., and Horwitz, M. (1980) Virology 101, 307–310
Bahch, A., and Nevins, J. R. (1981) Cell 26, 271–279
Carter, T. H., and Blanton, R. A. (1978) J. Biol. Chem. 253, 105–121
Challberg, M. D., and Kelly, T. J., Jr. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 655–659
D’Halluin, J. C., Cousin, C., Niel, C., and Boulanger, P. (1984) J. Gen. Virol. 65, 1305–1317
Field, J., Gronostajski, R. M., and Hurwitz, J. (1984) J. Biol. Chem. 259, 9487–9495
Friefeld, B. R., Krevolin, M. D., and Horwitz, M. S. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 1476–1480
Grondelle, R. (1989) Nature New Biol. 246, 685–687
Kaplan, L. M., Ariga, H., Hurwitz, J., and Horwitz, M. S. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4961–4967
Johnson, M. L., and Frasier, E. G. (1986) Methods Enzymol. 117, 301–342
Kaplan, L. M., Ariga, H., Hurwitz, J., and Horwitz, M. S. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5534–5538
Karpel, R. L., Henderson, L. E., and Oroszlan, S. (1987) J. Biol. Chem. 262, 4961–4967
Klein, R., Maltzman, W., and Levine, A. J. (1979) J. Biol. Chem. 254, 11051–11060
Klessig, D. F., and Grodzicker, T. (1979) Cell 17, 957–966
Kowalczykowski, S. C., Lonberg, N., Newport, J. W., and von Hippel, P. H. (1981) J. Mol. Biol. 145, 75–104
Kowalczykowski, S. C., Paul, L. S., Lonberg, N., Newport, J. W., McSwiggan, J. A., and von Hippel, P. H. (1986) Biochemistry 25, 1226–1240
Krevolin, M. D., and Horwitz, M. S. (1987) Virology 156, 167–170
Krujiver, W., van Schaik, F. M. A., and Sussenbach, J. S. (1981) Nucleic Acids Res. 9, 4438–4457
Krujiver, W., van Schaik, F. M. A., and Sussenbach, J. S. (1982) Nucleic Acids Res. 10, 4493–4500
Krujiver, W., van Schaik, F. M. A., Speijer, J. G., and Sussenbach, J. S. (1983) Virology 128, 140–153
Kuil, M. E., van Amerongen, H., van der Vliet, P. C., and van Grondelle, R. (1989) Biochemistry 28, 9795–9800
Lassen, U. K. (1970) Nature 227, 680–685
Ledneva, R. K., Razkivin, A. P., Kost, A. A., and Bogdanov, A. A. (1978) Nucleic Acids Res. 5, 4225–4243
Lindenbaum, J. O., Field, J., and Hurwitz, J. (1986) J. Biol. Chem. 261, 10218–10227
Linne, T., Jornvall, H., and Philipson, L. (1977) Eur. J. Biochem. 76, 481–490
Maizel, J. V., Jr., White, D. O., and Scharff, M. D. (1968) Virology 30, 110–125
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 158–173, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Martin, G. R., Varoququier, R., Cousin, C., D’Halluin, J. C., and Boulanger, P. A. (1978) J. Gen. Virol. 41, 303–314
McGhee, J. D., and von Hippel, P. H. (1974) J. Mol. Biol. 96, 1305–1317
McSwiggen, J. A., and von Hippel, P. H. (1986) Biochemistry 25, 4646–4652
Melnikov, B., and Leon, G. (1987) Virology 156, 647–650
Melnikov, B., and Leon, G. (1987) Virology 156, 647–650
Nevins, J. R., and Winkler, J. J. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1983–1987
Newport, J. W., Lonberg, N., Kowalczykowski, S. C., and von Hippel, P. H. (1981) J. Mol. Biol. 145, 105–121
Nicolas, J. C., Sarnow, P., Girard, M., and Levine, A. J. (1983) Virology 126, 228–239
Prellich, G., and Stillman, B. W. (1986) J. Virol. 57, 883–892
Schechter, N. M., Davies, W., and Anderson, C. W. (1980) Biochemistry 19, 2802–2810
Sharp, P. A., Moore, C., and Haertel, J. V. (1976) Virology 75, 442–456
Stillman, B. W., White, E., and Grodzicker, T. (1984) J. Virol. 50, 598–605
Tsamoglou, D., Tsigita, A., Tucker, A. D., and van der Vliet, P. C. (1985) FEBS Lett. 188, 245–252
Vallellano-Leon, G., Flomenberg, P., and Horwitz, M. S. (1985) J. Virol. 56, 647–650
van Amerongen, H., van Grondelle, R., and van der Vliet, P. C. (1987) Biochemistry 26, 4646–4659
van der Vliet, P. C., and Sussenbach, J. S. (1975) Virology 67, 415–426
van der Vliet, P. C., and Levine, A. J. (1973) Virology 56, 647–650
van der Vliet, P. C., Keegstra, W., and Jansz, H. S. (1978) Eur. J. Biochem. 86, 389–398
Purification and functional characterization of adenovirus ts111A DNA-binding protein. Fluorescence studies of protein-nucleic acid binding.
M L Meyers, K M Keating, W J Roberts, K R Williams, J W Chase and M S Horwitz

J. Biol. Chem. 1990, 265:5875-5882.

Access the most updated version of this article at http://www.jbc.org/content/265/10/5875

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/10/5875.full.html#ref-list-1