Isolation and Analysis of Adenovirus Type 5 Mutants Containing Deletions in the Gene Encoding the DNA-Binding Protein

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A genetic system is described which allows the isolation and propagation of adenovirus mutants containing lesions in early region 2A (E2A), the gene encoding the multifunctional adenovirus DNA-binding protein (DBP). A cloned E2A gene was first mutagenized *in vitro* and then was introduced into the viral genome by *in vivo* recombination. The E2A mutants were propagated by growth in human cell lines which express an integrated copy of the DBP gene under the control of a dexamethasone-inducible promoter (D. F. Klessig, D. E. Brough, and V. Cleghon, *Mol. Cell. Biol.* 4:1354-1362, 1984). The protocol was used to construct five adenovirus mutants, Ad5d1801 through Ad5d1805, which contained deletions in E2A. One of the mutants, Ad5d1802, made no detectable DBP and thus represents the first DBP-negative adenovirus mutant, while the four other mutants made truncated DBP-related polypeptides. All five mutants were completely defective for growth and plaque formation on HeLa cell monolayers. Furthermore, the two mutants which were tested, Ad5d1801 and Ad5d1802, did not replicate their DNA in HeLa cells. The mutant Ad5d1804 encoded a truncated DBP-related protein which contained an entire amino-terminal domain derived from the host range mutant Ad5hr404, a variant of Ad5 which multiplies efficiently in monkey cells. While results of a previous study suggest that the amino-terminal domain of DBP could act independently of the carboxyl-terminal domain to enhance late gene expression in monkey cells, the Ad5d1804 polypeptide failed to relieve the block to late viral protein synthesis in monkey cells. The mutant Ad5d1802 was used to study the role of DBP in the regulation of early adenovirus gene expression in infected HeLa cells. These experiments show that E2A mRNA levels are consistently reduced approximately fivefold in Ad5d1802-infected cells, suggesting either a role for DBP in the expression of its own gene or a *cis*-acting defect caused by the E2A deletion. DBP does not appear to play a significant role in the regulation of adenovirus early regions 1A, 1B, 3, or 4 mRNA levels in infected HeLa cell monolayers since wild-type Ad5- and Ad5d1802-infected cells showed very little difference in the patterns of expression of these genes.

The early region 2A gene (E2A) of human adenovirus types 2 and 5 (Ad2 and Ad5, respectively) encodes a 72-kilodalton (kDa) protein which possesses single-stranded DNA binding activity (34, 46). This polypeptide, commonly called the adenovirus DNA-binding protein (DBP), is thought to contain two physical (25, 43) as well as functional (4, 31, 40) domains. A large number of functions have been ascribed to DBP. The best characterized of these is the role of the protein in viral DNA replication. DBP is required for the strand elongation reaction of viral DNA synthesis (12, 16) and may play a role in the initiation reaction as well (17, 37, 47). This polypeptide has also been implicated in the regulation of adenovirus early gene expression. Results of studies involving the microinjection of purified adenovirus genes or mRNAs into cells have suggested that DBP may stimulate both early region 1B (E1B) (42) and early region 4 (E4) (41) gene expression. DBP has also been proposed to have a negative influence on early gene expression at intermediate and late times after infection. Evidence for this comes primarily from studies with the temperature-sensitive (*ts*) E2A mutant Ad5ts125. This mutant, unlike wild-type (WT) adenovirus, fails to turn down early gene expression as viral infection proceeds and thus accumulates higher

amounts of early mRNAs than WT virus (10, 11). The negative regulation mediated by DBP is thought to affect both the cytoplasmic stabilities of early region 1A (E1A) and E1B mRNAs (7) as well as the rate of transcription of E4 (38). More recently, repression of E4 transcription by DBP has been demonstrated *in vitro* (23). A further function for DBP in late viral gene expression has been revealed by studying abortive infections of human adenovirus in monkey cells. WT virus fails to grow in monkey cells because of a complex block to viral late gene expression (8, 14, 26). This block involves a reduced rate of transcription across the late viral gene block (J. M. Johnston, K. P. Anderson, and D. F. Klessig, submitted for publication), altered splicing of the mRNA encoding the fiber polypeptide (3, 28), and poor *in vivo* translation of the fiber mRNA (2). Adenovirus host range (*hr*) mutants which overcome these blocks and grow productively in monkey cells contain mutations in the amino-terminal segment of the E2A gene which cause a substitution of tyrosine for histidine at amino acid 130 of DBP (1, 9, 32). DBP also can affect the efficiency with which adenovirus transforms rat cells, as Ad5ts125 transforms cells at a three- to eightfold enhanced frequency compared with WT virus (19, 49). Finally, results of a recent study have suggested that DBP may play a role in the assembly of virus particles (39).

Although the small number of *ts* and *hr* mutants isolated to date have been invaluable in ascribing functions to DBP, it is apparent that a larger collection of mutants will be needed to understand this multifunctional protein in more detail. How-

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ever, since several of the activities of DBP are essential for virus growth, many potential E2A mutants are expected to have a nonconditionally lethal phenotype and thus cannot be isolated. To circumvent this problem, our laboratory has engineered several human cell lines, designated gmDBP cells, which contain and express integrated copies of the E2A gene (27). Since it was suspected that expression of DBP is toxic to cells (30), the E2A gene in these cell lines was under the control of a glucocorticoid hormone-inducible promoter. In the presence of dexamethasone, a synthetic glucocorticoid hormone, gmDBP1 and gmDBP2 cells induce DBP synthesis 50- to 200-fold to a level that is 5 to 20% of the peak synthesis level seen in adenovirus-infected HeLa cells (27).

The gmDBP cell lines therefore can theoretically serve as a permissive system for the isolation and propagation of E2A mutants. A similar strategy was used to isolate a wide variety of mutants mapping in E1A and E1B. These mutants are efficiently complemented by the human 293 cell line which expresses an integrated copy of E1A and E1B (20). DBP differs from the gene products encoded by E1A and E1B, however, in that it is probably required in very large amounts (up to 10^7 molecules per cell) during a viral infection. Although the gmDBP cell lines complement the growth of E2A *ts* mutants (27), the *ts* mutations probably inactivate only a subset of the functions of DBP (40). Thus it was unclear whether these cell lines would allow the isolation of DBP-negative adenovirus mutants.

In this report we describe a genetic system which has allowed the construction of five E2A deletion mutants. All of the mutants can be propagated in the gmDBP cell lines in the presence of dexamethasone. Since at least one of the mutants makes no functional DBP, the gmDBP cell lines should serve as a useful system to isolate E2A mutants of any desired genotype. The phenotypes of the five deletion mutants have shown, as expected, that DBP is an essential adenovirus protein required for viral DNA replication. Experiments are presented which utilize the E2A deletion mutants to investigate the role of DBP in the regulation of early and late viral gene expression.

MATERIALS AND METHODS

Cells, viruses, and infections. The human 293 and HeLa cell lines were obtained from J. F. Williams. CV₁ and CV_c cells, established lines of African green monkey kidney cells, were obtained from J. Mertz and P. Tegtmeier, respectively. Isolation of the E2A-complementing gmDPB1 and gmDBP2 cell lines has been described recently (27). The gmDBP2a cell line was isolated as a subclone of gmDBP2 cells. Monolayer cultures of 293, HeLa, and gmDBP cells were cultivated in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% calf serum (Irvine Scientific), 100 µg of streptomycin per ml, 100 µg of penicillin per ml, and 292 µg of glutamine per ml. For CV₁ and CV_c cultivation, 5% fetal calf serum (Flow Laboratories) was used instead of 10% calf serum.

Ad2 and Ad5 were originally obtained from U. Pettersson and J. F. Williams, respectively. Ad2hr400 and Ad5hr404 have been isolated by Klessig and Grodzicker (29). All of the viruses identified above were propagated in suspension cultures of HeLa cells. Ad5ts125 and Ad5d1434 were obtained from J. F. Williams and D. Solnick, respectively. Ad5ts1+2 was isolated after ligating the *Bam*HI A restriction fragment (coordinates 0 to 59.5) derived from Ad5ts2 (48) to the *Bam*HI B restriction fragment (coordinates 59.5 to 100)

derived from Ad5ts1 (48). The ligated DNA was used to transfect 293 cells at 33°C. Plaques were picked and screened for the inability to complement either of the parental viruses at the nonpermissive temperature. A positive isolate was plaque purified and designated Ad5ts1+2. Ad5ts125 and Ad5ts1+2 were propagated in monolayers of HeLa cells at 33°C, and Ad5d1434 was propagated in 293 cells at 37°C. Simian virus 40 (SV40) strain 776 was obtained from J. Sambrook, and stocks were prepared in CV₁ cells. Plaque assays for all virus except Ad5d1801 through Ad5d1805 were performed as described previously (22). The isolation, propagation, and assay of the E2A deletion mutants Ad5d1801 through Ad5d1805 are described in detail below.

All virus infections were done on confluent cell monolayers. Virus inocula were adsorbed at 37°C for 75 min in phosphate-buffered saline. The cell monolayers were washed, overlaid with media, and incubated at 37°C unless otherwise indicated.

Construction of plasmids containing E2A deletions. The first set of E2A deletions (d1801 and d1802) was constructed in the plasmid pAd2SA (obtained from U. Pettersson) which contains the Ad2 *Sma*I A fragment (adenovirus coordinates 56.9 to 75.8) cloned in the *Pst*I site of pBR322. This plasmid contains a unique *Xho*I restriction site at coordinate 65.9 in the amino-terminal portion of the DBP-coding region. It was necessary to use an Ad2 rather than an Ad5 clone because of Ad5 E2A gene does not contain this *Xho*I site. One microgram of plasmid DNA was first linearized at its unique *Xho*I site (coordinate 65.9 in the adenovirus insert) and then digested in a volume of 50 µl with 1 U of *Bal* 31 nuclease (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) for 20 s at 30°C. The DNA was extracted with phenol and chloroform-isoamyl alcohol, religated with T4 DNA ligase, and used to transform *Escherichia coli*. Plasmid DNA isolated from individual transformants (24) was screened by restriction enzyme digestion to identify those plasmids which contained deletions entirely within the DBP-coding region. Two such plasmids were obtained and designated pAd2SA-801 and pAd2SA-802.

The second set of E2A deletions (d1803 through d1805) was constructed with the plasmid pAd5hrKE, which was derived by cloning the Ad5hr404 *Kpn*I E fragment (coordinates 61.3 to 71.4) into the *Pvu*II site of pBR322 with *Kpn*I linkers (New England Biolabs, Inc., Beverly, Mass.). Ad5hr404 is an *hr* mutant of Ad5 which has acquired the ability to grow productively in monkey cells because of a point mutation in the amino-terminal segment of the E2A gene (29, 32). The pAd5hrKE plasmid contains three *Sst*II restriction enzyme sites, all of which reside in the DBP gene at coordinates 62.2, 64.4, and 65.7. One hundred micrograms of pAd5hrKE DNA was digested to completion with *Sst*II. The three resulting fragments were separated by electrophoresis in a 1.6% agarose gel and purified by electrophoresis into dialysis bags and passage over DEAE-Sephacel (35). Both purified insert fragments (the fragments at coordinates 62.2 to 64.4 and 64.4 to 65.7) were individually ligated into the vector backbone, and the DNA was used to transform *E. coli*. DNA from transformants was screened by restriction enzyme analysis to identify clones which contained single inserts in the proper orientation or no insert. Clones containing the deletions from coordinates 62.2 to 65.7, 64.4 to 65.7, and 62.2 to 64.4 were designated pAd5hrKE-803, pAd5hrKE-804, and pAd5hrKE-805, respectively.

The five E2A deletions were next introduced into the pAd5HA plasmid, which consists of the *Hind*III A fragment

of Ad5 cloned into pBR322. To accomplish this, the deletion plasmids were digested with *KpnI*, and the insert fragments (coordinates 61.3 to 71.4) were purified as described above. These were then cloned in the proper orientation into the purified pAd5HA vector backbone (pAd5HA minus its own insert) to give rise to the plasmids pAd5d1801 through pAd5d1805.

The extents of the deletions in pAd5d1801 and pAd5d1802 were analyzed by DNA sequence analysis (36). The pAd5d1801 plasmid contains a 313-base-pair (bp) deletion which fuses bp 22 of the DBP-coding region (the A of the initiating ATG is defined as bp 1) to bp 336. The pAd5d1802 plasmid contains a 242-bp deletion which fuses bp 64 to bp 307. The plasmids pAd5d1803 through pAd5d1805 contain precise deletions of one or both *SstII* fragments as the new joints were digestible with *SstII* and therefore are deleted for 1272, 802, and 470 bp, respectively. The deletions in pAd5d1803 through pAd5d1805 fuse bp 216 to bp 1489, bp 686 to bp 1489, and bp 216 to bp 687, respectively.

Construction, propagation, and plaque assay of the adenovirus E2A deletion mutants. Genomic DNA-terminal protein complex was prepared from Ad5ts1+2 by the method of Chinnadurai (13), dialyzed extensively in 10 mM Tris (pH 7.8)–1 mM EDTA–1 mM β -mercaptoethanol, and stored at 4°C. The DNA-protein complex (0.5 to 1 μ g) was mixed with 6 adenovirus molar equivalents of *HindIII*-digested pAd5d1801 through pAd5d1805 and transfected by the calcium phosphate procedure (21, 29) into 293 or dexamethasone-treated gmDBP1 cells. The transfected cells were incubated at 33°C for 4 days. Progeny virus were released from the cells by freeze-thawing and sonication. Four five-fold serial dilutions of each lysate were made in phosphate-buffered saline, and 0.5 ml of each dilution was used to infect monolayers (60 mm) of gmDBP1 or gmDBP2 cells. The infections were incubated for 3 to 6 days at 40°C in media containing 0.6 μ M dexamethasone. The progeny of these infections were used to infect fresh monolayers of gmDBP1 cells, and this process was continued until the cultures exhibited cytopathic effects (CPE). Large stocks of the deletion mutants were made by infecting gmDBP2 or gmDBP2a monolayers (60 to 100 100-mm plates) in the presence of 0.6 μ M dexamethasone. The infected cells were incubated at 37°C and harvested at 68 to 80 h postinfection (p.i.).

The infectious titer of the E2A deletion mutant stocks was determined by plaque assay on HeLa cells using Ad5d1434 as a helper virus. Plaque assays were performed as usual, except the HeLa cell monolayers were simultaneously infected with Ad5d1434 (6×10^6 PFU in 0.5 ml of phosphate-buffered saline per 60 mm plate). It was empirically determined that the E2A mutant stocks gave the greatest number of plaques when the HeLa cell monolayers were infected with Ad5d1434 at a multiplicity of 2 PFU per cell. Under these conditions, the E2A deletion stocks formed plaques with apparent one-hit kinetics and varied in titer between 2×10^8 and 2×10^9 PFU per ml. Analysis of several of these plaques (data not shown) indicated that they contained predominately WT virus, and thus presumably arose after recombination between the two deletion mutant genomes gave rise to a WT virus. Since the helper virus assay depends on a recombination event to generate a viral plaque, it may underestimate the amount of infectious virus in the E2A mutant stocks. This does not appear to be the case, however, since a given number of E2A deletion mutant PFU contain approximately the same amount of adenovirus DNA as does the same number of WT PFU which have been

titrated in the normal plaque assay without helper virus (S. Rice, unpublished data).

Analysis of viral proteins, DNA, and RNA. The synthesis of viral proteins was assayed by labeling infected cell monolayers with 40 to 75 μ Ci of [^{35} S]methionine per ml and analyzing the products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (26). Immunoprecipitation of DBP and fiber protein from infected cell lysates was performed as described previously (2, 30).

Analysis of adenovirus DNA synthesis and preparation of low-molecular-weight DNA from adenovirus-infected cells have been described previously (40). To compare the numbers of adenovirus templates in infected cells, isolated nuclei remaining after cytoplasmic RNA extraction (see below) were lysed in 3.8 ml of 50 mM Tris (pH 7.4)–10 mM EDTA–100 mM NaCl–0.5% SDS, and then 0.2 ml of 10 mg of pronase per ml was added. The lysates were incubated at 37°C overnight. After two extractions with phenol-chloroform-isoamyl alcohol (24:24:1) and one extraction with chloroform-isoamyl alcohol (24:1), the nucleic acid was precipitated with 2.5 volumes of ethanol. Ten micrograms of each DNA preparation was digested with *KpnI*, fractionated on a 1% agarose gel, and blotted to a nitrocellulose filter. The filter was hybridized with a nick-translated ^{32} P-labeled Ad5 DNA probe at 42°C in 50% formamide–0.4 M NaCl–0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.0)–5 mM EDTA–0.2% SDS–5 $\times$ Denhardt solution (1 $\times$ Denhardt solution is 0.2% [wt/vol] bovine serum albumin, Ficoll [molecular weight, 400,000], and polyvinyl pyrrolidone)–100 μ g of denatured salmon sperm DNA per ml. The filters were washed twice in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature, and three times in 0.1 $\times$ SSC–0.1% SDS at 55°C.

For comparison of steady-state levels of early adenovirus mRNAs, HeLa monolayers were infected with virus at a multiplicity of 50 PFU per cell. The overlay media included 10 mM hydroxyurea (Calbiochem-Behring, La Jolla, Calif.) to block DNA replication. Cytoplasmic RNA was prepared from the cells by phenol and chloroform extraction of the 0.5% Nonidet P-40 supernatant fractions followed by ethanol precipitation (26). For Northern blot analysis, equal amounts (usually 20 μ g) of each preparation were denatured and electrophoresed on a 1.2% formaldehyde-agarose gel as described by Maniatis et al. (35). For dot blot analysis, 5 μ g of each cytoplasmic RNA preparation was denatured in formaldehyde as described above for Northern analysis, mixed with an equal volume of cold 20 $\times$ SSC, and aspirated onto nitrocellulose through a 96-well dot blot manifold (Bethesda Research Laboratories). DNA probes specific for adenovirus early regions were labeled by nick translation with [^{32} P]dCTP to a specific activity of 1×10^8 to 5×10^8 cpm per μ g. Four of the probes were plasmid pBR322 derivatives containing Ad2 DNA inserts. Plasmids containing the *Ball* L (coordinates 6.0 to 7.7), *EcoRI* B (coordinates 58.5 to 70.7), and *HindIII* F (coordinates 89.5 to 97.1) fragments were probes for E1B, E2A, and E4, respectively. The plasmid pHEB4 (coordinates 0.8 to 4.5), a clone of a *Ball*-*HpaI* double digest, was used as the probe for E1A. The purified *EcoRI* C restriction fragment from Ad5 (coordinates 75.9 to 84.0) was used as the probe for early region 3 (E3). The nitrocellulose filters were hybridized and washed as described above for the DNA blots. For quantitation of the dot blot data, individual dots were cut out and counted in 3 ml of Aquasol-2 (New England Nuclear Corp., Boston, Mass.). The radioactivity bound to duplicate dots differed by

FIG. 1. Strategy for introducing a cloned E2A allele into the adenovirus genome. The upper line represents the Ad5ts1+2 genome, with the cross-hatched bars indicating the coordinates to which the ts2 hexon and ts1 100K mutations were mapped. The bottom line represents the Ad5 *Hind*III A restriction fragment. The arrows in the figure indicate the position and direction of transcription of the E2A gene. The two pairs of crossed lines show the recombination event which crossed out both *ts* mutations and introduced the cloned E2A allele.

less than 20%, and usually by less than 10%. The number of counts per minute binding to dots containing 5 μ g of HeLa cell RNA was considered background and was subtracted before quantitation.

RESULTS

Strategy for constructing adenovirus E2A mutants. To obtain and study DBP-defective adenovirus mutants, deletions were introduced into the E2A gene. Due to the large size of the adenovirus genome, however, it was impractical to engineer such deletions in vitro with genomic adenovirus DNA. Instead, deletions were first constructed in cloned copies of E2A, and then the altered alleles were introduced into the intact viral genomes by in vivo recombination. Our strategy for introducing a cloned E2A gene into the viral chromosome (Fig. 1) is a variation of the genetic mapping procedure called marker rescue (5, 18). In this technique a subgenomic fragment of adenovirus DNA is transfected with intact genomic DNA into permissive cells. A small but significant fraction of the progeny are recombinants which have acquired some or all of the subgenomic fragment. To select for the desired E2A recombinants, our strategy makes use of a parental virus which contains *ts* mutations in two late viral genes which flank E2A. This virus, designated Ad5ts1+2, contains both the *ts*1 mutation in the 100-kDa gene (mapped between coordinates 70.0 and 71.4) and the *ts*2 mutation in the hexon gene (mapped between coordinates 54.7 and 57) (48, 50).

The feasibility of the scheme was tested in a pilot experiment in which DNA-terminal protein complex derived from Ad5ts1+2 plus various plasmid DNAs were transfected into human 293 cells. The transfections were allowed to proceed through one infectious cycle at either the permissive or nonpermissive temperature, before a subsequent passage in HeLa cells at the nonpermissive temperature to select for non-*ts* recombinants. Only those cells which received plasmid DNA containing an adenovirus insert large enough to rescue both *ts* mutations yielded non-*ts* virus (Table 1). The rescuing plasmid, pAd5HA, contains the Ad5 *Hind*III A fragment (coordinates 50.1 to 72.8) cloned into the *Hind*III site of pBR322. This experiment also demonstrated that the state of the rescuing plasmid DNA and the conditions for selection are important. Plasmid pAd5HA DNA which had been digested with *Hind*III rescued much more efficiently than either undigested pAd5HA DNA or *Sal*I-digested pAd5HA DNA. Since *Hind*III digestion releases the viral insert while *Sal*I linearizes the plasmid but leaves pBR322 sequences at the ends, this result suggests that homologous ends increase the efficiency of recombination. It was also critical that the transfection be carried out at the permissive

temperature of 33°C, as no recombinants arose from transfected cells incubated at 40°C (see below).

Construction of adenovirus E2A deletion mutants. Five derivatives of the pAd5HA plasmid were constructed which contained various deletions in the DBP coding region (see above). These plasmids were designated pAd5d1801 through pAd5d1805 and are illustrated in Fig. 2. The viral inserts were released from pAd5d1801 through pAd5d1805 by *Hind*III digestion and then individually transfected with intact Ad5ts1+2 DNA-terminal protein complex into either 293 or dexamethasone-induced gmDBP1 cells. After one infectious cycle at 33°C, the progeny of the transfections were passaged multiple times (usually 3 days per passage) at 40°C in dexamethasone-induced gmDBP1 or gmDBP2 cells to select for non-*ts* recombinants. After three or four passages, most of the cultures which had originally been transfected with Ad5ts1+2 DNA-terminal protein complex plus the deleted E2A plasmids showed pronounced CPE, while the cultures which had originally been transfected with Ad5ts1+2 DNA-terminal protein complex alone showed no CPE. Restriction enzyme analysis of low-molecular-weight DNA isolated from these cultures indicated that the monolayers showing CPE did in fact contain adenovirus DNA, but that the E2A gene was apparently WT (i.e.,

TABLE 1. Rescue of Ad5ts1+2 by plasmid DNAs^a

Rescuing DNA ^b	Adenovirus coordinates ^c	Transfection temp (°C)	Total PFU ^d
None		33	<10
pHB1 (<i>Hind</i> III- <i>Bam</i> HI)	50.1-59.5	33	<10
pAd2HpB (<i>Bgl</i> II)	63.6-77.9	33	<10
pAd5HA (<i>Hind</i> III)	50.1-72.8	33	200,000
pAd5HA (undigested)	50.1-72.8	33	1,000
pAd5HA (<i>Sal</i> I)	50.1-72.8	33	400
pAd5HA (<i>Hind</i> III)	50.1-72.8	40	<10

^a Human 293 cells were transfected with Ad5ts1+2 DNA-terminal protein complex alone or with 10 adenovirus molar equivalents of various plasmid DNAs. The transfected cells were allowed to undergo one infectious cycle (4 days at 33°C; 3 days at 40°C). One-tenth of the progeny of the transfection was passaged in HeLa cells for 3 days at 40°C to select for non-*ts* recombinants.

^b The plasmid DNAs were first digested with the restriction enzymes indicated in parentheses. The plasmid pHB1 consists of a *Hind*III-*Bam*HI fragment of Ad2 (coordinates 50.1 to 59.5) cloned into pBR322. The plasmid pAd2HpB consists of the Ad2 *Hpa*I B fragment (coordinates 57 to 85) cloned into the *Pvu*II site of pBR322.

^c Coordinates of the potentially rescuing adenovirus fragment are indicated. For pAd2HpB, the coordinates of the internal viral fragment released by *Bgl*II digestion are indicated.

^d HeLa cell lysates were titrated on HeLa cells at 40°C to assay non-*ts* virus. The numbers shown are the total PFU in each lysate (approximately 3×10^6 cells).

FIG. 2. Structure of the deleted E2A alleles. The lower portion of the diagram indicates the structure of the WT E2A gene, its RNA, and its protein. At the bottom, adenovirus coordinates are indicated as are the restriction sites used in the mutant construction; X denotes the Ad2 *Xho*I site at coordinate 65.9, and S denotes the Ad5 *Sst*II sites at coordinates 62.2, 64.4, and 65.7. The large horizontal arrow indicates the coding exon of E2A mRNA, and the large open bar represents the open reading frame which encodes the 529-amino-acid (aa) DBP. The five lines above indicate the structures of the E2A genes in the deleted alleles. Parentheses denote the extent of the DNA sequences deleted, while the bars above each line represent the open reading frame predicted to be used by each mutant. Open bars correspond to translation in the normal DBP reading frame, and the stippled bars indicate that translation is in an alternate reading frame. The numbers above each bar correspond to the number of amino acids in each segment of the predicted polypeptide, while to the left of each bar is indicated the nonsense codon which should terminate translation. The asterisk above the open bar of d1804 represents the histidine to tyrosine alteration at amino acid 130 of the expected d1804 polypeptide. In the intact DBP, the alteration allows the virus to express its late genes in monkey cells.

undeleted; data not shown). Southern blot analysis of the DNA preparations, however, indicated that a very small percentage of the adenovirus DNA molecules in some cultures contained the deleted E2A alleles (data not shown).

Since cells transfected with Ad5ts1+2 DNA alone never gave rise to virus after selection at 40°C, the apparent WT virus obtained after transfection with Ad5ts1+2 plus the deleted plasmid DNA must have arisen by recombination. The most probable explanation for their origin was a double recombination event or events in which plasmid DNA sequences rescued both Ad5ts1+2 *ts* mutations, but left the Ad5ts1+2 E2A gene unaltered. Although this event is probably less frequent than the desired single recombination event depicted in Fig. 1, these WT recombinants might have a significant growth advantage over recombinants containing E2A deletions, even in the induced gmDPB cell lines. Thus by the third or fourth passage the WT recombinants may be the most abundant virus in the cultures.

This hypothesis predicts that the progeny of the original transfection would include more recombinants containing the desired E2A deletion than recombinants containing the WT E2A allele. Therefore, serial fivefold dilutions of the transfection lysates were made and passaged at 40°C in the

gmDBP cell lines as described above. Most of the cultures which received the lower dilutions (5- and 25-fold) again showed pronounced CPE on the third or fourth passage and were presumed to contain WT recombinants. Cultures which received the higher dilutions (125- and 625-fold), however, appeared healthy until the fifth or sixth passage, at which time they showed definite but less pronounced CPE. Low-molecular-weight DNA was isolated from these latter cultures, digested with *Kpn*I and electrophoresed on an agarose gel. Figure 3 shows the results of this analysis for the cultures which had originally been cotransfected with pAd5d1803 through pAd5d1805. Each of these cultures appeared to contain only the desired adenovirus deletion mutant, as none of the viral DNAs contained a wild-type *Kpn*I E restriction fragment (arrow, Fig. 3). Instead, each

FIG. 3. *Kpn*I digestion of low-molecular-weight DNA isolated from infected gmDPB2 cells. Low-molecular-weight DNA was isolated from monolayers (60 mm) of gmDPB2 cells which were infected with WT Ad5 or with the cell lysate from fifth-passage gmDPB2 cultures originally infected with dilutions of the lysates of cells that had been cotransfected with pAd5d1803, pAd5d1804, or pAd5d1805. One-third of each DNA preparation was digested with *Kpn*I, electrophoresed on a 1% agarose gel, and stained with ethidium bromide (labeled Ad5, d1803, d1804, and d1805). To see if the viral deletions corresponded to the deletions in the parental plasmids, *Kpn*I-digested plasmid DNA (approximately 0.3 µg) from WT pAd5HA and each of the three deletion derivatives was run in the lanes immediately to the right of each corresponding viral DNA (labeled pAd5HA, pAd5d1803, pAd5d1804, and pAd5d1805). The arrow indicates the position of the wild-type *Kpn*I E restriction fragment.

restriction pattern included a smaller fragment that comigrated with the deleted *Kpn*I E fragment of the parental plasmid. Similar results were obtained for the cultures which had originally been cotransfected with pAd5d1801 and pAd5d1802 (data not shown).

Large stocks of each of the five E2A deletion mutants were made in dexamethasone-induced gmDBP2 cells, starting from the dilution cultures which contained the appropriate deletion mutant DNA. The five adenovirus deletion mutants were designated Ad5d1801 through Ad5d1805. Although we did not sequence the E2A alleles of the five mutant virus, extensive restriction enzyme analysis of the viral DNAs indicated that each mutant virus had an E2A deletion consistent in structure with that of the parental plasmid (data not shown).

When the five deletion mutant stocks were titrated on HeLa cell monolayers, no viral plaques were observed. This result was consistent with the expectation that E2A defines an essential adenovirus gene. Unfortunately, no viral plaques were obtained when the deletion mutant stocks were

TABLE 2. Yields of virus in HeLa and gmDBP2a cells^a

Virus	Cell line	PFU/cell ^b
Ad5	HeLa	2,000
Ad5d1801	HeLa	0.1
Ad5	gmDBP2a	9,000
Ad5d1801	gmDBP2a	2
Ad5d1801	gmDBP2a (+ dex) ^c	90

^a Confluent monolayers were infected at a multiplicity of 20 PFU per cell and incubated for 68 h at 37°C.

^b Progeny virus were harvested by freeze-thawing and sonication. The yields of Ad5 were determined by titration on HeLa cells. The yields of Ad5d1801 were determined by titration on HeLa cells using Ad5d1434 as a helper virus.

^c The overlay media included 0.6 μ M dexamethasone (dex).

titrated on gmDBP1 or gmDBP2 cells in the presence of dexamethasone, even though these monolayers efficiently supported plaque formation by WT virus (see below). In an attempt to assay the infectivity of the deletion mutants, we titrated the mutant stocks on HeLa cells in the presence of a helper virus (see above). The helper virus used was Ad5d1434, an E1A-E1B deletion mutant (lacking sequences between viral coordinates 2.6 and 8.7; D. Solnick, unpublished data) which is itself defective for plaque formation and growth in HeLa cells. When HeLa cell monolayers were uniformly infected with Ad5d1434 at a multiplicity of 2 PFU per cell, the E2A deletion mutant stocks formed plaques with apparent one-hit kinetics and varied in titer between 2×10^8 and 2×10^9 PFU per ml.

DBP-related polypeptides encoded by the E2A deletion mutants. Since the DNA sequence of both the Ad2 and Ad5 E2A genes is known (32, 33), it is possible to predict the effects of the deletion mutations on E2A coding potential (Fig. 2). Four of the five deletions cause frameshift mutations in the DBP-coding region. The exception is the Ad5d1803 deletion which fuses in frame amino-terminal and carboxyl-terminal coding regions. To test these predictions, HeLa cell monolayers were infected with WT or mutant virus, and at 24 h p.i. they were pulse-labeled with [³⁵S]methionine. The labeled protein extracts were immunoprecipitated with a polyclonal antiserum raised against purified DBP (Fig. 4). As expected, none of the mutant-infected HeLa cells synthesized an intact 72-kDa DBP. With one exception, the mutants produced DBP-related proteins that were consistent in size with the predictions shown in Fig. 2. The exception was Ad5d1801, which was predicted to encode a 22-amino-acid polypeptide. Unexpectedly, Ad5d1801-infected cells synthesized two DBP-related polypeptides of 40 and 37 kDa. The origin of these Ad5d1801 proteins is not understood at this time (see below). Ad5d1802 was predicted to encode a small (47 amino acid) and probably undetectable DBP-related protein. Consistent with this, no detectable protein was immunoprecipitated from the Ad5d1802 infections. HeLa cells infected with Ad5d1803 through Ad5d1805 synthesized DBP-related proteins of approximately 17, 38, and 18 kDa, respectively, which are in reasonable agreement with the predicted 12-, 30- and 21-kDa polypeptides. These sizes are not inconsistent with expectation, since the WT DBP has a molecular weight of 59 kDa, as deduced by its amino acid sequence, but migrates on SDS-polyacrylamide gels as a 72-kDa protein, possibly as a result of the high proline content near the amino terminus of DBP (32, 33). In some additional experiments, the DBP-related proteins encoded by Ad5d1803 and Ad5d1804 were resolved into triplet and doublet species, respectively (data not

FIG. 4. DBP-related polypeptides synthesized in E2A deletion mutant-infected HeLa cells. Confluent monolayers of HeLa cells (60 mm) were infected at a multiplicity of 100 PFU per cell with WT Ad5 or with Ad5d1801 through Ad5d1805. At 20 h p.i. the monolayers were labeled for 1 h with 75 μ Ci of [³⁵S]methionine per ml. One-fourth of each labeled protein extract was immunoprecipitated with polyclonal rabbit antisera raised against purified DBP, and the immunoprecipitates were electrophoresed on a 15% SDS-polyacrylamide gel. An autoradiogram of the gel is shown. To the right of the panel, the position at which molecular weight marker proteins migrated is indicated (in thousands [K]). The position of the 72-kDa DBP is indicated to the left of the panel. The approximately 100-kDa protein which is seen in most lanes is a cellular protein which is sometimes immunoprecipitated by the anti-DBP serum.

FIG. 5. Viral protein synthesis in E2A deletion mutant-infected cells. Confluent monolayers of HeLa cells (a) or gmDBP2a cells (b) were infected at a multiplicity of 20 PFU per cell with WT Ad5, Ad5d1801, or Ad5d1802. Mutant-infected gmDBP2a cells were incubated either in the absence (-) or presence (+) of 0.6 μ M dexamethasone. At 40 h p.i., the monolayers were labeled for 1 h with 40 μ Ci of [^{35} S]methionine per ml. Equal fractions of each cell lysate were electrophoresed on a 15% SDS-polyacrylamide gel, and the dried gel was subjected to autoradiography. The positions of several late adenovirus proteins are indicated.

shown), but the origin of this protein heterogeneity has not been investigated.

E2A deletion mutants are defective for viral growth and DNA replication. As mentioned above, none of the five E2A deletion mutant stocks formed plaques on HeLa cells, suggesting that these mutants were defective for viral growth. To test this directly, monolayers of HeLa or gmDBP2a cells (a subclone of the gmDPB2 cell line) were infected with WT Ad5 or Ad5d1801, and the infections were allowed to proceed through an infectious cycle at 37°C. Virus yields from these infections are shown in Table 2. As expected, Ad5d1801 is defective for growth in HeLa cells, producing less than 1 PFU per infected cell. Although a small amount of progeny Ad5d1801 virus were produced in gmDBP2a cells in the absence of dexamethasone (2 PFU per cell), hormone stimulation was essential for efficient propagation of this mutant (90 PFU per cell). Note that even with dexamethasone stimulation, Ad5d1801 grew approximately 100-fold less efficiently in gmDBP2a cells than did WT Ad5.

Growth of adenovirus can also be evaluated by monitoring late viral protein synthesis. Therefore, monolayers of HeLa or gmDBP2a cells were infected with WT Ad5, Ad5d1801, or Ad5d1802 and pulse-labeled for 1 h with [^{35} S]methionine at 40 h p.i. The labeled proteins were electrophoresed on an SDS-polyacrylamide gel to assay for the synthesis of late viral proteins (Fig. 5). No late viral proteins could be detected in either mutant-infected HeLa cells or mutant-

infected gmDBP2a cells in the absence of dexamethasone. When gmDBP2a cells were infected with Ad5d1801 or Ad5d1802 in the presence of dexamethasone, however, synthesis of late viral proteins was easily detected, although shutoff of host protein synthesis did not occur as efficiently as in the corresponding WT infections. Similar results were obtained for mutants Ad5d1803 through Ad5d1805 (data not shown).

Since DBP is known to be required for adenovirus DNA synthesis (16, 47), it was strongly suspected that the E2A deletion mutants were defective for viral DNA replication. To test this, HeLa or dexamethasone-induced gmDBP2a monolayers were infected with WT Ad5, Ad5d1801, or Ad5d1802, and labeled from 28 to 34 h p.i. with [^{3} H]thymidine. Low-molecular-weight DNA was extracted from the cells, digested with *Kpn*I, and electrophoresed on an agarose gel. No viral DNA synthesis could be detected in mutant-infected HeLa cells (Fig. 6), even after very long exposures of the fluorogram. The two deletion mutants did replicate their DNA in gmDBP2a cells in the presence of dexamethasone, but at a reduced rate (approximately 30- to 50-fold) compared with that of WT. Although the mutants Ad5d1803 through Ad5d1805 were not directly tested for viral DNA replication, they were probably also defective since they failed to produce the intact carboxy-terminal segment of the DBP which contains *in vitro* DNA replication activity (4).

FIG. 6. Viral DNA synthesis in E2A deletion mutant-infected cells. Confluent monolayers (60 mm) of HeLa or gmDBP2a cells were infected at a multiplicity of 20 PFU per cell with WT Ad5, Ad5d1801 or Ad5d1802. The gmDBP2a infections were incubated in media containing 0.6 μ M dexamethasone. The monolayers were labeled from 28 to 34 h p.i. with [^{3} H]thymidine, and low-molecular-weight DNA was isolated. One-fourth of each DNA sample was digested with *Kpn*I and electrophoresed on a 1% agarose gel. Shown is an autoradiogram of the dried gel after treatment for fluorography. The labeled band comigrating with the Ad5 *Kpn*I F fragment was seen in mock-infected cells and probably represents synthesis of mitochondrial DNA.

FIG. 7. Failure of the Ad5dl1804 DBP-related polypeptide to enhance late viral gene expression in monkey cells. Monolayers of CV_c cells were either singly infected (40 PFU per cell) or doubly infected (20 PFU per cell of each virus except for one infection [indicated by an asterisk] in which 20 PFU of Ad2 per cell and 100 PFU of Ad5dl1804 per cell were used) with adenovirus mutants. At 36 h p.i. the monolayers were labeled for 2 h with 40 μ Ci of [³⁵S]methionine per ml. Equal fractions of each labeled protein extract were immunoprecipitated with polyclonal rabbit antisera raised against purified native fiber protein (A) or purified native DBP (B). In panel A, note that the Ad2 and Ad5 fiber polypeptides can be distinguished, as can Ad2 and Ad5 hexon polypeptides which are also partially immunoprecipitated by the fiber antiserum. In panel B, the positions of the 72-kDa DBP and the Ad5dl1804-specific, DPB-related polypeptide are indicated (arrow). SV40 coinfection also allows WT adenovirus to grow productively in monkey cells (8).

Expression of the truncated DBP-related polypeptide of Ad5dl1804 does not enhance late viral gene expression in monkey cells. We have previously presented data which suggests that the amino-terminal domain of DBP may act as an independent functional domain which can enhance late viral gene expression and viral growth in monkey cells (40). If so, then the DBP-related polypeptide encoded by Ad5dl1804 (Fig. 2) may retain this function since this truncated polypeptide should contain an amino-terminal domain derived from Ad5hr404, an E2A mutant able to express its late genes in monkey cells (29, 32). If the Ad5dl1804 DBP-related polypeptide is functional, then Ad5dl1804 should enhance WT Ad2 late gene expression when both viruses are coinfecting into monkey cells. Since Ad2 is not defective for DNA replication in monkey cells, the coinfecting cells should progress into the late phase of infection in which the DBP fragment containing the *hr* mutation might help overcome the block to late gene expression. Therefore, confluent monolayers of CV_c cells, a line of African green monkey kidney cells, were infected with various virus inocula, incubated until well into the late phase (36 h p.i.), and then labeled for 2 h with [³⁵S]methionine. Although synthesis of most late viral proteins is depressed in abortively infected monkey cells, the fiber polypeptide is the most dramatically affected, being reduced some 100- to 200-fold (2, 26). The labeled protein extracts therefore were immunoprecipitated with a polyclonal antibody prepared against purified fiber protein, and the immunoprecipitates were electrophoresed on an SDS-polyacrylamide gel (Fig. 7A). Note that Ad2 and Ad5 fiber polypeptides can be distinguished in this gel system, as can Ad2 and Ad5 hexon polypeptides which are also partially precipitated with the fiber antiserum. As expected, Ad2-infected CV_c cells produced very little fiber

compared with that of productive CV_c cell infections (Ad2-SV40, Ad2hr400, and Ad5hr404). As a positive control, CV_c cells were coinfecting with Ad5hr404 and Ad2, and as expected the hr404 DBP functioned in *trans* to enhance fiber synthesis. Ad5dl1804, however, could not enhance either fiber or hexon synthesis, even when very high Ad5dl1804 multiplicities of infection (100 PFU per cell) were used (asterisk, Fig. 7). Lack of enhancement was not due to the failure of the coinfecting cells to express the Ad5dl1804 DBP-related protein, as it was easily detected when the labeled extracts were immunoprecipitated with anti-DBP serum (Fig. 7B). However, immunoblot analysis of the protein extracts demonstrated that the steady-state levels of the Ad5dl1804 polypeptide were reduced between 10- and 40-fold in the coinfections compared with the level of DBP in a WT infection (data not shown). Determination of virus yields of parallel infections showed that Ad5dl1804 could not enhance Ad2 growth in CV_c cells (data not shown), which is consistent with its inability to enhance fiber synthesis. Very similar results were obtained when these experiments were repeated with CV₁ cells (data not shown), a monkey cell line in which the block to Ad2 growth is less stringent than in CV_c cells (40).

The role of DBP in adenovirus early gene regulation. To study the role of DBP in viral early gene regulation, steady-state levels of early mRNAs were monitored in WT Ad5-, Ad5ts125-, and Ad5dl1802-infected HeLa cells. Since Ad5dl1802 produces no DBP or detectable DBP fragment, this experiment allows one to observe adenovirus early gene expression in the complete absence of any DBP-mediated regulation. HeLa monolayers were infected at a multiplicity of infection of 50 PFU per cell with each virus and incubated at 40°C. To validate comparisons between WT Ad5 and the

FIG. 8. Analysis of early adenovirus gene expression in DBP mutant-infected cells. Confluent monolayers of HeLa cells were infected at a multiplicity of 50 PFU per cell with WT Ad5, Ad5d1802, or Ad5ts125. The infections were incubated at 40°C in media containing 10 mM hydroxyurea. (A) Northern blot analysis of early viral RNA expression in mutant-infected cells. At various times after infection (indicated in hours by the number immediately above each lane) cytoplasmic RNA was extracted. Equal amounts of each RNA preparation were denatured, electrophoresed on a 1.2% formaldehyde-agarose gel, and subjected to Northern blot analysis with ^{32} P-labeled DNAs specific for various early regions (indicated next to each panel) as probes. (B) Comparison of the number of viral templates in infected cell nuclei. Total nuclear DNA was prepared from the cells used for the cytoplasmic RNA extractions at 20 h p.i. Ten micrograms of each DNA preparation was digested with *Kpn*I and subjected to Southern blot analysis with ^{32} P-labeled Ad5 DNA used as a probe.

two DNA replication-defective mutants, the potent DNA replication inhibitor hydroxyurea was added to all the infections. At various times after infection, cytoplasmic RNA was prepared from the infected cells. As a control, to make sure that there were approximately equal numbers of viral templates in each infection, nuclear DNA was prepared from the infected cells at the last time point (20 h p.i.). Equal amounts of the DNA preparations were digested with *Kpn*I and subjected to Southern blot analysis with ^{32} P-labeled Ad5 DNA as a probe (Fig. 8B). This analysis confirmed that there were approximately equal numbers of viral DNA molecules in each infected cell nucleus. To compare the steady-state levels of messages from various early regions, equal amounts of the cytoplasmic RNA preparations were subjected to Northern blot analysis with ^{32} P-labeled DNAs specific for E1A, E1B, E2A, E3, and E4 as the hybridization probes (Fig. 8A).

Three general conclusions can be drawn from the results of this experiment and two repeat experiments of the same design (data not shown). First, DBP does not appear to be a significant positive regulator of either E1B or E4 mRNA expression in infected HeLa cells, since WT and Ad5d1802 show similar patterns for both the induction and continued expression of these two early genes. Second, we observed that E2A mRNA levels were reduced significantly in Ad5d1802 infections (Fig. 8A). Also note that E2A mRNA from Ad5d1802 infections migrates slightly faster than does E2A mRNA from WT infections, consistent with the expectation that it contains a 242-nucleotide internal deletion. Quantitation of mRNA levels by dot blot hybridization

analysis (data not shown) indicated that E2A mRNAs were reproducibly reduced about fivefold in Ad5d1802 infections as compared with WT Ad5 infections. These results are consistent either with a positive role for DBP in the *trans*-acting regulation of its own gene or with a *cis*-acting defect caused by the Ad5d1802 deletion. Third, DBP does not appear to negatively regulate expression of E1A, E1B, E3, or E4 mRNAs when DNA replication is tightly blocked since Ad5d1802 shows little if any overexpression of these regions when compared with that of WT Ad5. In some experiments (e.g., E4 in Fig. 8A), slight overexpression (approximately twofold) of early mRNAs in Ad5d1802-infected cells was noted, but these results were not reproducible in other experiments. Ad5ts125-infected cells, however, did show a reproducible tendency to overexpress early mRNAs at late times (12 to 20 h p.i.) when compared with that of WT Ad5 (e.g., E1B in Fig. 8A). The observed effect, however, was quite variable and never dramatic (maximally about fourfold). Much more reproducible was the tendency for Ad5ts125-infected cells to express early viral genes sooner than WT Ad5 or Ad5d1802-infected cells and at higher levels at the earlier times (4 to 9 h p.i.; this can be observed for all early regions in Fig. 8A). Since the DBP-negative mutant Ad5d1802 behaves much more similarly to WT Ad5 than to Ad5ts125, we conclude that DBP does not have a significant negative effect on the accumulation of E1A, E1B, E3, or E4 mRNAs in infected HeLa cells monolayers when DNA replication is tightly blocked. No conclusions can be drawn concerning the negative regulation of E2A by DBP, since Ad5d1802 fails to properly initiate E2A expression.

DISCUSSION

In this report a genetic system is described which can be used to construct Ad5 mutants of any desired E2A genotype. The E2A mutations were first engineered into a cloned E2A gene by techniques of *in vitro*, site-directed mutagenesis. Next the cloned, mutated alleles were transfected into human cells with genomic DNA-terminal protein complex derived from an adenovirus mutant, Ad5ts1+2, which contains *ts* mutations in two genes flanking E2A. Homologous recombination occurs in the transfected cells, creating viral progeny which crossed out both *ts* mutations and crossed in the mutant E2A allele. These recombinants can be genetically selected and propagated by growth at the nonpermissive temperature in DBP-producing cell lines (27). This protocol was used to isolate and propagate five Ad5 E2A deletion mutants, designated Ad5d1801 through Ad5d1805.

In the course of these experiments, it was discovered that the transfections need not be carried out in the DBP-producing cell lines. Four of the five deletion mutants described here were isolated after transfection of the noncomplementing but highly transfectable 293 cell line. Our results are most easily explained by assuming that recombinants constitute only a minor fraction of the viral templates in any given transfected cell. Thus the E2A mutants need not be complemented by the cell line since they are efficiently complemented by the DBP produced by the many Ad5ts1+2 templates. Consistent with this model is the observation that no recombinants were recovered when the transfection protocol was carried out at the nonpermissive temperature (Table 1). Recombinant genomes probably are formed at the nonpermissive temperature, but are not packaged due to the large amount of defective 100K and hexon polypeptides produced by the numerous Ad5ts1+2 templates.

Although the protocol described allowed the construction of several E2A mutants, two factors limit its general efficiency as a means of isolating large numbers of DBP mutants. First, the system requires the E2A mutations be introduced into the pAd5HA plasmid, since it is the smallest E2A-containing plasmid which can also rescue both mutations of Ad5ts1+2. The pAd5HA plasmid, however, is relatively large and therefore is not easily manipulated *in vitro*. We currently are testing other strategies for introducing a cloned E2A allele into the adenovirus genome which may allow us to use smaller E2A-containing plasmids. The second factor which has limited the utility of this system is the fact that E2A-defective mutants fail to form viral plaques on the complementing gmDBP cell lines. This failure is probably due to the relatively inefficient growth of the E2A mutants in the gmDBP cell lines (see Table 2). Since several rounds of viral replication normally are required to create a visible adenovirus plaque, the reduced yield of the mutants combined with the limited viability of the gmDBP monolayers during a plaque assay (about 9 days at 37°C) is probably responsible for the inability to detect plaques.

The failure of the DBP-defective mutants to form plaques results in two important problems with respect to the study of these mutants. First, the number of infectious virus in a mutant stock cannot be directly titrated in a plaque assay. Fortunately, we obviated this problem by titrating the mutant stocks on HeLa cells using a helper virus. The second and more serious problem is that, without plaque formation, E2A mutants cannot be directly isolated from a virus population. This is particularly serious since rescue of Ad5ts1+2 with E2A deletion plasmids often creates a small class of double recombinants which are WT for E2A and can rapidly

outgrow the desired E2A mutants. Plaque formation would also allow the transfection protocol to be carried out with a collection of mutagenized plasmids, rather than a single mutant plasmid. Large numbers of individual viral mutants could be isolated from a single mutagenesis and transfection experiment and screened for desired phenotypes.

The E2A deletions in Ad5d1801 and Ad5d1802 extended very near to the amino terminus of DBP-coding sequences. Since these deletions also caused frameshift mutations, both mutants were predicted to encode small proteins with very limited identity to DBP. This expectation was upheld for the mutant Ad5d1802, which made no detectable DBP-related polypeptide and thus represents the first DBP-negative adenovirus mutant. Ad5d1801, however, unexpectedly produced two DBP-related proteins of approximately 40 and 37 kDa. Although we have not yet investigated the origin of these proteins, it is interesting that Asselbergs et al. (6) have identified rare E2A mRNAs which can be translated *in vitro* into DBP-related proteins of similar size.

All five of the E2A deletion mutants appear to be absolutely defective for growth in HeLa cells, thus verifying the expectation that DBP is an essential adenovirus protein. It was also demonstrated that Ad5d1801 and Ad5d1802, and probably Ad5d1803 through Ad5d1805, are completely defective for viral DNA replication. Since most previously isolated adenovirus mutants which are DNA replication defective are to some extent leaky, it seems likely that the E2A deletion mutants described here are the tightest DNA replication-negative mutants in existence. As such, they may be useful for the study of the regulation of those adenovirus functions, such as late gene expression (44), which are dependent on viral DNA replication.

The *hr* mutant DBP carries out an activity in monkey cells which enhances the expression of late viral genes (29). The large T antigen of SV40 is functionally similar to DBP in that it can also enhance late adenovirus gene expression in monkey cells (8). In the case of T antigen, the enhancement activity is carried out by an independent functional domain at the carboxyl terminus of the protein, since this domain can function even when attached to other polypeptides (15, 45). We have suggested previously that the amino-terminal portion of DBP defines an analogous, and perhaps independent, functional domain (40). Evidence for this came from the study of the E2A double mutant Ad2ts400 which contains both an amino-terminal *hr* mutation and a carboxy-terminal *ts* mutation. Ad2ts400 was able to enhance late viral gene expression in monkey cells at the nonpermissive temperature, even when the Ad2ts400 DBP was nonfunctional for viral DNA replication. The mutant Ad5d1804 was constructed to test directly whether an amino-terminal portion of DBP containing the *hr* mutation could function as an independent domain. This mutant encodes a protein in which the amino-terminal 228 amino acids of the Ad5hr404 DBP are fused to 43 amino acids translated from an alternate reading frame. When Ad5d1804 was coinfecting with Ad2 into monkey cells, however, enhancement of late viral gene expression did not occur. Several interpretations of this result are possible. First, the amino-terminal portion of DBP may not define a functionally independent domain, requiring some or all of the carboxy-terminal portion for activity. Alternately, the amino-terminal portion may define an independent functional domain, but the particular conformation of the Ad5d1804 polypeptide, perhaps because of its abnormal carboxy terminus, renders this particular protein nonfunctional. Another possibility which cannot be ruled out is that the Ad5d1804 protein failed to enhance late gene ex-

pression because it was not produced in sufficient quantities. Indeed, immunoblot analysis indicated that the steady-state level of the Ad5d1804 protein in the coinfecting cells was reduced approximately 10- to 40-fold compared with the level of DBP in a WT infection. Additional DBP mutants, which efficiently express only the N-terminal domain, will be required to distinguish among these possibilities.

Isolation of the DBP-negative mutant Ad5d1802 allowed us to study adenovirus early gene expression in the absence of any DBP-mediated regulation. Results of previous studies which involved the microinjection of purified adenovirus genes or mRNAs into mammalian cells suggest that DBP may be required for or have a stimulatory effect on the expression of E1B (42) and E4 (41). Results of our experiments, however, indicate that DBP does not positively regulate the mRNAs of these genes in infected HeLa cells. It is important to note that since both microinjection studies assayed gene expression at the protein level, it remains possible that DBP stimulates translation of E1B and E4 mRNAs in HeLa cells.

These experiments unexpectedly show that Ad5d1802 fails to normally express E2A, as steady-state levels of Ad5d1802 E2A mRNAs were consistently reduced approximately fivefold compared with that of WT infections. Two hypotheses can be proposed to explain these data. First, DBP may be a positive *trans*-acting regulator of its own gene. Second, and perhaps more likely, is the possibility that the E2A deletion in Ad5d1802 causes a *cis*-acting defect which prevents efficient E2A expression. The sequences removed by the deletion may be required for efficient E2A transcription or RNA processing, or perhaps the altered Ad5d1802 E2A mRNA has a shorter half-life. Preliminary analysis has indicated that Ad5d1801-infected HeLa cells exhibit a similar deficiency in the expression of E2A mRNA.

Results of studies with *ts* E2A mutants such as Ad5ts125 have suggested that DBP negatively regulates expression from several early regions, since Ad5ts125-infected cells accumulate abnormally high levels of early mRNAs at intermediate and late times after infection (7, 10, 11, 39). In our experiments overaccumulation of E1A, E1B, E2A, E3, and E4 mRNAs was sometimes observed in Ad5ts125-infected cells at later times (12 to 20 h p.i.), but the effect was variable and never dramatic. A more reproducible effect of the Ad5ts125 mutation was the tendency of Ad5ts125-infected cells to express early mRNAs sooner than WT Ad5 and in higher amounts at these early times (4 to 9 h p.i.). Although we do not understand the discrepancy between these and previous results concerning Ad5ts125, perhaps variables such as the cell lines used are important. These studies used monolayer cultures of HeLa cells while previous studies which reported larger differences used HeLa cell suspension (7, 40) or KB suspension (10, 11) cultures. It may also be important that we used hydroxyurea to inhibit viral DNA replication, since cytosine arabinoside, the drug used in previous studies, in our hands often was not completely effective at inhibiting DNA synthesis. Repression of early gene expression by DBP may depend on a low level of DNA replication or certain physiological conditions which may occur in the presence of cytosine arabinoside but not in the presence of hydroxyurea.

If DBP were an important negative regulator of early mRNA levels, one would expect that the DBP-negative mutant Ad5d1802 would have a more dramatic phenotype, i.e., different from that of WT Ad5, than the *ts* DBP mutant Ad5ts125. This was not the case. Although Ad5d1802-infected HeLa cells sometimes showed slightly enhanced

levels of early mRNAs compared with WT-infected cells, most often little (<twofold) or no difference was observed. In no experiment did the DBP-negative mutant accumulate higher amounts of early mRNAs than did Ad5ts125. While it is impossible to exclude subtle effects of DBP, we conclude that DBP does not play a significant role in the negative regulation of E1A, E1B, E3, or E4 mRNA levels in infected HeLa cell monolayers when DNA replication is tightly blocked.

The E2A mutants Ad5ts125 and Ad5d1802 thus appear to differ somewhat in phenotype with respect to early gene expression. However, since the overaccumulation of early RNAs observed in Ad5ts125-infected cells was not dramatic and quite variable, we do not wish to overemphasize this difference between the two mutants. Ad5ts125 and Ad5d1802 can be easily distinguished with respect to another phenotype, however. At 38.5°C Ad5ts125 transforms rat embryo cells at a three- to eightfold enhanced rate compared with that of WT virus (19, 49), but Ad5d1802 as well as the other E2A deletion mutants transform at the same rate as WT (S. Rice, D. Klessig, and J. Williams, unpublished data). While it is not immediately apparent why an E2A *ts* mutant should have a phenotype not possessed by an E2A deletion mutant, it may reflect the multifunctional nature of DBP. Perhaps the temperature-sensitive DBP acquires altered and even pathological activities at the nonpermissive temperature because of the differential inactivation of functional domains.

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