JOURNAL OF VIROLOGY, May 1990, p. 2103–2109
0022-538X/90/052103-07$02.00/0

Vol. 64, No. 5

The Adenovirus DNA-Binding Protein Stimulates the Rate of Transcription Directed by Adenovirus and Adeno-Associated Virus Promoters

LONG-SHENG CHANG AND THOMAS SHENK*

Department of Biology, Howard Hughes Medical Institute, Princeton University, Princeton, New Jersey 08544

Received 6 November 1989/Accepted 16 January 1990

The DNA-binding protein (DBP) encoded by the E2A region of adenovirus type 5 was found to enhance the expression of a reporter gene controlled by several different promoters within transfected cells. The rate of synthesis of correctly initiated transcripts was increased by the DBP. The adeno-associated virus P5 promoter and the adenovirus E1A and E2A early and major late promoters responded to the DBP by increases in expression ranging from 6- to 27-fold, while the adenovirus E4 promoter was slightly inhibited by DBP. The adenovirus major late promoter showed a greater response to DBP than to the E1A transactivator protein, suggesting that the DBP plays a central role in activation of the late promoter.

The adenovirus DNA-binding protein (DBP), encoded by the viral E2A gene, is a 529-amino-acid phosphoprotein that is located predominantly in the nucleus of infected cells. Although it was first isolated by virtue of its ability to bind tightly to single-stranded DNA (55), it also binds to the ends of double-stranded DNA (12, 48, 54) and to RNA (1, 10, 50).

The DBP is a multifunctional polypeptide that plays a variety of roles during the Ad replication cycle. It is required for elongation during viral DNA replication (reviewed in references 31 and 53) and has been reported to play a role in efficient initiation of replication (32), although this is controversial (45). It influences the levels to which early viral mRNAs accumulate (6, 7), apparently by reducing the half-life of at least some early mRNAs (3) and by inhibiting transcription from the early region 4 promoter (19, 39). The DBP has been inferred to play a role in transcription of the adenovirus major late unit since its rate of transcription is reduced in monkey cells and this defect can be corrected by mutations within the protein (27, 33). Finally, the protein has been implicated in the assembly of virus particles since a mutant virus expressing a variant DBP produced viral DNA and late proteins but not virions (41).

Here we demonstrate that the DBP can enhance the rate of transcription directed by a variety of promoters. The adenovirus type 5 (Ad5) E1A, E2A, and major late promoters as well as the adeno-associated virus (AAV) P5 promoter were stimulated by cotransfection with a plasmid carrying the DBP gene. The major late promoter was stimulated to a greater extent by the DBP than by the E1A transactivation protein, suggesting that the DBP plays a major role in the activation of this promoter. In contrast to other Ad5 promoters tested, the E4 promoter was not stimulated in transfection assays by the DBP.

MATERIALS AND METHODS

Plasmids and cells. A DNA segment comprising the AAV P5 5′-flanking region (nucleotides 190 to 310; *Xba*I-*Hha*I cleavage product) was subcloned from p*sub*201 (47) and then inserted into the chloramphenicol acetyltransferase (CAT) expression vector (15) to generate pAAVP5-CAT190 (8). Deleted derivatives of the P5 5′-flanking region were constructed by using *Bal* 31 nuclease, and deletion endpoints were mapped by DNA sequencing. The rabbit β-globin-coding region from pSV2-β-globin (52) was substituted for the CAT-coding region in pAAVP5-CAT190 to produce pAAVP5-βG190 (8). pE1A-CAT (30) contains the E1A promoter (sequence −499 to +113 relative to the E1A cap site), pE2A-CAT (38) contains the E2 early promoter (sequence −284 to +62 relative to the E2 early cap site), pE4-CAT contains the E4 early promoter (sequence −324 to +36 relative to the E4 early cap site), and pMLP-CAT (8) contains the major late promoter (sequence −400 to +30 relative to the major late cap site) fused to the CAT-coding region.

pE1A-E1B (22) contains the Ad5 E1 region (nucleotides 1 to 5778), pE1A (22) contains the Ad5 E1A region (nucleotides 1 to 1767), pE1B (42) contains the Ad5 E1B region (nucleotides 1339 to 5778), pE2A contains the Ad5 E2A gene (59.5 to 66.3 map units), pE2AΔN was derived from pE2A by deleting a *Bgl*II fragment between 63.6 and 69 map units, pE2AΔC was derived from pE2A by deleting a *Bgl*II fragment between 60.2 and 63.6 map units, pE4 contains the Ad5 E4 gene (84.3 to 100 map units), pE4Δ366 contains a deleted E4 gene lacking the sequence between 92.1 and 98.4 map units (18), pVA RNA contains the Ad5 VA RNA genes (26.5 to 31.5 map units), and pPRV-IE (27) contains the pseudorabies virus immediate-early gene.

HeLa cells (American Type Culture Collection, Rockville, Md.) were grown in medium containing 10% calf serum.

Transfection and CAT assays. HeLa monolayer cultures were split 1:5 in medium containing 10% fetal calf serum at 20 to 24 h before transfection. Calcium phosphate-DNA coprecipitate mixtures were generated as previously described (16). Cells on a 100-mm-diameter dish were cotransfected with 2 μg of a CAT plasmid and 18 μg of a plasmid containing an activator gene or a control plasmid (pBR322 or pAT153). The precipitate was left on the cells in medium for about 12 h, after which the mixture was replaced with fresh medium containing 10% calf serum. At 48 h after transfection, cells were harvested and lysed in 0.25 M Tris hydrochloride (pH 7.8) by freeze-thawing, and portions of the lysates were assayed for CAT activity (15). To quantify relative levels of CAT activity, areas of silica gel containing acetylated and unacetylated ^{14}C-labeled chloramphenicol

* Corresponding author.

were scraped off thin-layer chromatography plates and radioactivity was determined by liquid scintillation counting.

RNA preparation and analysis. Steady-state levels of β-globin RNA encoded by pAAVP5-βG190 were assayed by isolating total cytoplasmic RNA (22) at 48 h after transfection of HeLa cells and performing RNase protection analysis (35). The ^{32}P-labeled RNA probe corresponded to the AAV P5 control region plus the 5′ half of the β-globin-coding region. Protected bands of about 400 nucleotides were generated, and they were quantified by densitometric scanning of appropriate autoradiographic exposures produced with preflashed Kodak XAR-5 film.

The half-life of β-globin mRNA was estimated by treating HeLa cell cultures with actinomycin D (5 μg/ml) at 48 h after transfection. Samples were harvested at various times after drug treatment, and RNA was prepared and assayed by RNase protection as described above.

For measurement of transcription rates, nuclei were prepared from HeLa cells at 48 h after transfection. Nuclei were incubated for 15 min at 30°C in the presence of [^{32}P]UTP, and labeled RNA was isolated and hybridized to probe DNAs immobilized on nitrocellulose filters (25). Probe DNAs were prepared by digestion of pAAVP5-βG190 with *Xba*I plus *Bam*HI and digestion of pLK22a (human β-actin cDNA [17]) with *Bam*HI, separation of the resulting fragments by electrophoresis, and transfer of the entire pattern to nitrocellulose strips. After hybridization, the radioactivity in individual bands was quantified by scintillation counting.

Measurement of DNA stability. Analysis of transfected plasmid DNA stability was as described by Alwine (2). Briefly, low-molecular-weight DNA was prepared (24) at 48 h after transfection and equivalent portions of each sample were digested with *Xba*I and analyzed by DNA blot analysis (51) with a ^{32}P-labeled β-globin-specific probe DNA.

RESULTS

Induction of pAAVP5-CAT expression by DBP. We found that the adenovirus DBP could influence the expression of other genes during an investigation of the spectrum of Ad gene products that influence expression from the AAV P5 promoter. A plasmid was constructed that contained a 190-base-pair segment from the AAV P5 5′-flanking region appended to the CAT-coding region (pAAVP5-CAT190), and HeLa cells were cotransfected with that plasmid plus other plasmids encoding potential activator proteins (Fig. 1). pAAVP5-CAT190 expression was stimulated by the Ad5 E1A and pseudorabies virus immediate-early proteins, presumably at the level of transcription (reviewed in reference 11), and by Ad5 VA RNAs, supposedly at the level of translation (reviewed in reference 49). Since these activators have been well studied with many other genes, they were not investigated further. Unexpectedly, however, cotransfection with plasmids carrying the Ad5 E2A or E4 gene also enhanced pAAVP5-CAT190 expression.

To ascertain whether the proteins encoded by the E2A and E4 genes were responsible for the increase observed in pAAVP5-CAT190 expression, we did an additional cotransfection experiment. HeLa cells were cotransfected with pAAVP5-CAT190 plus either plasmids encoding intact E2A or E4 gene products or plasmids carrying deleted coding regions (Table 1). pE4 and pE4Δ366 induced pAAVP5-CAT190 expression to the same extent. Since pE4Δ366 does not encode any intact E4 polypeptides, the activation observed for the E4 plasmids presumably results from the titration of a factor or factors by the E4 transcriptional control region within transfected cells. In contrast, whereas pE2A induced pAAVP5-CAT190 expression by a factor of 17, neither pE2AΔN nor pE2AΔC increased the level of CAT activity produced by pAAVP5-CAT190. Since these two variants contain nonoverlapping deletions, one in the N-terminal coding region and the other in the C-terminal coding region of the E2A gene, they would not both be expected to lack a binding site for an inhibitory factor that could have been titrated from pAAVP5-CAT190 by pE2A DNA. Therefore, we conclude that the DBP is acting either directly or indirectly to enhance pAAVP5-CAT190 expression.

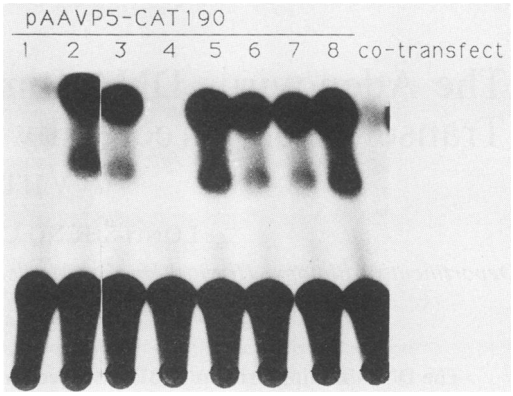

FIG. 1. Response of the AAV P5 promoter to various activators in a CAT assay. HeLa cells were cotransfected with pAAVP5-CAT190 DNA plus either pAT153 DNA or plasmid DNA encoding a potential activator. Cotransfecting plasmids are indicated by number above individual tracks. Extracts were prepared 48 h later and assayed for CAT expression. Lanes: 1, pAT153; 2, pE1A-E1B; 3, pE1A; 4, pE1B; 5, pE2A; 6, pE4; 7, pVA RNA; 8, pPRV-IE.

DBP enhances rate of transcription directed by AAV P5 promoter. A nuclear run-on experiment was performed to test directly for the ability of DBP to stimulate transcriptional activity of the AAV P5 promoter. HeLa cells were cotransfected with pAAVP5-βG190 (identical to pAAVP5-CAT190 except that the β-globin-coding region is substituted for the CAT sequence since CAT-encoding mRNAs were found to be unstable) plus a second plasmid which was a control, nonactivating plasmid (pBR322), a plasmid encoding the known pseudorabies virus immediate-early transactivator protein (pPRV-IE), or pE2A (Table 2). Transcription

TABLE 1. Expression of pAAVP5-CAT190 was enhanced by DBP[a]

Cotransfecting plasmid	pAAVP5-CAT190 expression	
	% CAT conversion	Induction
pAT153	0.5	1.0
pE4	3.0	5.6
pE4Δ366	2.9	5.4
pE2A	9.1	16.9
pE2AΔN	0.4	0.7
pE2AΔC	0.9	1.7

[a] Extracts were prepared at 48 h after transfection of HeLa cells and assayed for CAT activity. The values reported are the averages obtained by analysis of duplicate extracts. Induction was calculated as the percent conversion of CAT obtained for indicated cotransfected plasmids divided by the percent conversion obtained for pAT153, which carries no insert.

TABLE 2. Rate of pAAVP5-βG190 transcription was increased by DBP[a]

Cotransfecting plasmid	Cpm hybridized (induction) to:	
	β-Globin DNA	Actin DNA
pBR322	18 (1.0)	406 (1.0)
pPRV-IE	265 (14.7)	530 (1.3)
pE2A	302 (16.8)	338 (0.8)

[a] HeLa cells were cotransfected with pAAVP5-βG190 plus pBR322, pPRV-IE, or pE2A, and nuclei were prepared 48 h later. Run-on transcription was performed in the presence of [α-^{32}P]UTP, and nuclear RNA was prepared and hybridized to β-globin or actin (cellular control gene) probe DNAs which were immobilized on nitrocellulose membranes. Membranes were washed and radioactivity was quantified. Induction was calculated as the counts per minute obtained for indicated cotransfecting plasmids divided by the counts per minute obtained for pBR322, which carries no insert.

of β-globin sequences was stimulated by a factor of 16.8 when DBP was present.

Two control experiments were performed to be certain that the nuclear run-on experiment reflected induction of transcriptional initiation by the AAV P5 promoter. First, the transcriptional induction was shown to result from initiation at the AAV P5 start site. RNA was prepared at 48 h after transfection of HeLa cells with pAAVP5-βG190 plus pBR322, pE1A-E1B, pPRV-IE, or pE2A, and β-globin mRNAs were assayed by RNase protection analysis (Fig. 2A). There was a 15-fold increase in the level of properly initiated β-globin RNA (represented by a 400-nucleotide fragment) when pAAVP5-βG190 was cotransfected with either pE1A-E1B or pPRV-IE and a nearly 20-fold increase when pE2A was the cotransfecting plasmid. A faster-migrating fragment whose levels were also modulated by the activator proteins was also observed in the analysis. Shortened β-globin transcripts have been reported previously (37, 52), and they are likely due to initiation downstream of the major start site. In the second control experiment, the stability of pAAVP5-βG190 DNA was monitored subsequent to cotransfection with either pBR322 or plasmids encoding activator proteins. Low-molecular-weight DNA was prepared from the same experiment in which steady-state β-globin RNA levels were analyzed, and pAAVP5-βG190 DNA levels were assayed by DNA blot analysis (Fig. 2B). Two- to threefold more pAAVP5-βG190 DNA persisted in cells that received plasmids encoding activator proteins compared with those that received pBR322. However, the increase in DNA stability was not sufficient to account for the increased level of β-globin-specific transcription.

The DBP has been reported to decrease the half-life of E1A and E1B mRNAs in Ad-infected cells (3). It appeared unlikely that RNA stability was altered in transfected cells by DBP since the induction of pAAVP5-βG190 by DBP at the level of transcription (16.8-fold, Table 2) fit well with the increase in β-globin mRNA (15-to 20-fold, Fig. 2A). Nevertheless, the β-globin mRNA half-life was monitored directly. HeLa cells were cotransfected with pAAVP5-βG190 plus pBR322, pE1A-E1B, or pE2A, and cells were harvested at various times after new transcription was terminated by the addition of actinomycin D. β-Globin-specific RNA was quantified by RNase protection analysis (Fig. 3). The half-life of β-globin RNA was approximately 4 h in the presence or absence of activator proteins.

In sum, pAAVP5-βG190 DNA was not markedly stabilized by DBP, β-globin RNA molecules encoded by the plasmid were initiated at the AAV P5 start site, and the half-lives of these RNAs were not altered by DBP. Given the enhanced rate of β-globin transcription in the presence of pE2A, we conclude that the AAV P5 promoter is stimulated by E2A gene products.

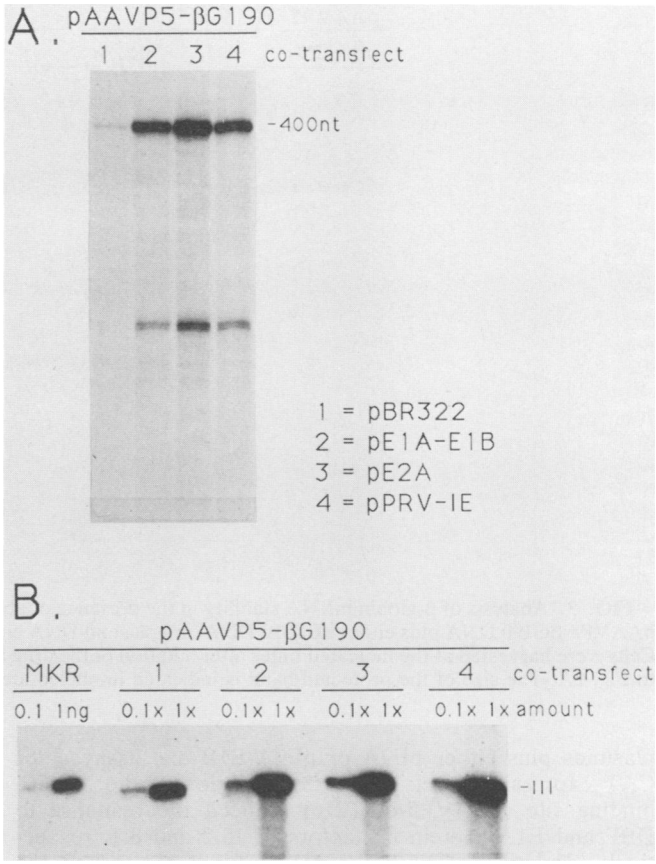

FIG. 2. Steady-state levels of RNA transcribed by the AAV P5 promoter and stability of transfected plasmid DNA. (A) RNase protection analysis of β-globin RNA in the cytoplasm of HeLa cells at 48 h after transfection with pAAVP5-CAT190 plus either pBR322 or a plasmid encoding a transactivator. The probe RNA corresponded to the P5 control region plus the 5′ region of the β-globin-coding sequence. The size of the protected band is indicated in nucleotides (nt). The faster-migrating band results from initiation within β-globin-coding sequences. Cotransfecting plasmids are indicated by number above each lane. (B) DNA blot analysis of pAAVP5-βG190 DNA isolated 48 h after transfection of HeLa cells. ^{32}P-labeled β-globin-specific DNA served as the probe. The relative amounts of DNA applied to each lane are indicated, and cotransfecting plasmids are designated by number. The position of linear (form III) pAAVP5-βG190 DNA is indicated. MKR, *Xba*I-cleaved pAAVP5-βG190 DNA.

AAV P5 sequence elements that respond to adenovirus E1A products. The AAV P5 5′-flanking region contains two sequence elements that we have previously shown respond to the adenovirus E1A transactivator protein (8). The first is a binding site for the major late transcription factor (MLTF), which was originally described within the adenovirus major late promoter, located between AAV sequence positions 204 and 215. The second is a directly repeated 10-base-pair sequence located between AAV sequence positions 217 and 236. Analysis of a series of pAAVP5-CAT190 derivatives that contain deletions extending from AAV position 190 toward the P5 start site at position 287 demonstrated that the same sequence elements respond to the DBP as to the E1A protein. HeLa cells were transfected with pAAVP5-CAT

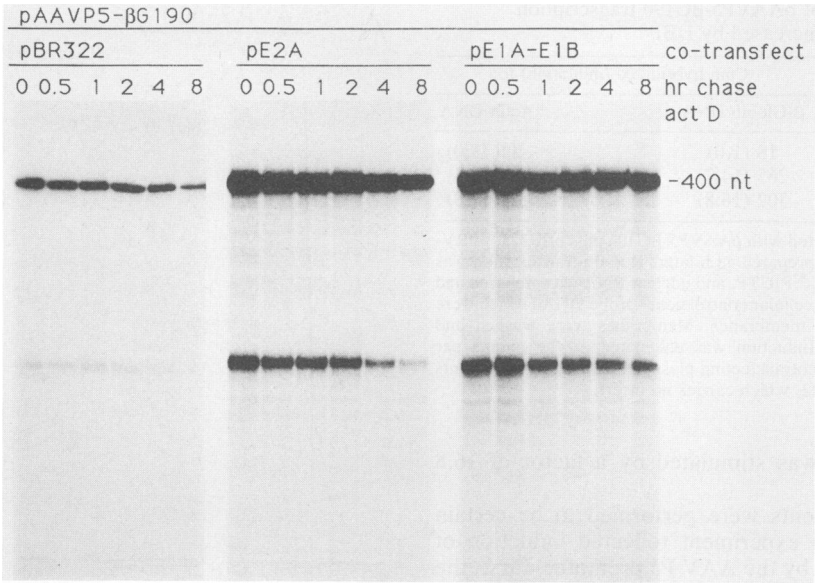

FIG. 3. Analysis of β-globin mRNA stability in the presence or absence of activator proteins. At 48 h after transfection of HeLa cells with pAAVP5-βG190 DNA plus either pBR322 DNA or a plasmid DNA encoding an activator protein, actinomycin D (act D) (5 μg/ml) was added. Cells were harvested at the indicated times after addition of the drug and assayed by RNase protection with the probe described in the legend to Fig. 2A. The size of the protected band is indicated in nucleotides (400 nt).

plasmids plus either pE2A or pE1A-E1B and assayed for CAT expression levels (Table 3). Deletion of the MLTF-binding site (pAAVP5-CAT216) reduced the response to DBP and E1A protein by factors of 10.2 and 8.1, respectively. Deletion of both the MLTF-binding site and the repeated sequence (pAAVP5-CAT243) completely abolished the response to both activating proteins.

Some but not all Ad5 promoters are induced by DBP. Next, the ability of the DBP to activate different Ad5 promoters was assayed. HeLa cells were cotransfected with pE2A or pE1A-E1B plus plasmids containing constructions in which CAT expression was controlled by the E1A (pE1A-CAT), E2 early (pE2-CAT), E4 (pE4-CAT), or major late (pMLP-CAT) promoter (Table 3). Each of the Ad5 promoters except the E4 promoter was stimulated by the DBP. The E4 promoter was modestly inhibited. As expected, all four Ad5 promoters were stimulated by the E1A protein (Table 3).

TABLE 3. Activation of Ad5 and AAV promoters by DBP[a]

Promoter test plasmid	Induction by cotransfecting plasmid		
	pE2A	pE1A-E1B	pPRV-IE
pAAVP5-CAT190	27.5	34.8	31.5
pAAVP5-CAT216	2.7	4.3[b]	ND[c]
pAAVP5-CAT230	1.8	4.7[b]	ND
pAAVP5-CAT243	1.0	1.0[b]	ND
pAAVP5-CAT262	1.2	1.5[b]	ND
pE1A-CAT	6.0	2.5	7.0
pE2-CAT	13.5	36.6	40.0
pE4-CAT	0.7	7.0	6.0
pMLP-CAT	14.7	4.7	ND

[a] Extracts were prepared at 48 h after transfection of HeLa cells and assayed for CAT activity. The values reported are the averages obtained by analysis of duplicate extracts. Induction was calculated as the percent conversion of CAT obtained for indicated cotransfected plasmids divided by the percent conversion obtained for pAT153, which carries no insert.

[b] These inductions are taken from an experiment reported earlier (8).

[c] ND, Not determined.

The major late promoter was more efficiently stimulated by the DBP than the E1A protein.

DISCUSSION

The main conclusion of this work is that the DBP can induce expression directed by a variety of AAV and adenovirus promoters within transfected cells (Fig. 1; Tables 1 and 3). The induction occurs at the level of transcription since the rate of synthesis of correctly initiated RNAs (Fig. 2A) was increased (Table 2), while the stability of transfected DNA was only slightly increased (Fig. 2B), and there was no change in RNA half-life in the presence of DBP (Fig. 3). As yet, we have not shown that the DBP enhances transcription within adenovirus-infected cells. This would likely be difficult to demonstrate given the variety of Ad gene products that contribute to overall transcription rates (E1A, reviewed in reference 11; E1B-21kd, references 23 and 58; E4-17kd, references 4, 20, and 43). Nevertheless, it seems reasonable that the DBP influences the rate of transcription within virus-infected cells. In fact, such a role has been inferred from the observation that a mutation in the E2A gene encoding the DBP (33) can enhance the normally depressed level of adenovirus late unit transcription in monkey cells (28). In addition, Rossini (46) has reported that the DBP has a stimulatory effect on the synthesis of E1B products within microinjected hamster fibroblasts.

The response of the E1A and major late promoters to the DBP was about threefold greater than their response to the E1A protein (Table 3), suggesting that the DBP plays a major role in the regulation of expression from these promoters as an Ad infection proceeds. In contract to the other promoters tested, the E4 promoter was slightly inhibited by the E2A product (Table 3). This fits with earlier reports that E4 transcription is inhibited by the DBP both in vivo (39) and in vitro (19). The extent of the inhibition of the E4 promoter correlated with the amount of DBP added to cell extracts, i.e., increased amounts of the protein increased the level of

the inhibition (19). This raises the possibility that the magnitude of both stimulatory and inhibitory DBP effects could change during an adenovirus infection as the intracellular concentration of the protein increases. Consistent with this notion, E4 transcription is substantially shut down and major late transcription becomes very active during the late phase of the infectious cycle when the DBP has accumulated to maximal levels. Further, cells infected at the nonpermissive temperature with the Ad5 DBP mutant *ts*125 accumulate elevated levels of a variety of early viral mRNAs (3, 5, 6, 7, 40, 44), while infection with the Ad5 DBP mutant *dl*802 generates normal amounts of early mRNAs (44). These results are likely due to the fact that the DBP is overproduced (41) in *ts*125-infected cells (presumably, the mutant protein can still activate transcription), while no DBP is synthesized in *dl*802-infected cells (44). Overexpression of E1A and E1B products could also explain the enhanced transforming ability of *ts*125 at the nonpermissive temperature (13, 57).

As yet, the mechanism underlying transcriptional enhancement by the DBP is unclear. It might influence the binding of cellular transcription factors at the promoter. Such a role seems possible given the recent observation that the DBP can increase binding of nuclear factor I to the origin of DNA replication (9, 54) and the fact that nuclear factor I is a member of a family of factors that function in DNA replication as well as in transcription (29). Binding of the MLTF is one possible target for DBP modulation since its recognition site is present in two of the promoters most strongly induced by the protein of those tested, the AAV P5 and adenovirus major late promoters (Table 3). If this model for DBP function is correct, however, it must influence the binding behavior of a variety of polypeptides since some promoters affected by the protein don't contain MLTF-binding sites.

The DBP could influence the binding of other factors either by interacting directly with them or by altering the DNA template. There is precedent for the latter in bacteriophage N4 transcription. Its virion-associated RNA polymerase requires that its promoters exist in a single-stranded form that is induced by the *Escherichia coli* single-stranded DBP (21). Perhaps, then, the DBP binds to and stabilizes, if only transiently, single-stranded regions that occur within promoters as the DNA duplex undergoes helix-coil transitions. Such a change in DNA structure could, then, facilitate the binding of certain transcription factors. Alternatively, the potential ability of DBP to stabilize single-stranded regions could help to drive the denaturation of the DNA duplex that is required for initiation of transcription (discussed in reference 56). It is also possible that DBP alters the superhelical density of DNA within cells as a result of its binding and thereby enhances the rate of transcription. Experiments are in progress to determine which of these possible modes of action underlies the ability of the DBP to influence the activity of transcriptional control regions.

The ability of cotransfected pE4 DNA to activate expression of the AAV P5 promoter (Fig. 1) deserves mention. As shown above, this activation did not require expression of an E4 gene product since pE4Δ366, which lacks the E4 coding region but retains E4-specific 5′- and 3′-flanking sequences, is able to mediate the activation (Table 1). Presumably, activation of the AAV promoter results from the ability of pE4 and pE4Δ366 DNAs to bind an inhibitory protein that otherwise binds to AAV P5 and interferes with its activity. Goding et al. (14) have previously reported that coinfection with a plasmid containing the adenovirus E4 gene can stimulate expression of the adenovirus E2 early promoter. Although it was not demonstrated in the report by Goding and colleagues, it is likely that an E4 gene product was involved in the stimulation. An E4 protein (4, 20, 43), specifically the E4 17-kilodalton protein (26, 34), can alter the binding behavior of the cellular transcription factor E2F; and this alteration leads to enhanced transcription from the E2 promoter that contains a pair of E2F-binding sites. Apparently, pE4 mediates activation of the AAV P5 promoter (no E2F-binding sites) and the adenovirus E2 early promoter (two E2F-binding sites) through different mechanisms.

While this article was under review, Morin et al. (36) reported that a mutant Ad expressing an altered DBP accumulated reduced levels of E2A mRNA. This observation led the authors to propose that DBP can enhance the accumulation of DBP mRNA within infected cells, a proposal consistent with our results (Table 3).

ACKNOWLEDGMENTS

We thank members of our laboratory for critical reading of the manuscript.

L.-S. Chang was a postdoctoral fellow of the Leukemia Society of America, and T. Shenk is an American Cancer Society professor.

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