Functional Analysis of the Adenovirus Type 5 DNA-Binding Protein: Site-Directed Mutants Which Are Defective for Adeno-Associated Virus Helper Activity

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We generated four point mutations in the DNA-binding protein (DBP) gene of adenovirus type 5 by oligonucleotide-directed site-specific mutagenesis. The sites mutated were in the three conserved regions (CR; amino acids 178-186 [CR1], 322-330 [CR2], and 464-475 [CR3]) identified previously by comparative sequence analysis (G. R. Kitchingman, *Virology* 146:90-101, 1985). The mutations resulted in changes in amino acids 181 (Trp to Leu), 323 (Arg to Leu), 324 (Trp to Leu), and 469 (Phe to Ile). The mutated DBP genes were put under the control of the simian virus 40 early promoter and analyzed by transfection for their ability to help adeno-associated virus replicate its DNA in COS-1 monkey cells. Mutations in the aromatic amino acids 324 and 469 reduced the amount of AAV DNA replication approximately 10-fold, while the mutation in Arg 323 produced a reduction of approximately fourfold. The Trp-to-Leu mutation in amino acid 181 had no effect on AAV DNA replication. The decreased helper activity of the 323, 324, and 469 mutations was not caused by any effect of the mutation on the stability of the DBP. These results suggest that CR2 and CR3 are involved in AAV helper activity, specifically in AAV DNA replication. The relevance of these findings to the identification of residues important for the functions of DBP in adenovirus infection is discussed.

Single-stranded (ss) DNA-binding proteins (DBPs) are produced by all cells, and many viruses code for their own DBPs. In addition to binding to ssDNAs in a sequence-independent manner, DBPs also bind tightly to RNA (reviewed in reference 39). All DBPs examined to date have a role in DNA replication, and, at least for DBPs encoded by viruses, they perform other functions in the replication of the virus. For example, the bacteriophage T4 gene 32 protein is involved in repair (4) and recombination (6) of viral DNA and is a translational repressor of its own mRNA (46). The DBP encoded by adenovirus (67) is thought to be involved in several aspects of viral mRNA metabolism, including transcription (27, 53), stability (3, 70), and perhaps splicing (1, 36). Similarly, the herpes simplex virus DBP is involved in the regulation of synthesis of early viral RNAs (22). These molecules therefore are multifunctional proteins which regulate viral and presumably cellular gene expression at many levels. Whereas the T4 gene 32 protein has been studied in considerable detail, comparatively little is known about the eucaryotic ss DBPs.

Adenovirus early region 2a codes for an ss DBP which is 529 amino acids in length for adenovirus type 5 (Ad5) (41). There is structural and functional evidence that the adenovirus DBP contains two functional domains (38). Mild chymotrypsin treatment of the DBPs from Ad2 and Ad5 produces fragments of 174 and 355 amino acids (35), representing the amino-terminal and carboxy-terminal portions, respectively, of the molecule. The carboxy-terminal fragment has been shown to bind to ssDNA (35) and to complement H5ts125 (2, 18), a temperature-sensitive replication-negative mutant of Ad5, for DNA replication *in vitro*. The region(s) of the protein that is involved in repression of transcription from the adenovirus early region 4 promoter

(27), in mRNA stability (3, 70), and in the regulation of E2a mRNA levels (54) is also affected by the ts125 mutation (11). This mutant has been sequenced and contains a point mutation resulting in a single change at amino acid 413 (aa 413) in the DBP (41). The amino-terminal domain of the DBP is involved in the host range of the virus; that is, viruses with wild-type DBP do not grow well in monkey cells (37). Viruses that can grow well in monkey cells, such as Ad2hr400 (37), have acquired a mutation at aa 130 (41). The amino-terminal domain is also highly phosphorylated and contains 9 to 11 phosphate residues as phosphothreonine and phosphoserine (35, 47).

We have been interested in identifying the amino acid residues in the two domains of the DBP that are involved in each of the functions of the DBP. By using classical genetic methods, only two types of mutants have been isolated, those with mutations at aa 413 that lead to the ts125 phenotype and those with mutations at aa 130 that lead to the hr400 phenotype. As a first step in our studies, we determined the amino acid sequence of the DBP of two adenoviruses from different subgroups, Ad4 (subgroup E; 34) and Ad7 (subgroup B; 58) and compared them to the sequences determined for the Ad5 (subgroup C; 41) and Ad12 (subgroup A; 40) DBPs. The overall homology among the four serotypes was 17.2% in the amino-terminal domain and 47.8% in the carboxy-terminal domain (34). The most striking feature of the pattern of homology among the serotypes was three regions of 9, 9, and 12 amino acids that were exactly conserved among the four serotypes. Based on the conservation of these sequences, we postulated that these regions contained important functional residues of the protein. We further postulated, based on the bacteriophage fd gene 5 DBP model (9), that the exactly conserved regions might be involved in binding to ssDNA (34). Binding of this protein to ssDNA has been shown to involve interaction of aromatic amino acid residues with the bases and interaction of positively charged amino acid residues with the phosphate

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Construction of vectors for the expression of the DBP under the control of the SV40 early promoter. The large *Kpn*I (nucleotide 274)-to-*Eco*RI (nucleotide 1782) fragment of simian virus 40 (SV40) containing all of the early gene region

FIG. 2. Scheme for the expression of mutagenized DBP genes. At the upper right is a map of the early regions of the adenovirus genome. The E2a region was cloned by insertion of a *Bam*HI-*Eco*RI fragment of Ad5 into pBR322. The derived plasmid, pH5B/R, was used for the oligonucleotide-directed site-specific mutagenesis. Construction of the SV40 vector used for expression of the E2a gene in monkey cells is shown at the left. A *Kpn*I-*Eco*RI fragment of SV40 was cloned into the vector pKB111 (pSV40 Ig). The sequences between the *Hind*III site at 5108 and the *Eco*RI site were replaced by the polylinker from M13mp18 (pSV40/mp18). The E2a expression plasmid was prepared by ligating the *Xho*I-*Bam*HI fragment of the E2a gene to the SV40 plasmid cut with *Sal*I and *Bam*HI (pSVB/R). This construct contains the SV40 origin of replication, the early promoter, and most of the 5' untranslated region of T antigen. The adenovirus sequences include the second and third (coding) exons of the gene as well as the E2a polyadenylation signal.

was cloned into the vector pKB111 (5). The sequences between the *Hind*III site at position 5107 in the early gene region and the *Eco*RI site were replaced by the polylinker from M13mp18 (55). The *Xho*I-to-*Bam*HI fragment of the wild-type or mutant pH5B/R plasmid (Fig. 2) was cloned into the *Sal*I and *Bam*HI sites of the expression vector. In this chimeric construct, the DBP gene is under the control of the SV40 early promoter and contains most of the T antigen 5' untranslated leader. The remainder of the gene consists of DBP sequences including splice junctions corresponding to the second intron of this gene, followed by the coding and polyadenylation sequences of the DBP. This vector contains the SV40 origin of replication and replicates to high copy numbers in COS-1 cells.

Transfection. COS-1 cells (20) were infected with a stock of AAV2 for 1 h before transfection. AAV stocks were prepared by coinfection of KB cells with AAV and Ad5,

followed by heat inactivation of the Ad5 at 56°C for 30 min. The quantity of AAV2 stock used was determined empirically in preliminary experiments to give the highest level of AAV replication. The infected COS-1 cells were then transfected by using the calcium phosphate procedure of Graham and van der Eb (23), modified to include a 20% glycerol shock (13). Generally, 10 μ g 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 with 2×10^6 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. The quantity of DNA per dish was constant in experiments comparing the helper activity of the wild-type and mutant

DBP genes but varied in the experiments analyzing the constellation of adenovirus genes required for AAV helper activity. Control experiments demonstrated that the use of different amounts of total plasmid DNA per dish did not affect the results.

DNA analysis. Low-molecular-weight DNA was prepared from the transfected cells by the procedure of Hirt (29). One-fifth of the DNA isolated from each dish was electrophoresed through a 1% agarose gel, transferred to nitrocellulose or nylon membranes (61), and probed with DNAs labeled by primer extension (17). An internal *KpnI* fragment of AAV (map units 40 to 80), derived from pAV2, was used as the source of AAV DNA for labeling, while the purified insert from pH5B/R was used as the source of DNA for region E2 of adenovirus. 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 Dupont 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 E2a DNA was also measured by cutting out and counting all bands hybridizing to the E2a-specific probe. The amount of AAV DNA replicated was normalized to the amount of E2a gene in the cell.

Analysis of the DBP. Transfected COS-1 cells were labeled between 48 and 66 h posttransfection with 100 µCi of [³⁵S]methionine per ml in alpha minimum essential medium which contained no methionine and 5% dialyzed calf serum. Extracts were prepared by lysing the cells in 50 mM Tris hydrochloride (pH 7.4)–0.6 M KCl–0.5% Triton X-100, freeze-thawing the extract, and centrifuging at 100,000 × *g* for 30 min at 4°C. Immunoprecipitates were prepared by mixing 5 × 10⁶ cpm of extract with 5 µl of a monoclonal antibody directed against the DBP. After incubation at room temperature for 1 h, 50 µl of a 30% protein-A Sepharose solution was added, and incubation was continued for another hour. The beads were washed three times with RIP buffer (50 mM Tris hydrochloride [pH 8.0], 1 mM EDTA, 150 mM NaCl, 0.25% bovine serum albumin) and three times in RIP buffer in which 0.1% Triton X-100 was substituted for bovine serum albumin. After being boiled, the immunoprecipitates were electrophoresed through 13% sodium dodecyl sulfate-polyacrylamide gels as described by Maizel (48). The gels were impregnated with sodium salicylate and exposed to Kodak XAR film in the presence of intensifying screens.

RESULTS

Isolation of mutants. We used oligonucleotide-directed, site-specific mutagenesis to change amino acids in the three conserved regions of the DBP either from aromatic to neutral or from charged to neutral amino acids. The amino acid and the nucleotide sequences of the conserved regions are shown in Fig. 1, as are the oligonucleotides used to generate the nucleotide changes. The strategy used for mutagenesis is outlined in Fig. 2 and described in detail in Materials and Methods. We changed the conserved aromatic amino acid residues in each of the three conserved regions (CR); in CR1 and CR2, Trp residues were changed to Leu,

FIG. 3. Effect of various combinations of adenovirus early genes on the replication of AAV DNA. Transfections into COS-1 cells were performed with 10 µg each of plasmids containing the adenovirus early gene listed at the top. Hirt supernatants were prepared 66 h after transfection, and a portion of the DNA was analyzed on 1% agarose gels. Nitrocellulose blots were probed with either AAV DNA (A) or with the *Bam*HI-*Eco*RI fragment of the E2a gene (B). The locations of the AAV double-stranded RF DNA, multimeric RF DNAs, and the ss genomic DNAs are indicated at the right. The band migrating slightly more slowly than the RF monomer (ss dimers?) was tentatively identified as a hydrogen-bonded ss DNA dimer. The closed circular (cc), open circular (oc), and linear (l) forms of the plasmid containing the DBP gene are indicated to the right of panel B.

and in CR3 a Phe residue was changed to Ile. The fourth point mutation was an Arg-to-Leu change in CR2. We also generated a series of mutants containing two point mutations by shuffling restriction fragments among the mutants. By cloning a *Bgl*II fragment (63.6 to 60.2) from the CR3 mutant clone into the CR1 and CR2 mutants, we generated mutants containing the CR1 + CR3 aromatic amino acid mutations, the CR2 + CR3 aromatic amino acid changes, and the CR3 aromatic + the CR2 charged amino acid mutations. Thus, four single-point and three double-point mutants were isolated.

Adenovirus helper activity for AAV replication. Several groups have shown that the adenovirus functions required by the replication-defective AAV can be supplied by microinjecting (59, 60) or transfecting (31) various combinations of adenovirus restriction fragments. These experiments led to the identification of the adenovirus genes required for helper activity: E1a or E1b (or both), E2a (DBP gene), E4, and the gene for the VA RNAs (31, 59, 60).

In Fig. 3 we show the effect of combinations of adenovirus genes on the replication of AAV DNA. For these experiments, COS-1 cells were first infected with AAV2 and then transfected with the adenovirus genes. By placing the DBP gene under the control of the SV40 early promoter, we were able to analyze the functions of the DBP in the absence of DBP gene modulation by E1a (7) and possibly E4 (21), and we were also able to take advantage of the amplification of SV40-origin-containing plasmids in COS-1 cells. Parallel

FIG. 4. Effects of mutations in the Ad5 DBP on the replication of AAV DNA. Experiments were performed as described in the legend to Fig. 3. The DBP mutants analyzed were CR1 (aa 181, Trp to Leu); CR2 R-L (aa 323, Arg to Leu); CR2 W-L (aa 324, Trp to Leu); and CR3 (aa 469, Phe to Ile). The mutants containing two point mutations (CR1 + CR3, etc.) were constructed by combining restriction fragments from the two appropriate point mutants. The identities of the AAV and E2 hybridizing bands are as described in the legend to Fig. 3.

blots were probed with AAV DNA to quantitate the levels of the various forms of AAV DNA and with DBP DNA to quantitate the levels of DBP gene-containing plasmid present in the cell. AAV viral DNA is ss, and in cells coinfecting with adenovirus the ssDNA is converted first to a double-stranded monomeric RF and then to concatemeric RF DNAs (10). In determining the effects of the mutations in the DBP gene on the replication of AAV DNA, we normalized the amount of AAV monomeric RF DNA to the amount of DBP gene present in the transfected cells. In the autoradiogram shown in Fig. 3A, the various forms of AAV DNA can be seen; in addition to the monomeric ss and RF DNAs, concatemeric forms of the RF DNA and perhaps a hydrogen-bonded dimer of ss AAV DNA accumulated. The lower part of the figure shows an identical blot probed with the DBP gene (Fig. 3B). When plasmids containing a single adenovirus gene were transfected into AAV-infected cells, only the DBP gene (lane 1) was able to provide helper function for AAV DNA replication. Transfection of E1, E4, or the VA RNA genes alone provided no helper activity (data not shown), nor is there any AAV DNA replication observed when cells are infected with AAV alone (data not shown). When combinations of adenovirus genes were used in conjunction with the DBP gene, it was demonstrated that addition of the E4 gene clone stimulated RF production about threefold compared with transfection with the DBP gene alone. The addition of a plasmid containing the VA RNA genes also stimulated the formation of RF DNA (about two- to fourfold in various experiments) and allowed the accumulation of AAV ssDNA. Addition of an E1a-containing plasmid had little effect on the formation of RF.

Thus, addition of the DBP gene, the E4 gene, and the genes for the VA RNAs is sufficient to provide helper activity for AAV DNA replication.

Effect of DBP mutations on AAV DNA replication. We analyzed the effects of the point mutations on the ability of the DBP to help AAV DNA replicate. For these experiments, we used AAV-infected cells transfected with plasmids containing the E4 gene, the VA RNA genes, E1a, and the appropriate mutant DBP gene under the control of the SV40 early promoter. Analysis of the AAV DNA present in Hirt supernatants is shown in Fig. 4, with the upper panel representing hybridization to an AAV probe and the lower panel representing hybridization to a DBP gene probe. A summary of the results is presented in Table 1.

The Trp-to-Leu mutation in CR1 did not affect the function of the DBP in AAV DNA replication. The level of AAV RF DNA formation was equivalent to that found in cells transfected with wild-type DBP (mean, 1.07 ± 0.20 ; n , 4). The Trp-to-Leu mutation in CR2, however, did affect the helper activity of the DBP. Averaging the results from five transfections, the helper activity of the CR2 mutant was reduced to about 17% (0.17 ± 0.04) of that of the wild-type DBP. Synthesis of both RF and AAV ssDNA was affected by the mutation. The third mutant examined, the Phe-to-Ile mutation in CR3, was perhaps the least functional of the four DBP point mutants; helper activity of this DBP was only about 12% (0.12 ± 0.02) of that of the wild-type DBP. Again, both RF and ssDNA synthesis were affected. The fourth mutant, the Arg-to-Leu mutation in CR2, was not as defective as were the aromatic-amino-acid residue mutants but reduced the helper activity to about 29% (0.29 ± 0.12) of that of wild-type DBP (Table 1).

The double-point mutants were analyzed in the same manner. The helper activities for AAV DNA replication of the two CR2 + CR3 mutants were similar to that of the CR3 point mutant (Table 1). The CR1 + CR3 mutant was more active in helper function (0.21 ± 0.12) than was the CR3 single-point mutant (0.12 ± 0.02). However, these results represent the mean of only two separate experiments, so they should be viewed with caution.

In other experiments, we determined that the mutant DBPs are not dominant, since cotransfection of mutant and wild-type DBP genes results in replication of AAV DNA consistent with the combined activity of the two DBPs (data not shown).

Analysis of mutant DBP proteins in transfected cells. The reduced helper activity of the mutant DBPs could be caused by the instability of the mutant proteins in the cell, resulting in lower steady-state concentrations of the DBP. To deter-

TABLE 1. Helper activity for AAV DNA replication of Ad 5 DBP mutants

Mutant	Relative helper activity ^a (n ; range)
Wild type	1.00
CR1 (W-L)	1.07 ± 0.20 (4; 0.80–1.24)
CR2 (R-L)	0.29 ± 0.12 (5; 0.17–0.49)
CR2 (W-L)	0.17 ± 0.04 (5; 0.13–0.22)
CR3 (F-I)	0.12 ± 0.02 (5; 0.10–0.17)
CR1 + CR3	0.21 ± 0.12 (2; 0.12–0.29)
CR2 (R-L) + CR3	0.11 ± 0.02 (3; 0.08–0.12)
CR2 (W-L) + CR3	0.13 ± 0.06 (2; 0.09–0.17)

^a Relative helper activity was calculated by first normalizing the amount of AAV DNA to the amount of DBP gene DNA and then by normalizing the ratios obtained for the mutants to that obtained for the wild-type DBP gene.

FIG. 5. Effect of the mutations in the Ad5 DBP on its stability. Transfected COS-1 cells were labeled at 48 to 66 h posttransfection with [35 S]methionine, and immunoprecipitates were prepared from cell extracts. The immunoprecipitates were electrophoresed through 13% sodium dodecyl sulfate-polyacrylamide gels and fluorographed. Shown in the autoradiogram are the results for transfection with the wild-type (wt) DBP gene, one of the point mutants (CR3), and all three mutants containing two point mutations.

mine whether the mutant DBPs were in fact stable, we labeled transfected cells between 48 and 66 h with [35 S]methionine and prepared immunoprecipitates from the cell extracts. The results for one of the single-point mutants and all three double-point mutants are shown in Fig. 5. The amounts of DBP in the mutant transfected cells are comparable to those in wild-type gene-transfected COS-1 cells. Similar results were obtained for the other three single-point mutants (results not shown). Pulse labeling of transfected cells for 2 h yielded similar results, indicating that the reduced helper activity of the mutant DBPs is not due to a reduced rate of synthesis or an increase in turnover rate for these proteins.

DISCUSSION

We identified specific amino acid residues within the carboxy-terminal domain of the Ad5 DBP that are involved in one of the functions of the protein, replication of viral DNA. The regions of the DBP mutated were previously identified as potentially important in DBP function by comparing the amino acid sequences of DBPs from four distantly related adenovirus serotypes (34, 40, 41, 58). The three highly conserved regions identified by that analysis were the regions used for site-directed mutagenesis. Three of the four point mutants isolated decreased the ability of the DBP to function in AAV replication; one mutant (aa 323, Arg to Leu) was about fourfold less efficient in helping AAV DNA replication, and two mutants (aa 324, Trp to Leu; aa 469, Phe to Ile) were about 10-fold less active than was wild-type. The fourth mutant, a Trp-to-Leu mutation in CR1 (aa 181), showed helper activity for AAV DNA replication comparable to that of the wild-type DBP gene. Determination of whether other amino acid residues in CR1 are involved in DNA replication or any of the other functions of the DBP will have to await the isolation of more mutants in this region and their complete functional characterization. Thus, two of the three regions identified by comparative sequence analysis do contain residues important for the function of the DBP.

Effect of mutations on the DBP. The mutations do not alter the *in vivo* stability of the DBPs. In both short- (2 h) and long-term (18 h) labeling experiments, the quantity of mutant DBPs in transfected cells was always comparable to that of wild-type DBP. These mutations are unlike the *ts125* muta-

tion, which alters both the function (2, 11, 12) and stability (19) of the DBP. It is likely that the *ts125* mutation, a Pro-to-Ser change in aa 413, results in a change in the structure of the DBP at the nonpermissive temperature and that the structural change is the cause of the lack of function of the protein. Based on the conservation of the amino acid sequence and the lack of effect on the stability of the DBP, we postulate that the CR2 and CR3 mutations do not result in structural changes but are in amino acid residues that are part of the binding site for ssDNAs or that recognize and stimulate the activity of the DNA replication complex proteins (52) (since many ss DBPs are known to bind directly to DNA polymerases to influence their activities [39]). It is likely that the recently characterized mutant, Ad2⁺ND1^{ts23}, also produces a DBP with an alteration in an amino acid involved in DNA or protein recognition (57). The DBP gene of this temperature-sensitive mutant contains a Leu-to-Phe change at aa 282, which results in a protein that binds less well to ssDNA and does not support elongation of adenovirus DNA replication (57). Like the mutants described in this paper, the *ts23* DBP is stable *in vivo*, and exhibits the same digestion pattern as the wild-type DBP when incubated at various temperatures with the protease thermolysin. aa 282 is not in a highly conserved region of the DBP (34), but the four sequenced serotypes have either a leucine or a valine at this site. Collectively, the CR2 and CR3 mutants along with *ts23* probably define for the first time functional residues in the carboxy-terminal domain of the DBP.

Role of the DBP in AAV replication. The replication of AAV in human cells requires a number of early adenovirus functions. We show in the present study that in the transfection system, adenovirus early-gene regions E2a and E4 and the late-gene products VAI and VAI RNAs are required to attain maximal synthesis of AAV DNA. These results are in agreement with studies from other laboratories examining the constellation of adenovirus genes required for AAV helper activity when the adenovirus genes were introduced either by transfection (31) or by microinjection (59, 60). On the basis of their microinjection experiments, Richardson and Westphal postulated that the adenovirus early genes required for AAV helper activity may depend on the doses of individual genes introduced into the cells (60). For example, adenovirus E1a or E1b gene products are necessary in the microinjection assay when the dosage of E2a and E4 is below 100 copies per cell but not necessary when 200 copies per cell of E2a and E4 are injected. Similarly, E1b and E4 can provide AAV helper activity in the absence of E2a, providing the copy number of these two genes is sufficiently high (60). If this suggestion is correct, then the high level of DBP expression in our transfection system, caused by the presence of the SV40 origin of replication and early promoter and the use of COS-1 cells, compensates for the lack of E1b and renders the system insensitive to the addition of E1a. Taken together, our results indicate that although E4 is absolutely required for AAV replication, the functions provided by E1b and E2a can apparently be used interchangeably. How can these two gene products function interchangeably for AAV helper activity? We propose that the requirement for either E1b or E2a is a manifestation of the need to protect AAV ssDNA from nuclease attack. The product of the E1b region is known to inhibit DNases (16, 43), whereas the product of E2a (the DBP) could physically protect the input ssDNA genomes and newly replicated AAV ssDNA from attack by DNases. High levels of E1b protein could inhibit the activity of nuclear DNases sufficiently to protect AAV ssDNA and permit conversion to a

double-stranded form. Laughlin et al. (44) have shown that AAV DNA is degraded in the absence of an adenovirus E1 gene product, probably E1b, strengthening the argument that the AAV DNA must be protected for DNA replication to occur. It should be pointed out that the E1b mutants used in their studies also contained deletions in E1a, so there was little or no transcription of the E2a gene and no production of the DBP to compensate for the absence of the E1b proteins.

The ability of either the E1b or E2a gene products to protect AAV ssDNA could explain the discrepancy between the results obtained in this study and the results obtained by McPherson et al. with the Ad5 mutant *ts125* (50). In that study, the *ts125* virus was as efficient a helper at the nonpermissive temperature as was the wild-type virus, implying that E2a was not required for AAV helper activity. This result can be explained by the fact that in the absence of a functional DBP the levels of E1b mRNAs and protein increase (11, 12), thereby potentially compensating for the lack of DBP with increased levels of DNase inhibitor. When the *ts125* DBP gene is placed into the expression vectors used in the present study and analyzed for AAV helper activity in cotransfection experiments with E4 and the VA RNA genes, no helper activity is observed at the nonpermissive temperature, consistent with the need for either an active E1b or E2a gene product.

On the basis of the preceding discussion, we propose that our results with the mutant DBPs indicate that the decrease in AAV DNA replication is, at least in part, the result of a decrease in the ability of the mutant proteins to bind to and protect AAV ssDNA. We have direct-binding data that supports this proposal; the CR2 and CR3 mutant DBPs, which are less efficient in helping AAV DNA replicate, bind less tightly to ssDNA (G. A. M. Neale and G. R. Kitchingman, manuscript in preparation). We cannot rule out effects of the mutations on other proposed functions of the DBP in AAV replication, including splicing of AAV mRNAs (51), translation of AAV polypeptides (32), and an effect on the expression of the adenovirus E4 gene (59). We know that the mutations in the DBP genes do not affect the accumulation of AAV mRNAs, since quantities of mRNAs are roughly equivalent in wild-type and mutant DBP transfected cells (G. R. Kitchingman, unpublished data). We are currently examining the structure of AAV mRNAs (24) synthesized in wild-type and mutant DBP transfected cells to determine whether the DBP mutations have any effect on the splicing of the AAV mRNAs (51).

Functions of other adenovirus gene products in AAV replication. Recently, the E4 region of adenovirus was shown to have a role in the replication of adenovirus DNA (26, 69), which is not surprising in light of its known function in AAV DNA replication (10, 31, 59). As shown here, the amplification of AAV RF DNA is absolutely dependent on the presence of a functional E4 gene region, consistent with the results of Carter et al. (10), who found a 10-fold reduction in the accumulation of AAV DNA when cells were coinfecting with AAV and Ad2d/807, an E4 deletion mutant. Infection of human cells with adenoviruses containing mutations in the E4 gene results in the synthesis of reduced quantities of viral DNA (26, 69), similar to the decrease observed in the transfection system for AAV DNA replication when E4 is not present. With the transfection assay described here, it might be possible to determine which of the complex array of 18 to 24 separate mRNAs encoded by the E4 region (65) produce proteins that are involved directly or indirectly in DNA replication.

The other adenovirus genes required for AAV helper activity (31, 44, 60) are most likely required to enhance the level of expression of E2a, E4, and the AAV genes required for replication. The E1a region is known to code for transcriptional enhancers which are required under conditions of normal infection to initiate transcription from the other adenovirus early promoters (7). The function of the VA RNA genes in AAV replication is probably to enhance the translation of AAV mRNAs (31) and perhaps adenovirus early mRNAs. The ability of the VA RNAs to enhance translation of viral and nonviral mRNAs has been documented by several laboratories (33, 63, 64), and in the transfection system used here, the accumulation of AAV ssDNA probably reflects the enhancement of translation of mRNAs for one or more AAV-coded polypeptides (28, 66).

In summary, we constructed by site-directed mutagenesis techniques a series of mutants with four point mutations and three double-point mutations in the DBP of Ad5. Three of the point mutations, located in two distinct regions of the carboxy-terminal domain of the DBP, affect the ability of the DBP to help AAV DNA replicate. The defect in AAV DNA replication results in the accumulation of lower amounts of RF DNA, with the synthesis of ssDNA progeny from the RF being unaffected by the DBP mutations. The mutations in the DBP are situated in the highly conserved regions originally identified by comparative DNA sequence analysis and probably represent functional residues of the protein.

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