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Conserved Region 3 of the Adenovirus Type 5 DNA-Binding Protein Is Important for Interaction with Single-Stranded DNA

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The adenovirus-encoded single-stranded DNA-binding protein (DBP) functions in viral DNA replication and several aspects of RNA metabolism. Previous studies (G. A. M. Neale and G. R. Kitchingman, J. Biol. Chem. 264:3153–3159, 1989) have defined three highly conserved regions in the carboxy-terminal domain of the protein (amino acids 178 to 186, 322 to 330, and 464 to 475) that may be involved in the binding of the protein to single-stranded DNA. We examined the role of conserved region 3 (464 to 475) by constructing nine classes of point mutants with from one to four amino acid changes. The point mutants were tested for their ability to assist adeno-associated virus DNA replication. All nine differed from wild-type DBP; seven were essentially nonfunctional, whereas two had 55 and 145%, respectively, of the wild-type DBP helper activity. Three of the mutants were found to be temperature sensitive, with significantly greater helper activity at 33°C than at 37°C. All nine mutants produced essentially wild-type levels of protein. One monoclonal antibody against the DBP, termed 2/4, did not immunoprecipitate the mutant DBPs as well as wild-type DBP, indicating either that the antibody recognized sequences around CR3 or that the conformation of the protein around the epitope recognized by 2/4 had changed. Two of the three temperature-sensitive DBP mutants bound to single-stranded DNA-cellulose with the same affinity as wild-type DBP at 4°C; the remaining mutants all showed reduced affinity. These results demonstrated that many of the residues within conserved region 3 of the DBP are important for interaction of the protein with nucleic acid.

Single-stranded (ss) DNA-binding proteins (DBP) are ubiquitous in nature (23) and are coded for by procaryotic and eucaryotic cells and viruses. The best-studied eucaryotic ssDBP is a protein of 529 amino acids (aa) produced during the early phase of the infectious cycle of human adenovirus type 5 (Ad5) (43). Our knowledge of the functions of this protein derives from a limited set of viral mutants that are either temperature sensitive (*ts*) for growth (9) or have an altered host range (1, 22). Studies performed with these mutants have shown that the adenovirus DBP is a multifunctional protein that regulates viral macromolecular synthesis at multiple levels.

This protein is involved in the initiation (45, 46) and elongation (41) phases of DNA replication in vivo. In vitro systems for adenovirus DNA replication with purified proteins (reviewed in reference 12) demonstrated that DBP is not essential for the first steps of DNA synthesis, although it facilitates this process. However, the DBP is absolutely required for elongation of the nascent DNA chain beyond several hundred nucleotides (11). The DBP is thought to play two roles in DNA synthesis: as an ssDBP that protects the nascent ssDNA strand from nuclease attack and separates it from the DNA replication complex, and as a facilitator of chain elongation through its interaction with another protein (presumably the adenovirus DNA polymerase) in the DNA replication complex (28). Evidence for the latter role is hypothetical; interaction of the DBP with other proteins has not been demonstrated (28).

Little is known about the regions of the DBP that are involved in these proposed activities. The three *ts* mutants in the DBP gene map to the codons for aa 280 (34), 282 (34), and 413 (24). The mutations in aa 280 and 282 are in a region of the DBP that closely resembles the consensus sequence of a zinc finger (47, 48), a structure thought to be important for protein binding to double-stranded DNA (2). These two mutants, Ad2*ts*111 (aa 280) and Ad2$^+$ ND1*ts*23 (aa 282), both produce DBPs that do not support DNA replication in vitro at the nonpermissive temperature (34, 40). The *ts*23 DBP, however, supports initiation in vitro at the nonpermissive temperature at a level almost equal to that of the wild-type DBP. Thus, the data for the *ts*23 DBP are most consistent with a wild-type protein-protein interaction, with the defect in elongation being due to the decreased affinity for ssDNA. The *ts*125 mutation (aa 413) is probably structural, as this Pro-to-Ser change results in a protein that is unstable at the nonpermissive temperature (14). The protein binds to ssDNA with the same affinity as the wild-type DBP at 4°C, supporting the suggestion that this mutation is in a region of the protein that is important for folding.

We have previously reported on a series of mutants isolated by site-directed mutagenesis of the cloned DBP gene (36). The mutations were localized to the three conserved regions of the DBP identified by comparative sequence analysis (20). Mutations in two of these three regions affected the ability of the DBP to bind to ssDNA and to support adeno-associated virus (AAV) DNA synthesis in vivo (36). The conserved region mutants that showed altered properties (CR2 mutations in aa 323 and 324, and a CR3 mutation in aa 470) produced stable proteins that were properly localized in the cell and properly modified (32). Thus, the mutations may be in functional residues and may identify regions of the DBP important for DNA-protein interaction. These aa, together with aa 280 and 282, represent the functionally important aa residues identified to date.

To expand our knowledge of important functional residues, we turned our attention to conserved region 3 (aa 464 to 475) to determine whether aa other than the lysine residue mutated in the previous study are important for the functions of the protein. We saturated this region with mutations by bisulfite-generated regional mutagenesis and isolated a series

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of nine mutants. Surprisingly, each of these mutants was functionally affected.

MATERIALS AND METHODS

Plasmids. The plasmids used in this work include pSVB/R, which has been described previously (35); pBalI (the *Bal*I fragment of Ad2 containing sequences between 0.8 and 6.0 map units [33]); pH5RIB (the *Eco*RI B fragment of Ad5, map units 84.0 to 100 [3]); and pVASVOd (a *Hin*dIII-*Sal*I fragment of Ad2, map units 26.55 to 31.66, containing the two virus-associated RNA genes [19]). The pH5Kpn5 plasmid (referred to as Kpn5 throughout this paper) is the pSVB/R plasmid into which a nucleotide change resulting in the loss of the *Kpn*I site at 61.5 has been introduced; this area is outside the DBP gene, and the DBP produced from this plasmid has the wild-type DBP aa sequence.

Mutagenesis of DBP gene. The 36 base pairs coding for the 12 aa of CR3 were precisely excised with an oligonucleotide (GCGTTTAGCAGTCCGCCCTGCG; synthesized on a DNA synthesizer (model 380; Applied Biosystems, Inc.]) that hybridized to either side of this region. Oligonucleotides were purified on denaturing 20% polyacrylamide gels and used as described previously (36). Deletion mutants were identified by restriction endonuclease analysis and confirmed by DNA sequencing (30). A mutant with a precise 36-base-pair deletion was identified and designated pSVB/RdeltaCR3.

Bisulfite mutants were generated essentially as described by Kalderon et al. (18). The pSVB/R plasmid containing the wild-type DBP gene was digested with *Bam*HI, which cuts once, and the deltaCR3 plasmid was linearized with *Hin*dIII. The linearized plasmids were mixed, denatured with 0.2 N NaOH at room temperature for 10 min, neutralized, reannealed at 63°C for 3 h, and precipitated with ethanol. Three volumes of a freshly made solution of 4 M sodium bisulfite (pH 6.0) were mixed with 1 volume of reannealed plasmids, followed by 0.04 volume of freshly prepared 50 mM hydroxyquinone. The reaction mix was overlaid with paraffin oil and incubated at 37°C in the dark for 4 h. The reaction mix was placed in a dialysis bag and dialyzed sequentially against 5 mM potassium phosphate (pH 6.8), 0.5 mM hydroxyquinone at 0°C for 2 h (twice), 5 mM potassium phosphate for 4 h, 0.2 M Tris hydrochloride (pH 9.2)–50 mM NaCl–2 mM EDTA at 37°C for 16 to 24 h, and 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA at 4°C for 4 h. The DNA was recovered by ethanol precipitation and solubilized in a small volume of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA.

Transformation of the mutated gapped duplexes into *Escherichia coli* BW313 (25) was performed as described by Kushner (26). Random colonies were picked, and plasmids were prepared by the alkaline lysis procedure (4). DNAs were analyzed for the presence of wild-type-length plasmid by *Hin*fI digestion. Wild-type-length plasmids were sequenced by the procedure of Maxam and Gilbert (30); plasmids were labeled by partial fill-in at an *Mlu*I site that is 7 base pairs away from CR3. Large-scale plasmid preparations were done by the procedure of Clewell and Helinski (8) with the modifications previously described (36). The entire coding sequence of the DBP gene was sequenced by using a series of oligonucleotide primers and the Sequenase protocol for double-stranded DNA.

Transfections. COS-1 cells (15) were infected with a stock of AAV2 for 1 h before transfection, essentially as described previously (36). The infected COS-1 cells were then transfected by the calcium phosphate procedure of Graham and van der Eb (16), modified to include a 20% glycerol shock (7). Generally, 10 μg of each plasmid was used per 100-mm dish. DNA in 1.5 ml of calcium phosphate precipitate was added to dishes seeded the previous day at 1.6×10^6 COS-1 cells. After 4 h, the DNA solution was removed and the cells were treated with 20% glycerol in alpha minimum essential medium for 1 min at 37°C. After removal of the glycerol, the cells were washed once, fed with 15 ml of alpha minimum essential medium containing 10% newborn calf serum, and incubated for 48 to 66 h at 37°C.

DNA analysis. Low-molecular-weight DNA was prepared from the transfected cells by the procedure of Hirt (17). One-fifth of the DNA isolated from each dish was electrophoresed through a 1% agarose gel, transferred to nitrocellulose or nylon membranes (39), and probed with DNAs labeled by primer extension (10). An internal *Kpn*I fragment of AAV (map units 40 to 80) derived from pAV2 (27) was used as the source of AAV DNA for labeling, while the purified insert from pSVB/R was used as the source of DNA for the DBP gene. Hybridization was for 16 h at 42°C in a buffer containing 50% formamide, 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES, pH 7.0), 0.8 M NaCl, 0.1% sarcosyl, 200 μg of denatured salmon testes DNA per ml, and 5× Denhardt solution. Filters were washed twice at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for a total of 30 min and then four times with 0.1× SSC–0.1% sodium dodecyl sulfate for a total of 2 h at 52°C. Autoradiography was performed with Kodak XAR film and Cronex Lightning-Plus intensifying screens. AAV DNA replication was quantified by cutting out and counting the band corresponding to the monomeric replicative form (RF). In every experiment, the amount of DBP DNA was quantified by cutting out and counting the RF band hybridizing to the DBP-specific probe. The amount of AAV DNA replication was normalized to the amount of DBP gene in the cell.

Analysis of DBP. COS-1 cells in 60-mm dishes were transfected with 10 μg of plasmid and labeled at 64 h posttransfection with either 100 μCi of $^{32}P_i$ per ml in phosphate-free alpha minimum essential medium containing 5% dialyzed calf serum or 100 μCi of [^{35}S]methionine per ml in methionine-free alpha medium. After labeling, the cells were washed twice with phosphate-buffered saline and lysed in 1 ml of 50 mM Tris hydrochloride (pH 7.4)–0.6 M KCl–0.5% Triton X-100. The extracts were freeze-thawed and then clarified by centrifugation at 16,000 × *g* for 30 min at 4°C. Immunoprecipitates were prepared (36) from each extract (10^6 cpm) by using a monoclonal antibody directed against the DBP. For sequential immunoprecipitations, the supernatants present after clearing of the extract with protein A-Sepharose were again incubated with antibody and cleared with protein A-Sepharose. After boiling, the immunoprecipitates were electrophoresed through 13% sodium dodecyl sulfate-polyacrylamide gels as described by Maizel (29). The gels were impregnated with sodium salicylate (^{35}S only), dried, and exposed to Kodak XAR film in the presence of Cronex Lightning-Plus intensifying screens.

ssDNA-cellulose column chromatography of DBPs. Transfected COS-1 cells were labeled 48 h posttransfection with $^{32}P_i$, and cell extracts were prepared as described by Klein et al. (21). Before sample loading, 2 ml of ^{32}P-labeled cell extract was mixed with a ^{3}H-labeled purified wild-type DBP (about 10^6 cpm). The reference ^{3}H-labeled wild-type DBP was purified by ssDNA-cellulose chromatography (43) of extracts from KB cells infected with wild-type Ad5 and

labeled with [4,5-^{3}H]leucine 48 h postinfection. The Ad5-infected KB cells were maintained in medium containing 25 μg of cytosine arabinoside per ml to inhibit the expression of viral late genes. ssDNA-cellulose chromatography was done at 4°C. The mixed extract was adjusted to 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA–2 mM β-mercaptoethanol–10% glycerol (buffer A) containing 0.1 M NaCl and loaded onto an ssDNA-cellulose column (8-ml volume) at a flow rate of 2 ml/h. The column was washed extensively with buffer A containing NaCl at the same concentration used in loading the sample. Bound proteins were eluted with a 50-ml linear gradient from 0.1 to 0.85 M NaCl in buffer A, and 1.25-ml fractions were collected. The elution profiles of the ^{32}P and ^{3}H radioactivity were determined by liquid scintillation counting. Fractions were also analyzed by immunoprecipitation with a monoclonal antibody directed against the DBP (B6) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were dried and exposed to Kodak XAR film in the presence of intensifying screens. Under these conditions, only ^{32}P radioactivity is detected, which permits determination of the elution profile of the ^{32}P-labeled DBPs. The ^{32}P-labeled DBP was quantified by scanning autoradiograms with a Hoefer scanning densitometer and associated software.

RESULTS

Previous results with a single mutant from conserved region 3 of the Ad5 DBP indicated that a single aa change, a Lys to Thr in aa 470, could substantially reduce the ability of the DBP to function in AAV DNA replication (36) and to bind to ssDNA-cellulose (32). To determine whether other amino acid residues in CR3 are involved in the functions of the DBP, we set out to isolate a large number of point mutants. We chose to use regional bisulfite mutagenesis because this method is effective in producing C-G to A-T transitions (18). Codons for 8 of the 12 aa in CR3 could potentially be altered to code for other aa by bisulfite mutagenesis.

We used the bisulfite mutagenesis technique described by Shortle and Nathans (38) as modified by Kalderon et al. (18) for gapped duplex molecules. The first step in this procedure is to create a deletion in the area to be mutated. This was accomplished through the use of an oligonucleotide that hybridized on either side of the 36 nucleotides of CR3. Isolation of a precise deletion was confirmed by DNA sequence analysis. DNA from this mutant, pSVB/RdeltaCR3, was linearized with *Hin*dIII and heteroduplexed to the wild-type plasmid that had been linearized with *Bam*HI. The resulting heteroduplexes were mutagenized with bisulfite as described in Materials and Methods and transformed into *E. coli* BW313. Random colonies were picked and checked for the presence of wild-type-length DBP genes by restriction endonuclease analysis. Candidate mutants were then sequenced from an *Mlu*I site adjacent to CR3. A total of 92 colonies were examined, and 31 of these contained point mutations in CR3. These 31 mutants could be classified into nine groups (Fig. 1). Two mutants, 11 and 19, contained identical aa changes but had several nucleotide differences. We found no differences in the properties of these two mutants. Because of the chance that mutagenesis occurred outside the gapped heteroduplex, we sequenced the entire DBP gene for a representative of each of the nine groups and subcloned a fragment of the DBP gene containing the mutation into a nonmutated plasmid background. One of the mutants , E2/20, was found to have extensive nucleotide changes about 500 base pairs upstream of CR3. The relevant portion of this mutant was therefore subcloned into a wild-type DBP background to produce a mutant with only changes in CR3.

Up to four aa changes were found in the mutants (Table 1). Some changes were fairly conservative (an Ala-to-Val change in mutant E2/5); others could be predicted to have significant effects on the DBP (changes in both Pro residues in mutants E2/11 and E2/19). Collectively, the mutants had changes in seven of the eight aa that could be affected by bisulfite mutagenesis.

Adenovirus helper activity for AAV DNA replication. The mutants were first characterized for their ability to help in the AAV DNA replication assay, a convenient method for functionally screening large numbers of mutants. We initially analyzed two independent representatives of each mutant group for their helper activity. COS-1 cells were infected with AAV and transfected with the other adenovirus genes required for AAV DNA replication (E1a, E4, and the VA RNAs) as well as the appropriate DBP mutant gene. Hirt supernatants were prepared at 66 h posttransfection, and duplicate samples of DNA were run on agarose gels. Hybridization of Southern blots containing Hirt DNA from wild-type and mutant DBP-transfected cells with probes for AAV and the DBP gene is shown in Fig. 2. Every mutant was affected in its ability to help AAV DNA replication, with activities ranging from 3 to 147% that of wild-type DBP (Table 1).

Mutants with single aa changes had 19% (Ala to Val; E2/5) and 147% (Asp to Asn; E2/20) of the wild-type AAV helper activity. For the three double mutants, AAV helper activities ranged from 55% (E2/13) to 10% for the E2/33 mutant and about 3% for the E2/42 mutant (Fig. 2). All mutants with more extensive changes were totally defective for AAV helper activity, yielding only background levels of AAV RF DNA (Fig. 2).

***ts* activity of mutant DBPs.** The AAV helper activity of the mutant DBPs was examined at 33, 37, and 40°C to ascertain whether any of the point mutants had a *ts* phenotype. Three of the mutants were more active at 33°C, with activities ranging from 54 to 109% of the wild-type activity (Table 2). The temperature sensitivity was most striking for E2/5 and E2/33; virtually no activity remained at 40°C (Fig. 3). The E2/13 mutant retained considerable activity at both 37 and 40°C (about one-half that of the wild-type DBP). For comparison, results for the non-*ts* E2/19 mutant are shown (Fig. 3).

Analysis of mutant DBP proteins in transfected cells. The reduced helper activity of the eight mutant DBPs could be due to instability of the mutant proteins or to a reduced affinity for substrate(s). To examine the first possibility, COS-1 cells transfected with the expression plasmids containing the mutant DBP genes were pulse-labeled for 2 h with either $^{32}P_i$ or [^{35}S]methionine and the DBP from cell extracts was immunoprecipitated with a monoclonal antibody directed against the carboxy-terminal portion of the protein. In our initial experiments, we used a monoclonal antibody isolated in this laboratory termed 2/4. The quantity of mutant proteins observed was much reduced compared with the wild-type DBP, and no protein was detected for six of the mutants. Scanning densitometry indicated that for the E2/5 and E2/33 mutants, about 10% as much protein was found as in wild-type DBP-transfected cells, and for the E2/13 mutant, about 25% as much protein was found (data not shown). This finding could be due to the lack of synthesis of the DBP, to a short half-life, or to an altered conformation

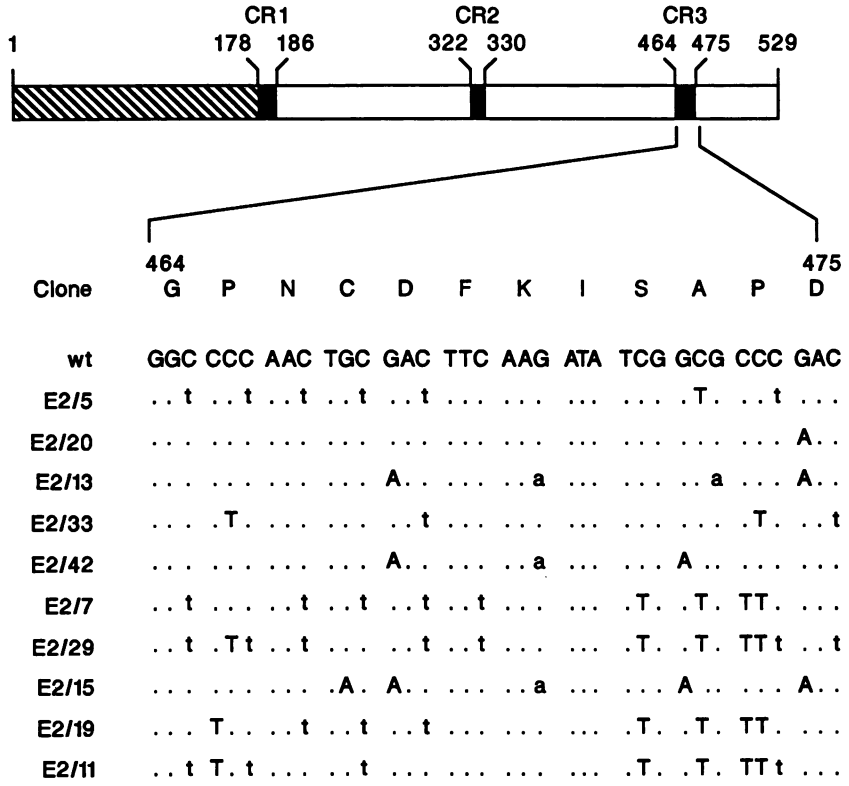

FIG. 1. Location of the conserved regions (CR) of the Ad5 DBP and the sequence of the CR3 bisulfite mutants. The DBP is 529 aa long and consists of an amino-terminal domain of about 174 aa (striped) and a carboxy-terminal domain (aa 175 to 529). Within the carboxy-terminal domain are three regions that are exactly conserved among adenovirus serotypes, CR1 to CR3. The aa sequence of the wild-type CR3 is given, as is the nucleotide sequence. Below the nucleotide sequence of the wild-type CR3, the sequences of 10 CR3 mutants are shown. Nucleotide changes leading to aa changes are shown in capital letters, while silent mutations are shown in lower-case letters. Dots indicate same nucleotide as wild type (wt). Note that while E2/19 and E2/11 have the same nucleotide substitutions leading to changes in aa, there are differences in the location of silent mutations.

not recognized by the monoclonal antibody. To examine these possibilities, we pulse-labeled COS-1 cells for 15 min to determine whether shortened pulse times could identify short-lived DBP species. The results were essentially identical to the 2-h pulse-labeling gel (data not shown). We used another monoclonal antibody (B6; kindly provided by Arnold Levine) to test the possibility that 2/4 was not recognizing the protein owing to an altered conformation. The

TABLE 1. Activity of DBP mutants in the AAV DNA replication assay

Mutant	aa sequence	n[a]	Mean[b]	SD	Range
wt[c]	GPNCDFKISAPD	20	1.00	NA[d]	NA
E2/5	.....V..	18	0.192	0.128	0.01–0.40
E2/20	.....N	7	1.47	0.150	1.27–1.64
E2/13	....N.....N	11	0.550	0.191	0.28–0.91
E2/33	.L.....L.	20	0.109	0.079	0.01–0.26
E2/42	....N....T..	10	0.028	0.031	0.01–0.10
E2/7	.....LVF.	20	0.054	0.053	0.01–0.19
E2/29	.L.....LVF.	19	0.031	0.034	0.01–0.11
E2/15	...WN....T.N	19	0.042	0.042	0.01–0.11
E2/19	.S.....LVF.	19	0.048	0.047	0.01–0.14
E2/11	.S.....LVF.	13	0.051	0.053	0.01–0.12

[a] n, Number of experiments.
[b] Mean values relative to wild-type DBP for helper activity for AAV DNA replication in vivo.
[c] The wild-type (wt) DBP gene was contained on the Kpn5 plasmid.
[d] NA, Not applicable.

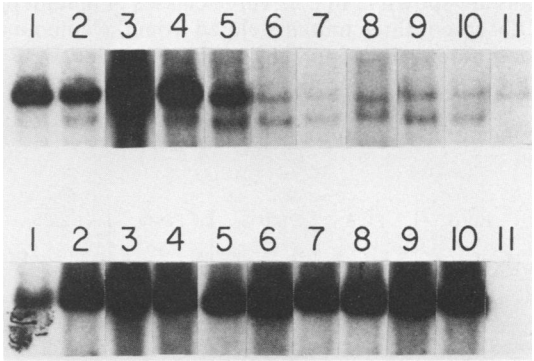

FIG. 2. In vivo AAV DNA replication. The ability of the various mutant DBPs to help AAV replicate its DNA was measured by transfection of the E1a, DBP, virus-associated RNA, and E4 genes of adenovirus into COS-1 cells that had been infected with AAV. At 66 h postinfection, Hirt supernatants were prepared and aliquots were run on parallel gels. In the top gel, a Southern blot was hybridized to an AAV DNA probe, and in the bottom blot, the parallel blot was hybridized to a DBP gene probe. Quantitation was performed by cutting out and counting the AAV RF DNA band and the DBP gene RF DNA. The lanes contain the following DBP genes: 1, Kpn5 plasmid (wild-type DBP); 2, E2/5; 3, E2/20; 4, E2/13; 5, E2/33; 6, E2/42, 7, E2/7; 8, E2/29; 9, E2/15; 10, E2/11; 11, no DBP.

TABLE 2. Effect of temperature on the activity of DBP mutants in the AAV DNA replication assay

Mutant	aa sequence	Activity relative to wild type[a]		
		33°C	37°C	40°C
wt	GPNCDFKISAPD	100	100	100
E2/5	.....V..	105	19	7
E2/13	....N.....N	105	55	54
E2/33	.L.....L.	54	11	4

[a] Activity relative to wild-type (wt) DBP gene (contained on the Kpn5 plasmid) for AAV DNA replication in vivo.

levels of mutant and wild-type DBP immunoprecipitated by B6 were very similar. These experiments indicated that the two antibodies might be recognizing different epitopes and that the epitope recognized by the 2/4 antibody might be absent or altered in the mutant DBPs. Therefore, we performed sequential immunoprecipitations using the 2/4 antibody first, followed by B6, and then the reverse sequence. The results for the 2/4-B6 sequence are shown in Fig. 4 (left side). Each protein extract (labeled for 2 h with $^{32}P_i$) was immunoprecipitated sequentially three times with both antibodies to ensure removal of all reactive material. Little protein was brought down by the 2/4 antibody for seven of the nine mutants (the exceptions are E2/20 and E2/13), but greater than 80% of the wild-type DBP was immunoprecipitated. With the B6 antibody, large quantities of each of the mutant proteins and the remaining 20% of the wild-type protein were immunoprecipitated. When the immunoprecipitation order was reversed, B6 immunoprecipitated essentially all the DBP in the extract (Fig. 4, right side), indicating that the epitope for the 2/4 antibody is altered or masked in the mutant DBPs.

Binding of mutant DBPs to ssDNA-cellulose. To compare directly the binding ability of the mutant and wild-type proteins, we mixed ^{32}P-labeled extracts from mutant-transfected cells with ^{3}H-labeled, purified wild-type DBP before chromatography. Elution profiles of representative mutant proteins are shown in Fig. 5. Three classes of mutant protein were observed; three mutants eluted from the column at salt concentrations similar to that of the wild-type DBP (e.g., E2/20; Fig. 5, top), two eluted at 0.1 to 0.2 M lower salt (e.g., E2/33; Fig. 5, middle), and four eluted at 0.2 to 0.3 M lower salt concentration than the wild-type DBP (e.g., E2/19; Fig. 5, bottom). The results for all the mutants are summarized in Table 3.

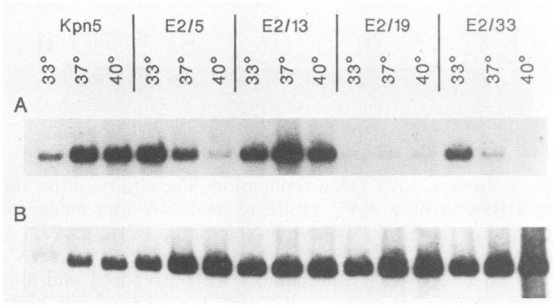

FIG. 3. AAV helper activity at various temperatures. The in vivo AAV DNA replication assay was performed as described in the legend to Fig. 2 except that incubation was performed at 33, 37, or 40°C for 66 h. (A) Autoradiogram of a Southern blot of Hirt supernatant DNA hybridized to an AAV DNA probe. (B) Parallel blot hybridized to a DBP gene probe. The blot was quantitated as described in the legend to Fig. 2. The Kpn5 plasmid contains the wild-type DBP gene.

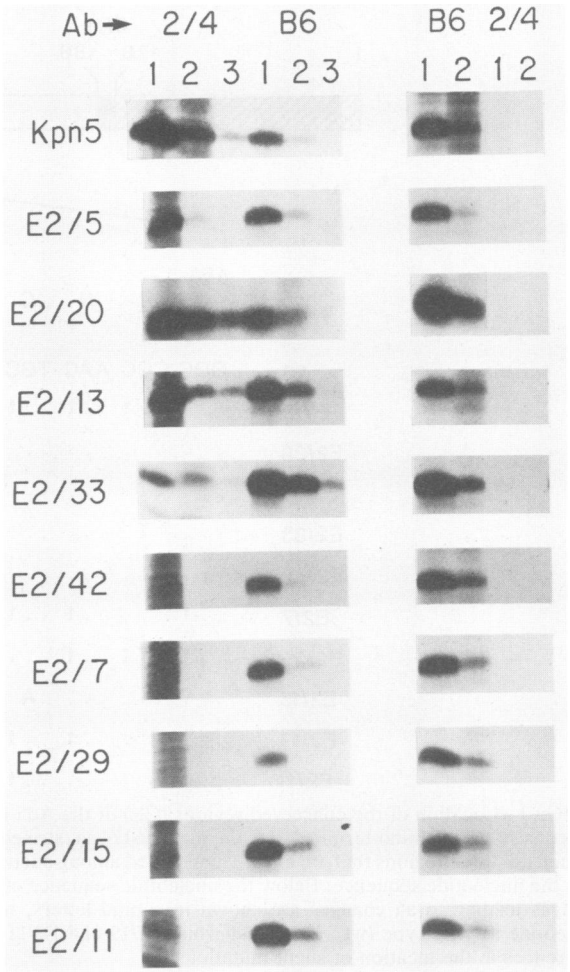

FIG. 4. Immunoprecipitation of mutant DBP with monoclonal antibodies (Ab). COS-1 cells were transfected with mutant DBP gene-containing plasmids and labeled between 64 and 66 h postinfection with ^{32}P. Extracts were prepared and immunoprecipitated sequentially with antibodies 2/4 and B6. When 2/4 was the first antibody used, three sequential immunoprecipitations were performed with this antibody, followed by three cycles with antibody B6. When B6 was used first, two cycles were performed with each antibody. The immunoprecipitates were separated on sodium dodecyl sulfate-polyacrylamide gels and autoradiographed. Results for 2/4 followed by B6 are shown on the left side of the figure, and those for B6 followed by 2/4 are shown on the right side. The Kpn5 plasmid contains the wild-type DBP gene.

DISCUSSION

The adenovirus ssDBP is a multifunctional protein that can serve as a paradigm for regulatory elements in eucaryotic cells whose site of action does not involve binding to specific DNA sequences. To understand how the DBP performs its multiple proposed functions, it is necessary to generate mutants that are affected for one or more of these functions. In this report, we expanded our original set of seven mutants and described some of the properties of nine mutants generated by mutagenesis of CR3 by bisulfite. The fact that all nine classes of mutants were functionally af-

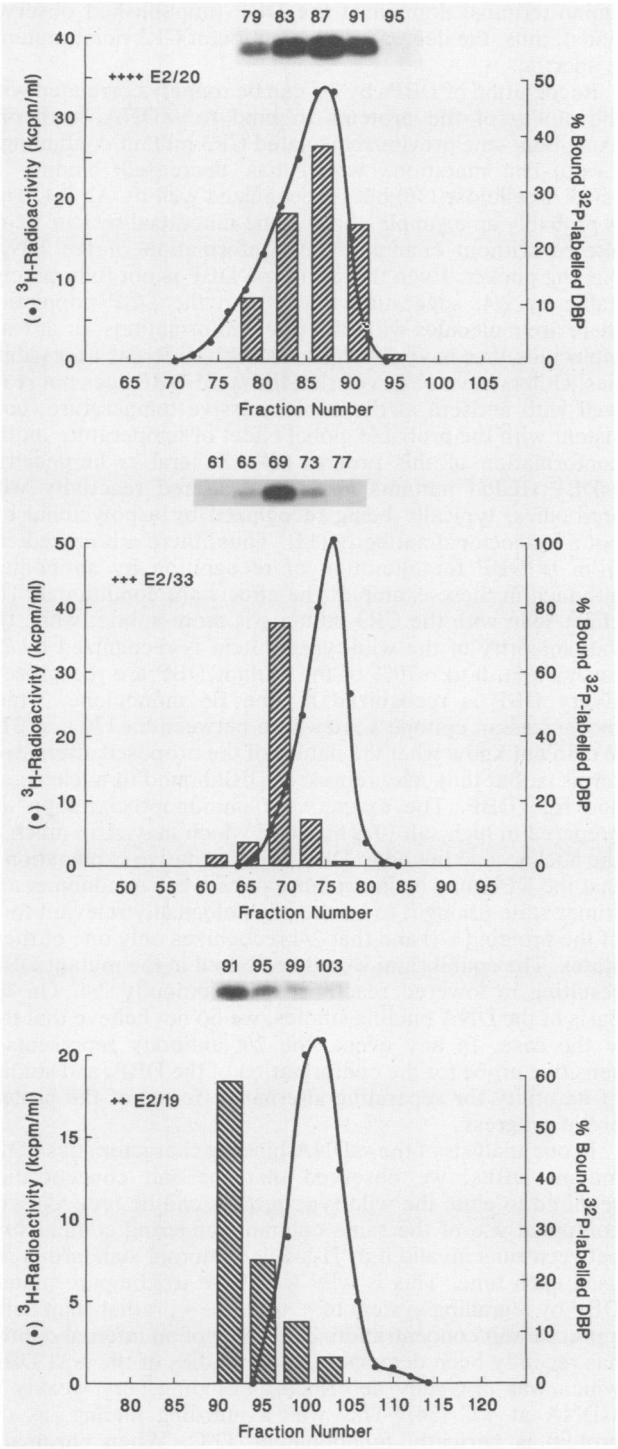

FIG. 5. ssDNA-cellulose column chromatography of mutant DBPs. Labeled DBP was prepared as described in the legend to Fig. 4, mixed with a small amount of ^{3}H-labeled wild-type DBP (derived from Ad5-infected KB cells), and passed over an ssDNA-cellulose column at 4°C. Proteins were eluted with a 0.1 to 0.85 M NaCl gradient. Fractions were counted to locate the ^{3}H-labeled wild-type DBP, and every fourth fraction was immunoprecipitated with the monoclonal antibody B6. Immunoprecipitates were separated on sodium dodecyl sulfate-polyacrylamide gels, and the mutant DBP was located by autoradiography. For simplicity, only the ^{3}H profile is shown along with the relevant portion of the autoradiogram. The autoradiograms were quantified, and the percentage of DBP found in each fraction is represented in the bar graph. Representatives of strong binding (E2/20), moderate binding (E2/33), and weak binding (E2/19) DBPs are shown in the three panels.

TABLE 3. Comparison of the properties of the conserved region mutant DBPs

DBP[a]	Posttranslational modification	Relative AAV helper activity[b]	Avg NaCl concn of elution peak from ssDNA-cellulose[c]
Wild type	++++	100	++++
CR1 W→L[d]	++++	107	++++
CR2 R→L[d]	++++	29	+++
CR2 W→L[d]	++++	17	+++
CR3 K→T[d]	++++	12	++
CR1 + CR3[d]		21	
CR2 + CR3[d]		11	++
CR2 + CR3[d]		13	
E2/5	++++	19	++++
E2/20	++++	147	++++
E2/13	++++	55	++++
E2/33	++++	11	+++
E2/42	++++	3	++
E2/7	++++	5	++
E2/29	++++	3	+++
E2/15	++++	4	++
E2/19	++++	5	++
*ts*23[e]			+++
*ts*111[f]	++++		++++
*ts*125[g]	±	25	++++

[a] All DBP were located in the nucleus.

[b] AAV helper activity of mutants is expressed relative to that observed with the wild-type DBP.

[c] ssDNA-binding affinities are expressed in terms of the decrease in NaCl concentrations needed to elute the protein from an ssDNA-cellulose column (++++, wild-type DBP; +++, 0.1 to 0.2 M decrease; ++, 0.2 to 0.3 M decrease). Binding was performed at 4°C.

[d] DBP mutants characterized by Neale and Kitchingman (32). The CR1 + CR3 and CR2 + CR3 mutants represent double point mutants in which the individual mutations have been combined.

[e] DBP mutant characterized by Prelich and Stillman (34); ssDNA-cellulose binding data are from M. Tsuji and G. R. Kitchingman (unpublished data).

[f] DBP mutant characterized by Stillman et al. (40); ssDNA-cellulose binding data are from M. Tsuji and G. R. Kitchingman (unpublished data).

[g] DBP mutant characterized by Klein et al. (21).

fected by the mutations strengthens our previous suggestion (32) that CR3 is important for the binding of the DBP to its substrates.

The bisulfite mutagenesis protocol was effective in generating point mutants; about one-third of the randomly picked plasmids contained mutated DBP genes. The clones contained from 1 to 12 nucleotide changes, most of which were silent and seemed to have no effect on the stability of the mRNA or its ability to be translated (based on the lack of an effect on the steady-state levels of mutant DBP protein and the levels of mRNA as analyzed on Northern [RNA] blots; unpublished observations). Seven of the eight possible aa changes were obtained, with only the initial glycine codon being refractory to change. There were no spurious mutations in the immediate vicinity of the deletion loop, although we did find one mutant with extensive changes about 500 nucleotides away. These mutations resulted in several aa changes and the generation of an amber codon in aa 271. This mutant DBP gene produced a truncated protein, presumably representing the amino-terminal 270 aa of the protein, that was inactive in the AAV helper activity assay. This protein was useful for localizing the epitopes for the

monoclonal antibodies used in this study (see below). The CR3 mutation from this mutant was moved into a wild-type background before further study.

Mutations in sequence-specific DBP have been categorized as structural or functional by Bowie and Sauer (5). Structural mutations lead to the synthesis of unstable protein which cannot function because it does not accumulate. Functional mutations result in the production of protein that has altered activity relative to the wild type. All 10 of the CR3 mutants we have isolated to date meet the criteria for functional mutations. Each one has altered functional activity in the AAV helper assay, and 7 of the 10 show a decreased affinity for ssDNA. The mutant DBPs are all stable: protein accumulates in a 2-h labeling in quantities similar to that of the wild-type DBP, and when examined, the half-lives of the mutant DBPs are identical to that of the wild-type protein (data not shown). There are, however, small structural changes in the protein that, while not affecting stability, do affect the ability of the protein to be recognized by a monoclonal antibody. The DBP mutants with lowered AAV helper activity probably represent proteins with small, localized changes in structure that do not affect the overall folding of the protein but do affect its ability to interact with ssDNA. For the E2/20 mutant, which shows a higher-than-wild-type DBP level of AAV helper activity, this increased activity could be due to the apparently higher levels of accumulation of this mutant DBP in the cell (compare levels of Kpn5 [wild type] and E2/20 immunoprecipitated by B6 in Fig. 4).

The changes in function could be due to alterations in nucleic acid binding properties or in protein-protein interactions. Because many of the DBP mutants are altered in their ability to bind to DNA, we favor the former interpretation. One caveat to this interpretation is that the active form of the DBP is probably a trimer (37), and therefore protein-protein interaction might be required for ssDNA binding. If lowered affinity for ssDNA is the explanation for the decreased ability of the mutant DBP to bind to ssDNA, this could result from changes in either (i) aa residues directly involved in interaction with the bases and phosphate backbone of the DNA or (ii) the conformation of the nucleic acid-binding pocket through mutation of residues not directly involved in binding but important for the local structure. The bacteriophage fd ssDBP model (6) suggests that aromatic residues are important for base stacking and that positively charged aa residues are important for interaction with the phosphate backbone of ssDNA. In our collection of 10 CR3 mutants, only the Lys-to-Thr (32, 36) mutant has an alteration in one of these types of aa. This mutant is quite impaired in AAV helper activity (32) and binding to ssDNA (36). Of the nine mutants here, none showed changes in the basic or aromatic aa thought to be important for ssDNA interaction, so alterations in function most likely are due to changes in the local structure of the DNA-binding pocket.

We propose that this possibility can be assessed by using the monoclonal antibody 2/4. Immunoprecipitation studies with DBP deletion and premature termination mutants have demonstrated that this antibody recognizes an epitope between aa 270 and 529 (G. R. Kitchingman, unpublished data). The 2/4 antibody shows a gradient of reactivity with DBP mutants, while the monoclonal antibody B6, which recognizes an epitope between aa 170 and 270, can efficiently clear wild-type and mutant DBP from extracts. Antibody 2/4 efficiently recognizes other DBP mutants, including those having point mutations in CR1 and CR2, a deletion mutation encompassing all of CR1 (aa 178 to 186), and deletions of the amino-terminal domain of the DBP (unpublished observations); thus, the decreased recognition of CR3 point mutants is specific.

Recognition of DBPs by 2/4 can be roughly correlated with the ability of the proteins to bind to ssDNA, with one exception—the previously isolated CR3 mutant containing a Lys-to-Thr mutation, which has decreased binding to ssDNA-cellulose (36) but is recognized well by Ab 2/4. This is probably an example of a specific functional residue being altered without changing the conformation of the DNA-binding pocket. Even the wild-type DBP is not fully precipitable by 2/4, suggesting that within the DBP population there are molecules with different conformations, or at least molecules that have 2/4 epitopes with different accessibilities. Others have observed that the *ts*125 DBP does not react well with antisera at the nonpermissive temperature, consistent with the probable global effect of temperature on the conformation of this protein (14). Several *ts* herpesvirus ssDBP (ICP8) mutants also show altered reactivity with antibodies, typically being recognized by a polyclonal but not a monoclonal antibody (13). Thus, there are precedents from *ts* DBP for alteration of recognition by antibodies, although in these examples the effects are conditional. The effect seen with the CR3 mutants is more subtle; while the vast majority of the wild-type protein is recognized by 2/4 (over 80%), 0 to ~70% of the mutant DBP are recognized. Every DBP is recognized by the B6 monoclone, which recognizes an epitope somewhere between aa 170 and 270. We do not know what the nature of the proposed alternative forms is, but they may represent DBP bound to nucleic acid and free DBP. The extracts for immunoprecipitation are prepared in high salt (0.6 M KCl), which may strip much of the nucleic acid bound to DBP. An alternative explanation is that the DBP may be in equilibrium between a monomer and trimer state (thought to be the physiologically relevant form of the protein [37]) and that 2/4 recognizes only one of these states. The equilibrium would be shifted in the mutant DBP, resulting in lowered reactivity with antibody 2/4. On the basis of the DNA binding studies, we do not believe that this is the case. In any event, the 2/4 antibody represents a sensitive probe for the conformation of the DBP, and studies of its utility for separating alternative forms of the protein are in progress.

In our analysis of the ssDNA-binding characteristics of the mutant DBPs, we observed that the salt concentration required to elute the wild-type protein can be reduced with continued use of the same column, rendering comparisons between runs invalid if a ^{3}H-labeled internal standard is not used each time. This is why we chose to compare mutant DBP by a grading system of + to ++++, rather than using a precise salt concentration. The value of an internal control has recently been demonstrated in studies of the *ts*23 DBP, which was originally described as binding very weakly to ssDNA at 4°C (34). This was a puzzling finding, as the protein is perfectly functional at 32°C. When chromatographed with an internal control, the *ts*23 DBP elutes at a position equivalent to +++ (M. Tsuji and G. R. Kitchingman, unpublished data), similar to the E2/33 mutant described here. Thus, the previously reported lack of binding probably reflected problems with decay of the column.

Bowie and Sauer (5) have observed that when the ARC repressor is mutated, some aa positions are fairly tolerant of substitutions, while others seem incapable of tolerating virtually any change. In the small ARC repressor (53 aa), about one-third of the residues are functionally important, while about one-half are structurally important. aa changes

in the amino-terminal part of that protein result in functionally defective proteins, although most are structurally tolerated. Our results with a much smaller panel of mutants in CR3 mutants are similar.

Overall, these mutants establish the importance of CR3 in the DNA binding properties of the DBP and therefore in its ability to function. What is the role of CR1 (aa 178 to 186) and CR2 (aa 322 to 330) in these processes? We previously demonstrated that two mutants in CR2 were functionally defective in the AAV assay and bound less tightly to ssDNA, while the CR1 mutant behaved like wild-type DBP in both assays. Mutations in either CR2 or CR3 can abolish almost entirely the binding of the protein to ssDNA. We speculate that these regions of the protein, separated by about 140 aa in the primary sequence, are close to one another in the native protein. They could together form the binding pocket for ssDNA, and mutations in either one might affect binding. Determination of the three-dimensional structure of the DBP (42) and cross-linking studies (31) will be required to assess this possibility. At present, we cannot rule out the possibility that other regions of the DBP are involved in binding to ssDNA. This will have to be examined by mutagenesis of regions outside of the three conserved regions that we have studied.

In summary, a series of nine mutants in the CR3 region of the Ad5 DBP were isolated and found to be functionally affected. Most showed reduced activity in an AAV helper assay, but one was more active than the wild-type DBP. In addition, three were *ts*. ssDNA-binding activities of the mutants ranged from poor to equivalent to wild type. The mutant proteins had an altered recognition site for one monoclonal antibody. These mutants provide some insight into the CR3 residues that are important to the functions of the DBP.

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