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Mutations That Affect Phosphorylation of the Adenovirus DNA-Binding Protein Alter Its Ability To Enhance Its Own Synthesis

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The multifunctional adenovirus single-strand DNA-binding protein (DBP) is highly phosphorylated. Its phosphorylation sites are located in the amino-terminal domain of the protein, and its DNA- and RNA-binding activity resides in the carboxy-terminal half of the polypeptide. We have substituted cysteine or alanine for up to 10 of these potential phosphorylation sites by using oligonucleotide-directed mutagenesis. Alteration of one or a few of these sites had little effect on the viability of virus containing the mutated DBP. However, when eight or more sites were altered, viral growth decreased significantly. This suggests that the overall phosphorylation state of the protein was more important than whether any particular site was modified. The reduction in growth correlated with both depressed DNA replication and expression of late genes. This reduction was probably the result of lower DBP accumulation in mutant-infected cells. Interestingly, although the stability of the mutated DBP was not affected, DBP synthesis and the level of its mRNA were depressed 5- to 10-fold for the underphosphorylated protein. These results suggest that DBP enhances its own expression and imply that phosphorylation of the DBP may be important for this function. Similarities to several eucaryotic transcriptional activators, which are composed of negatively charged activating domains and separate binding domains, are discussed.

It has become increasingly evident that phosphorylation is a major mechanism used to modulate the activity of many proteins. The importance of this posttranslational modification was first shown by the pioneering work of Krebs and Fisher (38) on the regulation of glycogen metabolism. Since then, many examples of activation or inhibition of protein function have been documented (for reviews, see references 17 and 28). For example, autophosphorylation of tyrosine kinases such as pp60$^{c\text{-}src}$ increase their activity (51), whereas eIF2α function is inhibited by this modification (for a review, see reference 56). However, the role of phosphorylation in the function of many proteins is unclear. For instance, the loss of individual autophosphorylation sites of the epidermal growth factor receptor does not appear to alter its biological properties (27). Similarly, altering the phosphorylation states of the adenovirus E1a 289-amino-acid protein (18, 54, 67) or E1b 176-amino-acid protein (45) had few detectable effects.

The adenovirus DNA-binding protein (DBP) is a 72-kilodalton phosphoprotein, which accumulates in nuclei of infected cells at both early and late times (7, 40, 65, 70). Numerous functions have been attributed to the DBP, and these are important in several steps of the infectious cycle. The protein contains at least two functionally distinct domains (amino terminal and carboxy terminal), which can be separated by chymotrypsin treatment (33, 43).

Several functions have been assigned to the carboxy-terminal domain. The carboxy-terminal 44-kilodalton fragment generated by chymotrypsin cleavage retains the ability to bind single-stranded DNA and RNA (15, 33) and is required for viral DNA replication (20, 48, 69). Mutants such as H5*ts*125, which contain temperature-sensitive mutations in this domain, fail to replicate their DNA. Moreover, the 44-kilodalton carboxyl fragment can complement the H5*ts*125 DBP for DNA replication in vitro (6). Studies of these *ts* mutants and their revertants have also suggested roles for this domain in the regulation of early viral gene expression (8, 12) as well as viral assembly (49).

The amino-terminal domain of DBP affects viral late gene expression and host range. Wild type (wt) human adenovirus infection of monkey cells is abortive because of reduced production of late mRNAs (29, 35) and altered splicing and poor translation of the fiber mRNA (3). A set of host range mutants carrying an identical histidine-to-tyrosine change at position 130 in the amino-terminal domain of DBP (1, 11, 37, 39) overcome the block to late gene expression and multiply efficiently in monkey cells.

Not only are the two domains of DBP functionally distinct, but also they exhibit an asymmetric distribution of amino acids. The carboxyl part of the protein is very highly conserved among different serotypes (31, 71). In contrast, the sequence of the amino-terminal region is only poorly conserved. This domain is very rich in proline residues and contains a high content of both basic and acidic amino acids. The amino-terminal domain contains most, if not all, of the estimated 1 to 15 phosphates bound per DBP molecule (33). Most of these phosphates appear to be linked to serine and threonine residues by a monoester bond (42).

The role of phosphorylation of DBP has been primarily a matter of conjecture. It has been proposed that the various phosphorylated forms of DBP may carry out the different roles of this multifunctional protein. Only the effect of this posttranslation modification on DNA binding has been directly tested. Linne and Philipson (43) found that removal of the majority of phosphates by alkaline phosphatase did not alter binding to single-stranded DNA. However, Klein et al. (33) inferred from their studies that the less phosphorylated species of DBP bound single-stranded DNA more tightly than the highly phosphorylated forms did.

To understand the function of this prevalent modification of DBP, we have systematically altered 10 potential phosphorylation sites of the adenovirus type 5 (Ad5) DBP. These 10 sites have been preliminarily mapped on the very closely related Ad2 DBP (2; R. Mann, Ph.D. thesis, New York

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University, New York, 1987). The mutant genes with one or more altered sites have been introduced into the viral genome by in vivo recombination. The phenotypes of these mutants suggest that the overall phosphorylation state of this protein may be important for its expression. This result, together with results of several other early studies (16, 53), argues that DBP enhances its own expression.

MATERIALS AND METHODS

Cells, viruses, and infections. The human 293 and HeLa cell lines were obtained from J. Williams, Carnegie Melon University. The gmDBP2 cell line, which contains and expresses DBP under the control of the mouse mammary tumor virus promoter, has been described elsewhere (36). All the cells were grown in Dulbecco modified Eagle medium (Flow Laboratories, Inc.) supplemented with 100 μg of streptomycin and 100 μg penicillin per ml, 2 mM glutamine, and 10% calf serum.

The wt Ad5 virus was originally obtained from J. Williams. Ad5*hr*404 was isolated by Klessig (34; also see reference 37). Viral stocks were produced in HeLa suspension cells. Plaque assays for all viruses were performed as described previously (23). Infections were carried out with 10 PFU per cell.

Plasmid constructs and site-directed mutagenesis. M13K801 through M13K807 (801 through 807 refer to a specific mutation [see Fig. 1]) vectors were constructed by inserting the Ad5*hr*404 *Sac*I-*Kpn*I fragment (61.3 to 68.9 map units [mu]), containing the coding region of the host range DBP gene, into the *Sac*I and *Kpn*I sites of M13Mp19 (72). The oligonucleotide site-directed mutagenesis was performed by the procedure described by Zoller and Smith (74). Single-stranded DNA was isolated by M13K801 through M13K807 infections of *E. coli* JM109. The oligonucleotides, designated as Ka through Kg, were prepared by the Center for Advanced Biotechnology and Medicine of New Jersey Network Laboratory and purified by polyacrylamide gel electrophoresis. Single-stranded DNA template (0.5 pmol) was annealed with 10 pmol of 5′-phosphorylated oligonucleotide(s) (see Fig. 1A) and 10 pmol of unphosphorylated M13 sequencing primer (Boehringer Mannheim Biochemicals). The oligonucleotides were extended with the Klenow fragment of DNA polymerase I (Boehringer Mannheim) and deoxynucleoside triphosphates. After ligation with T4 DNA ligase, the DNA was used to transform competent JM109. Plates with approximately 50 to 100 plaques were replicated on nitrocellulose filters. The filters were subsequently hybridized for 16 h at 55°C in hybridization solution (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 10× Denhardt solution) with appropriate mutant oligonucleotides labeled at their 5′ ends with [γ-^{32}P]ATP. Autoradiograms were performed first after washes at low stringency (40°C in 6× SSC) and then after washes at high stringency (60 to 70°C in 6× SSC to as low as 0.5× SSC, as described elsewhere [74]). Putative mutant bacteriophages were plaque purified a second time before their single-stranded DNAs were screened for mutations by Dot blot hybridization as described previously (74). By using this technique in a sequential manner, we inserted several mutations at different positions in the same gene. The introductions of the desired mutations were all confirmed by DNA sequencing by using the dideoxy technique (57) with the Sequenase kit (U.S. Biochemical Corp.).

Construction, propagation, and plaque assay of the viral mutants. Mutated DBP genes were inserted in pXE plasmid (a gift from the laboratory of J. S. Sussenbach). pXE contains the wt Ad5 DNA sequence from 60 mu (*Bam*HI site) to 100 mu (right end to which a *Sal*I linker was attached) cloned into pBR322. Part of the nonessential E3 region of Ad5 between the *Xho*I and *Eco*RI sites at 82.9 and 84 mu, respectively, has been deleted in pXE, which results in the loss of both sites (70a). The wt *Mlu*I fragment (62.8 to 68 mu) of pXE was substituted by the various (M13K801 through M13K807) mutant fragments. The resulting plasmids are referred to as pXEK801 through pXEK807. The adenovirus sequences in these plasmids were extended from 60 to 42 mu by insertion of the Ad2 *Bam*HI fragment (42 to 60 mu). The resulting Ad2-Ad5 hybrid plasmids, containing the adenovirus sequence between 42 and 100 mu in *Bam*HI and *Sal*I sites of pBR322, are referred to as BMSK801 through BMSK807. The procedures used for cloning were described by Maniatis et al. (44).

Ad5 DNA-terminal protein complex (prepared by the method of Chinnadurai et al. [14]) after digestion with *Bam*HI and *Eco*RI was mixed independently with each of the purified *Sal*I fragments (46 to 100 mu) of plasmids BMSK801 to BMSK807. The mixtures were used to transfect 293 and dexamethasone-treated gmDBP2 cells by the calcium phosphate procedure (21). Individual plaques were picked and plaque purified a second time before preparation of stocks on HeLa cells. Adenovirus DNA was prepared as described by Hirt (25), except that 1 mg of pronase per ml was included in the initial lysis buffer, and the plate was incubated at 37°C for 2 h prior to addition of NaCl to 1 M and incubation on ice. The Hirt DNAs were digested with *Eco*RI or *Xho*I to test for a new restriction enzyme pattern characteristic of the recombinant virus (i.e., loss of the 82.9-mu *Xho*I and 84-mu *Eco*RI sites and absence or presence of the Ad2 *Eco*RI site at 58.5 mu) and also subjected to Southern blot analyses (63) with the different mutant oligonucleotides used independently as probes. Hybridization was carried out for 6 h at 55°C in 10 ml of hybridization buffer (6× SSC, 10× Denhardt solution) with 2×10^6 cpm of oligonucleotide-labeled probe. Filters were first washed at low stringency and autoradiographed before and after washes at increasing stringencies (60 to 70°C in 6× SSC to as low as 0.5× SSC). The identified mutated viruses were grown to large stocks on HeLa cells as described by Rice and Klessig (53).

Radioactive labeling. Virus- and mock-infected cultures were labeled with [^{35}S]methionine (50 μCi/ml; Amersham Corp.) in Dulbecco modified Eagle medium without methionine (Flow Laboratories) and without serum for times ranging from 10 min to 4 h (as described below). Label was chased, when indicated, by washing the cells with prewarmed medium containing a 1,000-fold excess of unlabeled methionine and then incubating them in this medium with 2% calf serum for the times indicated.

Viral proteins were labeled with ^{32}P by incubating the infected cells with 200 μCi of carrier-free $^{32}P_i$ (Amersham) per ml in culture medium without phosphate and supplemented with 5% dialyzed calf serum. Cells were washed three times in phosphate-buffered saline, scraped in 10 mM NaCl–10 mM Tris hydrochloride (pH 7.4), and frozen at −20°C.

DNA was labeled with [^{3}H]thymidine (Du Pont, NEN Research Products) by incubating infected cells for 5 h in medium without serum and containing 100 μCi of [^{3}H]thymidine per ml. DNA was extracted by the method of Hirt (25).

Protein analyses. Rabbit polyclonal antibodies against DBP, fiber, and hexon proteins (4, 36) were used for immu-

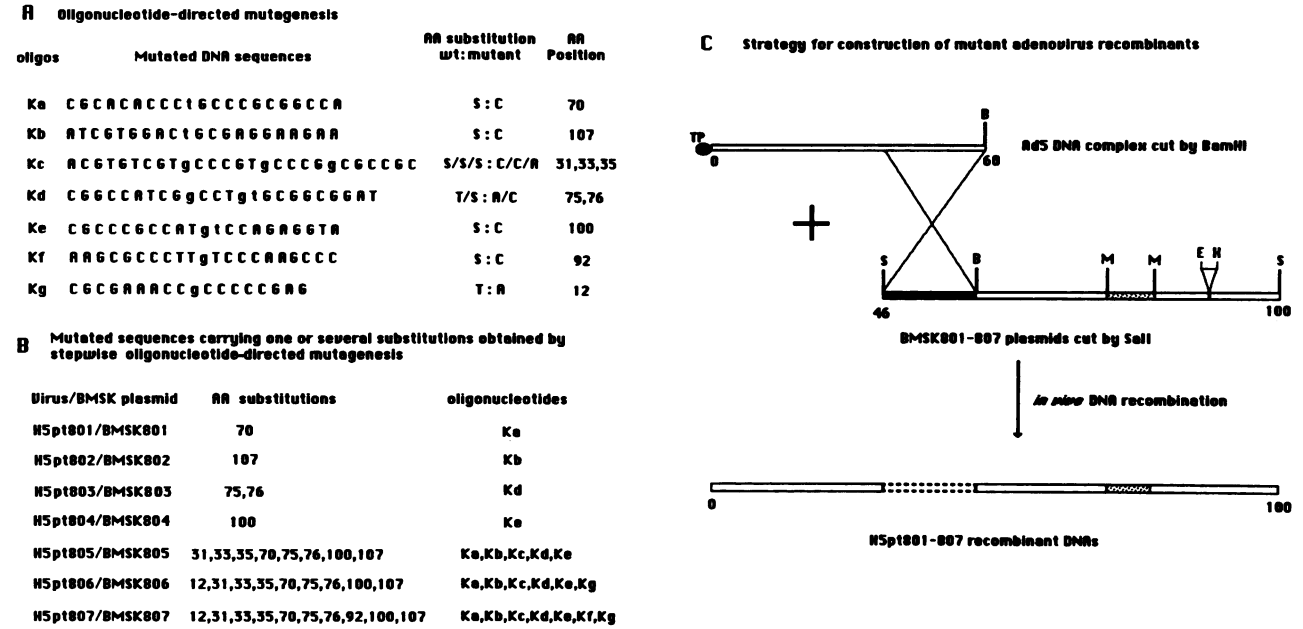

FIG. 1. Mutagenesis strategy. (A) The sequences of the primers used for mutagenesis are shown 5′ to 3′. The mutated nucleotides are shown in lowercase letters. The alterations these cause at the amino acid level are shown in the middle and right-hand columns. (B) The names of the mutant viruses and corresponding plasmids are indicated in the left-hand column. The locations of the mutations are given in the middle column, and the oligonucleotides used in the stepwise mutagenesis are listed in the right-hand column. (C) The strategy for construction of the mutant viruses is based on in vivo recombination between BMSK plasmids and terminal protein (TP)-Ad5 DNA complex. The coordinates given in map units on the Ad5-Ad2 genome are denoted by numbers, and relevant restriction enzyme sites are indicated as follows: S, *Sal*I; E, *Eco*RI; M, *Mlu*I; X, *Xho*I; B, *Bam*HI. Symbols: ■, Ad2 sequence; □, Ad5 sequence; ▩, mutated Ad5 sequence; ▽, deletion in the nonessential E3 region; ⊏⊐⊏⊐, Ad2 or Ad5 sequence depending on the site of homologous recombination.

nological analyses. Immunoprecipitations were carried out by the protein A method of Kessler (30), as described in detail elsewhere (10). Proteins were immunoblotted by a method described previously (4, 36), with the substitution of 5% nonfat dry milk (Carnation) for 5% bovine serum albumin. Two-dimensional gel electrophoresis was performed as described by O'Farrell (50); Pharmalyte 5-8 and 3-10 were purchased from Pharmacia Fine Chemicals and used in a ratio of 3:2; sodium dodecyl sulfate (SDS)–10% polyacrylamide gels were run for the second dimension.

RNA analyses. Cytoplasmic RNAs were prepared as described by Yen et al. (73). RNAs were fractionated on formaldehyde-agarose gels and transferred to nitrocellulose paper for Northern (RNA) analysis by the method of Maniatis et al. (44).

RESULTS

Construction of mutant DBP viruses. Anderson et al. (2; also see Mann, Ph.D. thesis) tentatively mapped a large number of phosphorylation sites on Ad2 DBP. At least 10 of these, located at amino acids 12, 31, 33, 35, 70, 75, 76, 92, 100, and 107, are shared with Ad5. Using oligonucleotide-directed mutagenesis, we have systematically altered these serine or threonine codons in the Ad5 DBP gene to those encoding the conservative amino acid substitution cysteine or alanine (Fig. 1A). Seven mutant DBP genes (Fig. 1B) were constructed and then introduced via several steps into a large plasmid, BMSK, which contained the right half of the adenovirus genome (see Materials and Methods). The resulting plasmids (BMSK801 through BMSK807) contained Ad2 sequences from 42 to 60 mu and Ad5 sequences from 60 to 100 mu with the altered DBP genes and a deletion of part of the nonessential E3 region (Fig. 1C). These plasmids, together with *Eco*RI- and *Bam*HI-cleaved terminal protein-Ad5 DNA complex, were transfected into 293 cells to generate mutant DBP viruses via in vivo recombination (Fig. 1C). This strategy (13, 26), based on recombination between two large fragments of the adenovirus genome (0 to 60 and 46 to 100 mu), resulted in efficient generation of mutant viruses with low background of wt virus. gmDBP2 cells, which contain and express a wt DBP gene (36), were also transfected to ensure the recovery of viruses carrying lethal DBP mutations.

The viral mutants are essentially Ad5 in DNA sequences, except for the region between mu 46 and 60, which is partially Ad2 and Ad5. The recombination events occurring in this region probably generated Ad2-Ad5 hybrid hexon genes, since the hexon gene occupies the majority of the region. To ensure that the hybrid hexon proteins were not responsible for the phenotypes of the DBP mutants, several viral recombinants were made with a wt Ad5 DBP gene. These recombinants had phenotypes identical to that of wt Ad5 (data not shown), except that the mobility of the hybrid hexon protein was sometimes more similar to that of Ad2 than Ad5, presumably depending on where recombination occurred.

Seven mutant DBP viruses (H5*pt*801 through H5*pt*807) were isolated from transfected 293 cells. Their identity was confirmed first by restriction enzyme analysis of their DNA (presence of an Ad2 site in the 46- to 60-mu region and absence of part of the E3 region) to ensure that they were generated by recombination and second by hybridization of their DNA with the appropriate mutant oligonucleotides to verify the presence of the altered phosphorylation sites in

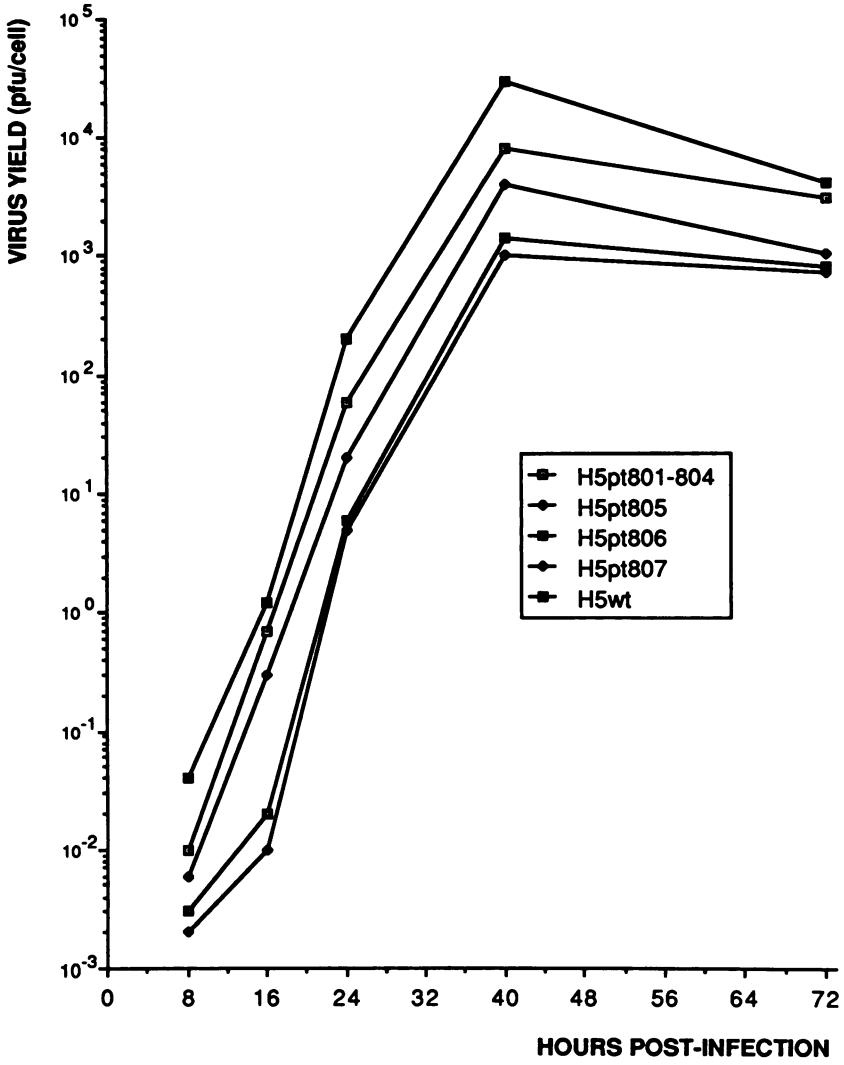

FIG. 2. Kinetics of growth of the mutant virus. HeLa cell monolayers, infected with the phosphorylation mutants H5*pt*801 through H5*pt*807 and wt Ad5 (H5wt) as a control, were harvested at 8, 16, 24, 48 and 72 h postinfection. Virus yields were determined by titration of the cell lysates on HeLa cell monolayers.

the DBP gene. These seven mutants contain 1 to 10 substituted codons that remove potential phosphorylation sites (Fig. 1B).

Kinetics of viral growth. All seven mutants were isolated on 293 cells, which implied that removal of up to 10 phosphorylation sites was not lethal. To determine whether the loss of these phosphorylation sites might have less dramatic yet detectable effects on virus viability, we compared their growth kinetics with those of wt Ad5 on HeLa cells (Fig. 2). None of the mutants showed delayed growth, as was recently observed for H5*dl*802r1, a mutant which contains a large deletion in the amino-terminal domain that removes many of the phosphorylation sites (16). Substitution of one or two sites (H5*pt*801 through H5*pt*804) had little effect on the final virus yield. However, as more sites were removed, viral growth was increasingly diminished. Substitution of 10 potentially phosphorylated amino acids in H5*pt*807 resulted in a 30-fold reduction in viral growth.

Synthesis, stability, and accumulation of mutant DBP. Synthesis of the mutant DBPs in infected HeLa cells was analyzed by labeling the proteins with [^{35}S]methionine and immunoprecipitating DBP with rabbit polyclonal antiserum. Although the mutants with one or a few phosphorylation sites removed exhibited rates of DBP synthesis similar to that of wt Ad5, mutants H5*pt*805 through H5*pt*807, with 8, 9, and 10 sites missing, respectively, showed depressed DBP synthesis (Fig. 3 and 4A).

During wt infection, DBP synthesis is enhanced as viral DNA replication commences, owing at least in part to increased template numbers (7, 40). If the DBP mutations affected DNA replication, this might account for the altered DBP synthesis. To test this, viral DNA replication was inhibited with hydroxyurea. DBP synthesis in H5*pt*801-infected cells was only slightly diminished, whereas that in H5*pt*802-, H5*pt*803-, and H5*pt*804-infected cells was comparable to that in wt Ad5 (Fig. 3, lanes 1 to 4). In contrast, synthesis of H5*pt*805 through H5*pt*807 DBP was depressed 5- to 10-fold even in the absence of DNA replication (Fig. 3, lanes 5 to 7).

Although this suggested that loss of phosphorylation sites had a direct effect on DBP synthesis, substitution of eight or more amino acids might alter the conformation of the protein

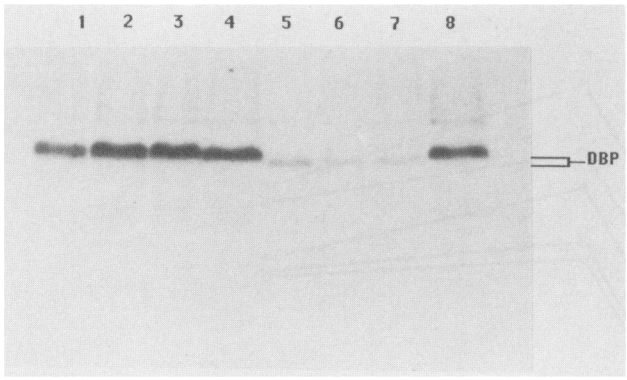

FIG. 3. DBP synthesis. Mutant DBP HeLa cells were infected with 10 PFU of mutant virus H5*pt*801 (lane 1), H5*pt*802 (lane 2), H5*pt*803 (lane 3), H5*pt*804 (lane 4), H5*pt*805 (lane 5), H5*pt*806 (lane 6), H5*pt*807 (lane 7), or wt Ad5 (lane 8) per cell, and 5 mM hydroxyurea was added 2 h postinfection. Infected cells were labeled 16 h later with [^{35}S]methionine for 25 min. DBP polypeptide was immunoprecipitated, fractionated by SDS-PAGE on a 15% gel, and autoradiographed.

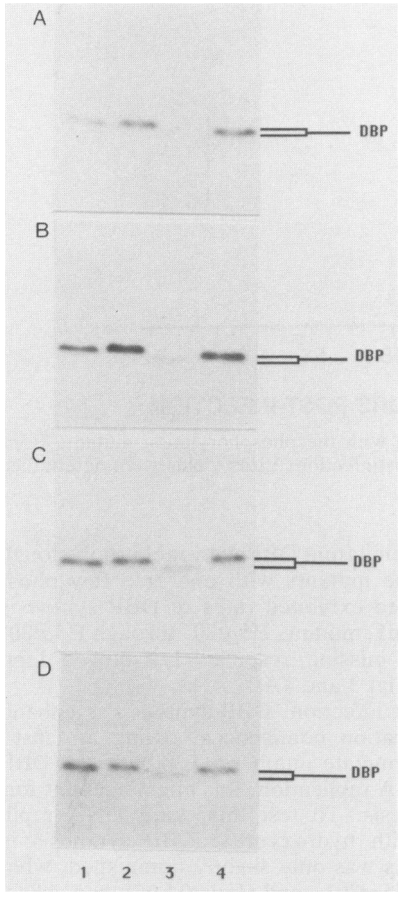

FIG. 4. Stabilities of mutant DBPs. HeLa cell monolayers were infected in the absence of hydroxyurea with H5*pt*801 (lane 1), H5*pt*804 (lane 2), H5*pt*807 (lane 3), and wt Ad5 (lane 4). The stability of DBP was determined by immunoprecipitating equal fractions of infected-cell lysates with anti-DBP serum after a 10-min [^{35}S]methionine pulse (A) at 20 h postinfection, followed by a 15-min (B), 45-min (C), or 120-min (D) chase. Products were fractionated by SDS-PAGE and autoradiographed.

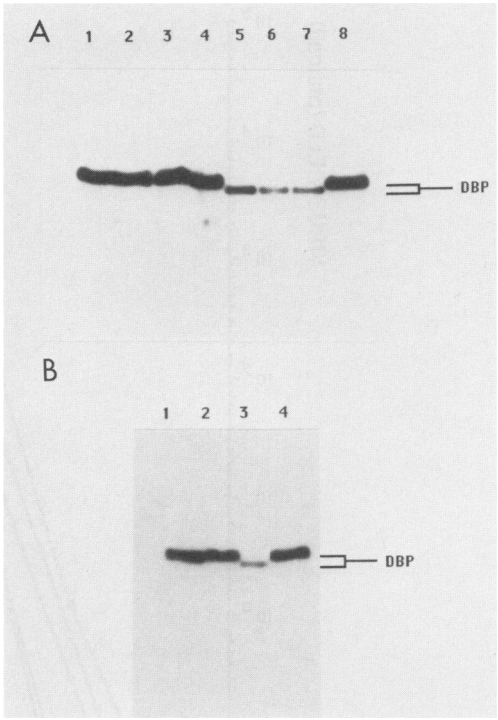

FIG. 5. Accumulation of Ad5 and mutant DBPs. (A) In a parallel experiment to that shown in Fig. 3, the levels of DBP which had accumulated by 16 h postinfection in the presence of hydroxyurea were determined by immunoblotting by using SDS-PAGE (on 10% gels), a rabbit polyclonal anti-DBP serum, and ^{125}I-labeled protein A (10 μCi). Lanes 1 through 7 correspond to the protein extracts from H5*pt*801- through H5*pt*807-infected cells, and lane 8 represents the wt Ad5 control. (B) In a parallel experiment to that shown in Fig. 4, the levels of mutant DBP (H5*pt*801 [lane 1); H5*pt*804 [lane 2), and H5*pt*807 [lane 3]) and wt DBP (lane 4) which had accumulated at 20 h postinfection in the absence of hydroxyurea were determined by immunoblot analysis.

and hence its stability. The stability of several mutant DBPs was compared with that of wt DBP by pulse-chase analyses (Fig. 4). At 20 h postinfection, HeLa cells were labeled with [^{35}S]methionine for 10 min and then chased with excess unlabeled methionine for up to 2 h. H5*pt*807 DBP synthesis during the 10-min pulse was again significantly reduced (ca. 10-fold), whereas H5*pt*801 DBP synthesis was only slightly depressed and H5*pt*804 produced similar levels to that of wt virus. The amount of labeled DBP for Ad5, H5*pt*801, and H5*pt*804 was partially reduced during the chase, indicating that these polypeptides were moderately unstable. In contrast, there was little change in the level of labeled H5*pt*807 DBP, suggesting that it was at least as stable as the wt protein.

Accumulation of DBP in mutant-infected HeLa cells during the infectious cycle was also determined by using immunoblot analysis. Mutant DBPs with only one or a few phosphorylation sites removed (H5*pt*801 through H5*pt*804) accumulated to wt levels in the presence (Fig. 5A) or absence (Fig. 5B) of hydroxyurea. However, accumulation of the more dephosphorylated DBPs (H5*pt*805 through H5*pt*807) was depressed ca. 10- to 20-fold compared with wt DBP, consistent with their reduced rates of synthesis (Fig. 3).

Level of mutant DBP mRNA. That the depressed level of DBP was due to reduced synthesis rather than decreased

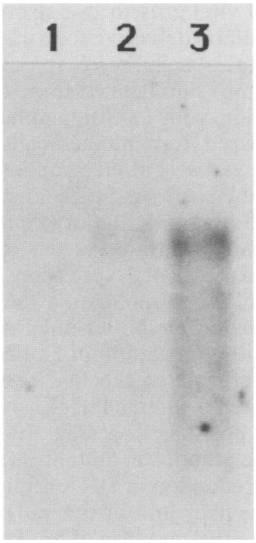

FIG. 6. Steady-state levels of DBP mRNA. HeLa cell monolayers were mock infected (lane 1) or infected with H5*pt*807 (lane 2) or wt Ad5 (lane 3); 5 mM hydroxyurea was added 2 h postinfection, and RNA was extracted for Northern blot analyses 22 h later. Cytoplasmic RNAs (10 μg each) were fractionated on formaldehyde-agarose gels, transferred to nitrocellulose filters, and hybridized to a probe specific to the DBP gene. Analysis of RNA extracted 24 and 48 h postinfection in the absence of hydroxyurea by RNase protection mapping (46) gave similar results.

stability of the mutant proteins was consistent with the analyses of the steady-state levels of their mRNA. The level of DBP mRNA was 5- to 10-fold lower in H5*pt*807- than Ad5-infected cells (Fig. 6) (in other experiments which used RNase mapping H5*pt*807 DBP mRNA was found to be depressed 10-fold or more). Thus, mutations which result in the loss of a large number of phosphorylation sites affect the accumulation and, more importantly, the synthesis of DBP and the level of its mRNA. This suggests that (i) DBP enhances its own synthesis and (ii) its state of phosphorylation may be important for this function.

Phosphorylation of the mutant DBPs. Several criteria were used to show that the mutations altered the phosphorylation

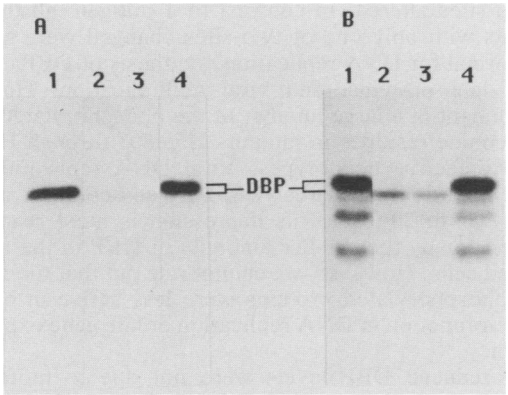

FIG. 7. Phosphorylation of mutant DBPs. Mutant (H5*pt*804 [lane 1], H5*pt*807 [lane 2], and H5*pt*806 [lane 3]) and wt (lane 4) Ad5-infected HeLa cell monolayers were labeled for 3.5 h at 24 h postinfection with either $^{32}P_i$ (A) or [^{35}S]methionine (B). Cell lysates were immunoprecipitated with anti-DBP serum prior to SDS-PAGE on 15% gels and autoradiography.

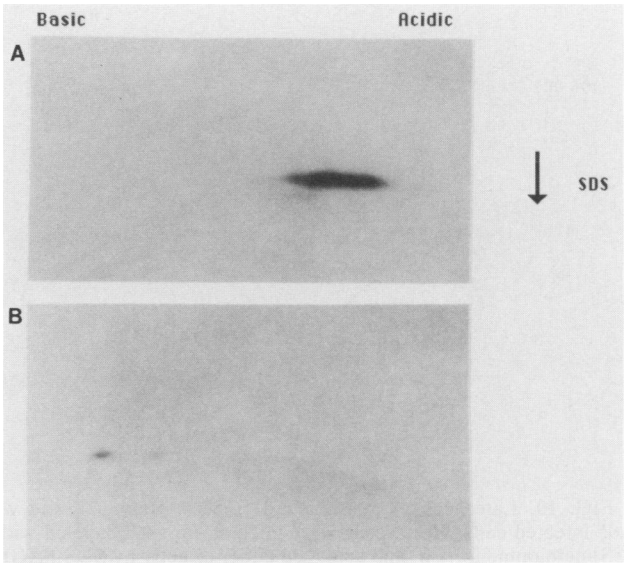

FIG. 8. Two-dimensional gel fractionation of wt Ad5 DBP (A) and H5*pt*807 DBP (B). At 24 h postinfection, HeLa cell lysates were fractionated by isoelectric focusing and SDS-PAGE on 12% gels, followed by immunoblot analysis with anti-DBP serum. The pH range in the first dimension (horizontal) was ca. 5 to 8. Shorter exposures of the autoradiograph in panel A shows distinct species (spots) of DBP.

of DBP. First, DBPs with a large number of sites removed (H5*pt*805 through H5*pt*807) exhibited increased mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5). This is consistent with the observation that removal of phosphate group in vitro with alkaline phosphatase resulted in a similar shift in mobility (33, 43).

Second, the amounts of phosphorylation of the various DBPs were compared by labeling with ^{32}P for 3.5 h at 24 h

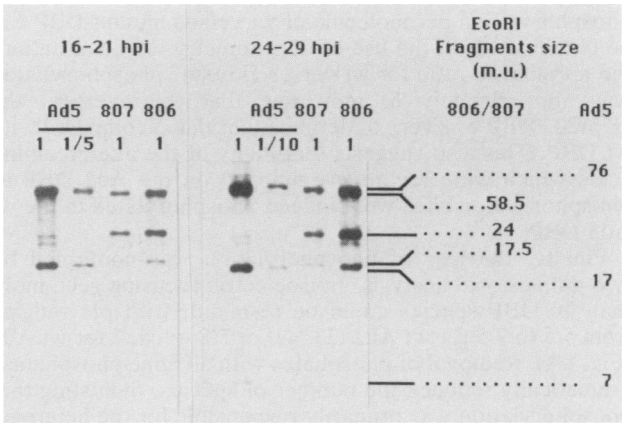

FIG. 9. Viral DNA replication in H5*pt*806-, H5*pt*807-, and wt Ad5-infected cells. Infected HeLa cells were labeled from 16 to 21 or 24 to 29 h postinfection (hpi) with [^{3}H]thymidine. DNAs purified by Hirt extraction were digested with *Eco*RI, separated on a 0.8% agarose gel, transferred onto a nitrocellulose filter, and fluorographed. Two different amounts of wt Ad5 DNA were loaded for easier quantitation. The size of the *Eco*RI-generated DNA fragments (in kilobases) are indicated on the right for mutants and Ad5. The mutants lack the Ad5 *Eco*RI site at 84 mu in the E3 region but contain the Ad2 *Eco*RI site at 58.5 mu. The levels of viral DNA were quantitated by comparison of the intensities of mutant 17.5-kilobase and wt 17-kilobase fragments.

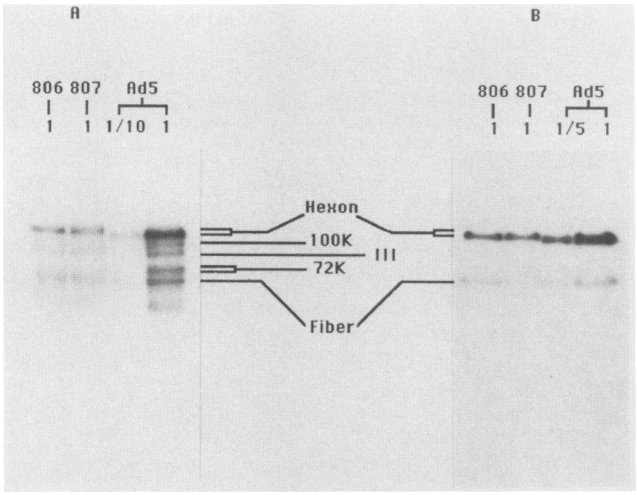

FIG. 10. Late gene expression in H5*pt*806-, H5*pt*807-, and wt Ad5-infected cells. HeLa cells were infected for 24 h, labeled with [^{35}S]methionine for 1 h, and analyzed either directly by SDS-PAGE on 15% gels (A) or after immunoprecipitation with a mixture of polyclonal anti-fiber and anti-hexon antibodies (B). Two different amounts of Ad5 protein extracts were loaded for easier quantitation. The positions of several viral polypeptides are indicated. The hexon polypeptides of H5*pt*806 and H5*pt*807 have a mobility intermediate between those of Ad2 and Ad5 wt hexon polypeptides.

postinfection (Fig. 7A). To take into account the reduced synthesis and accumulation of several of the mutant DBPs, in a parallel set of infections the level of newly synthesized DBP was monitored during the same period by labeling with [^{35}S]methionine (Fig. 7B). Phosphorylation is believed to occur within several hours of DBP synthesis, based on changes in its mobility and its increased heterogeneity in two-dimensional gels (33) (see below). Thus, by normalizing the ^{32}P ratio for wt versus mutant DBP with the [^{35}S]methionine ratio, an estimate of the relative level of phosphorylation per molecule of wt versus mutant DBP can be obtained. With the use of densitometry for quantitation, the normalized ratio for wt versus H5*pt*807 phosphorylation was approximately 6, indicating that, on average, the H5*pt*807 DBP was very underphosphorylated compared with wt DBP. This also suggests that many of the altered amino acids which were tentatively mapped on the Ad2 DBP as phosphorylation sites were indeed phosphorylated in the wt Ad5 DBP.

Finally, the loss of phosphorylation was confirmed by two-dimensional analysis. In isoelectric focusing gels, more than 10 DBP species could be resolved, with pIs ranging from 5.5 to 7.5 for wt Ad2 (33, 43) or 5.8 to ca. 7 for wt Ad5 (Fig. 8A). Removal of phosphates with alkaline phosphatase dramatically reduced the number of species, indicating that phosphorylation was primarily responsible for the heterogeneity. In contrast to wt Ad5, only two major species of H5*pt*807 DBP were detected (Fig. 8B). They had pIs of approximately 7.3 and 7.5, consistent with the shift in pIs resulting from alkaline phosphatase treatment (33).

Thus, by these criteria (mobility shift, ^{32}P labeling, reduced heterogeneity, and increased pI), many, if not most, of the phosphorylation sites have been removed in H5*pt*807. However, this mutant DBP can still be labeled to a low level with ^{32}P, indicating that at least one site remains.

Viral DNA replication. Since DBP is essential for DNA synthesis and as H5*pt*806 and H5*pt*807 were deficient for DBP synthesis and viral growth, we strongly suspected that these mutants would be defective for viral DNA replication. To verify this, HeLa cells were infected with wt Ad5, H5*pt*806, and H5*pt*807 and labeled from 16 to 21 or from 24 to 29 h postinfection with [^{3}H]thymidine. Low-molecular-weight DNAs prepared from infected cells were analyzed by *Eco*RI digestion, agarose gel electrophoresis, and fluorography of the blotted DNA (Fig. 9). Synthesis of H5*pt*806 was reduced 2- to 5-fold, whereas that of H5*pt*807 was depressed 5- to 10-fold compared with that of wt Ad5.

Viral late gene expression. Expression of adenovirus late genes requires viral DNA replication (66). In addition, the heavily phosphorylated amino-terminal region of DBP has been implicated in the regulation of late gene expression (4, 5, 11, 29, 37). Synthesis of the late proteins in wt Ad5-, H5*pt*806-, and H5*pt*807-infected HeLa cells was therefore compared at 24 h postinfection (Fig. 10A). The depressed synthesis of the late proteins in mutant-infected cells more or less paralleled the reduction in DNA replication. To provide better quantitation, fiber and hexon polypeptides were immunoprecipitated with a mixture of antibodies against these two proteins (Fig. 10B). Synthesis of both proteins was reduced 5- to 10-fold with the mutants compared with wt Ad5. This depressed DNA synthesis and late gene expression is consistent with the lower rate of DBP synthesis and can account for the reduced viral growth of the most dephosphorylated DBP mutants.

DISCUSSION

Like the adenovirus DBP, the multifunctional simian virus 40 large T antigen contains numerous phosphorylation sites (58) that are located outside the DNA-binding domain (59). Although removal of these PO_4^{3-} groups by alkaline phosphatase appears to enhance its DNA replication function but not its helicase activity, it is still unresolved whether dephosphorylation affects its DNA-binding capacity (9, 22, 32, 60, 61). To determine the importance of this posttranslational modification at individual sites, Schneider and Fanning (59) altered each site independently by oligonucleotide-directed mutagenesis. Although phosphorylation at some sites was fortuitous, substitution of a nonphosphorylatable alanine or cysteine at other sites dramatically affected the viability of simian virus 40.

Using a similar approach, we have constructed a series of seven Ad5 DBP mutant viruses that have 1 to 10 phosphorylation sites altered. In contrast to T antigen, all the DBP mutants with only one or two sites changed were more or less normal for DNA replication, synthesis of DBP, synthesis of late proteins, and viral multiplication. However, replacement of a large number of the phosphorylated serine or threonine residues in mutants H5*pt*805 through H5*pt*807 led to defective phenotypes. Viral DNA replication, late gene expression, and virus yield were all depressed approximately 5- to 20-fold. This depression is most readily accounted for by the smaller amounts of DBP in the mutant-infected cells. However, we cannot rule out that the mutant, underphosphorylated proteins were less active in carrying out their function in DNA replication or late gene expression or both.

The reduced DBP levels were not due to more rapid turnover as might be expected for these highly mutated proteins. Rather, the rates of DBP synthesis and its mRNA levels were diminished 5- to 10-fold compared with those of wt Ad5. These reductions were independent of viral DNA replication and hence template number, suggesting that DBP enhances its own expression in a more direct fashion.

Our studies (16, 53) of several other DBP mutants have led to a similar conclusion. The levels of DBP mRNA were depressed in cells infected with H5*dl*802, a deletion mutant of DBP. A frameshift caused by the deletion results in premature termination of the peptide very near the N terminus of DBP. A viable revertant H5*dl*802r1, which has a restored reading frame and produces a deleted DBP, also was deficient in DBP synthesis. The deletion in H5*dl*802r1 covers amino acids 23 to 105, the same region in which many of the phosphorylation sites were replaced in H5*pt*805 through H5*pt*807. The large deletion in both H5*dl*802 and its revertant might act in *cis* to either destabilize the mRNA or disrupt efficient transcription of the DBP gene. However, it is unlikely that several point mutations that alter DBP phosphorylation sites in H5*pt*805 through H5*pt*807 fortuitously would act in a similar manner to depress DBP expression.

A more probable explanation is that DBP activates its own gene. The low level of DBP mRNA in H5*dl*802-infected cells would then be due to the absence of any DBP. The delayed and reduced expression of the DBP gene in H5*dl*802r1-infected cells could result from either inefficient entry of the mutant DBP into the nucleus, owing to the deletion which removed the nuclear localization signals of the protein, or removal by the same deletion of a critical region (amino acids 23 to 105) for autoenhancement. The phosphorylation mutants (H5*pt*805 through H5*pt*807), which retain the region deleted in H5*dl*802r1, including the nuclear localization signals, show normal entry into the nucleus at least at 24 to 28 h postinfection, yet exhibit depressed DBP gene expression. This suggests that the underphosphorylation of DBP by substitution (or deletion) diminishes its autoenhancing activity.

The loss of autoenhancing activity could also result from local changes in the structure of the protein or loss of one or more critical amino acids, regardless of whether they can be phosphorylated. Although these possibilities cannot be excluded at present, we suspect that the overall state of phosphorylation is the critical factor influencing this function for the following reasons. First, all the mutations resulted in conservative amino acid substitutions. These are less likely to disrupt any critical secondary or tertiary structure of the amino-terminal domain, which itself exhibits tremendous sequence divergency between different adenovirus serotypes. Second, a series of small deletion mutants (H5*dl*806 through H5*dl*809) which spanned amino acids 26 through 81 exhibited normal DBP expression (70a). All of these mutants retained at least 7 of the 10 phosphorylation sites. Third, substitution of one or a few sites, as in H5*pt*801 through H5*pt*804, or removal of a few sites, as in H5*dl*807 and H5*dl*808 (deletions of amino acids 26 to 50 and 40 to 81, respectively), did not affect DBP synthesis. However, when eight or more of these same sites were replaced, as in H5*pt*807, expression of DBP was diminished. Moreover, H5*pt*807r1, isolated on the gmDBP2 cell line, was shown by DNA sequencing to have reverted the four mutated sites 70, 92, 100, and 107 but not 75 and 76 of H5*pt*807, suggesting that a double recombination event occurred with the endogenous copy of the DBP gene carried in the cell chromosome. H5*pt*807r1, which still lacks 6 of the 10 phosphorylation sites, again exhibited depressed DBP synthesis, although the level of repression was lower than for H5*pt*807 (N. Morin and D. F. Klessig, unpublished results).

If, as we suspect, the overall state of phosphorylation of the DBP amino-terminal domain is critical to its autoenhancing activity, several interesting parallels with a number of eucaryotic transcriptional activators are evident. These activators or factors contain two functionally distinct regions or domains, both of which can be interchanged between factors. The binding domain directs the factor to the DNA either directly by recognizing a specific DNA sequence or indirectly via interacting with another protein bound to the DNA. This enables its activating domain to interact with a component of the transcription complex to stimulate transcription. Many, but not all, activating domains are composed of acidic, relatively short regions (30 to 100 residues) that do not have rigid sequence requirements (for reviews, see references 47, 52, and 64).

Similarly for DBP, its DNA-binding domain (carboxy-terminal half of the molecule) and autoenhancement domain (amino-terminal fifth of the molecule) are separated. The amino-terminal domains encoded by different adenovirus serotypes are heterogeneous in both size and sequence (31, 71). In contrast to the acidic activating domains of many transcription factors, the amino-terminal autoenhancement domain of DBP is rich in both positively and negatively charged residues. However, phosphorylation of the prevalent serines converts it to a highly acidic region. Multiple phosphorylation to create an acidic domain has also been implicated in the activation by high temperatures of the yeast heat shock transcription factor (62).

Although the autoenhancing activity of DBP affects the level of DBP mRNA, it is not known whether it plays a regulatory role in transcription of this gene. Several studies, particularly those with H5*ts*125, which carries a temperature-sensitive mutation in the carboxy-terminal domain, suggest that DBP represses rather than enhances the expression of early viral genes, including itself. This may be mediated by transcriptional repression of the E4 region (24) and destabilization of other early mRNA during the late phase of infection (8). However, the failure of the DBP-negative mutant H5*dl*802 to overexpress its early genes calls into question the presumptive role of DBP in repressing early-gene expression (53). Furthermore, in contrast to many transcriptional activators, sequence-specific binding to either single- or double-stranded DNA has not been demonstrated for DBP (68). However, some transcriptional activators, such as adenovirus E1A 289-amino-acid protein (19, 41) and herpes simplex virus VP16 polypeptide (reviewed in reference 55), enhance transcription without directly binding to DNA.

Although there are several differences between DBP and a number of well-characterized transcriptional activators, the common feature of an acidic activating domain separated from a distinct binding domain is intriguing. However, regardless of whether this eventually proves to be a useful analogy, a highly phosphorylated and hence negatively charged region located in the amino-terminal portion of the DBP is necessary for DBP to enhance its own expression. The overall state of phosphorylation of this region may be important for this activity (although we cannot exclude the possibility that the multiple mutations introduced into the region alter the function of DBP independently of their effect on phosphorylation). This new function of DBP thus could be regulated by cellular protein kinases and phosphatases. Given the multifunctional role of DBP in many critical processes in the infectious cycle of the virus, regulation of the amount of this protein via phosphorylation (and perhaps of other proteins whose synthesis might also be affected by the phosphorylation state of DBP) may provide a delicate sensor by which the virus monitors its cellular environment

through the use of the kinases and/or phosphatases of the cell.

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