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Introduction, Stable Integration, and Controlled Expression of a Chimeric Adenovirus Gene Whose Product Is Toxic to the Recipient Human Cell

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The DNA-binding protein (DBP) encoded by human adenoviruses is a multifunctional polypeptide which plays a central role in regulating the expression of the viral genes. To gain a better understanding of the relationships between the various functions provided by DBP, an extensive collection of DBP mutants is essential. To this end we have constructed several permissive human cell lines which contain and express the DBP gene at high levels to allow propagation of otherwise lethal, nonrecoverable mutants of DBP. Because DBP is toxic to human cells, cell lines were constructed by using a vector in which the DBP gene is under the control of the dexamethasone-inducible promoter of the mouse mammary tumor virus. The low basal levels of DBP synthesis in the absence of dexamethasone allows isolation and propagation of these cells. Addition of dexamethasone enhances DBP production 50- to 200-fold, and within 8 h its synthesis from the single integrated copy of the chimeric gene is 5 to 15% of that observed during peak DBP synthesis in infected human cells in which hundreds of copies of the DBP gene serve as templates. At the nonpermissive temperature, adenovirus mutants with *ts* lesions in the DBP gene replicate their DNAs, express their late genes, and form infectious viral particles in these DBP$^+$ cell lines but not in the parental HeLa cells.

A number of multifunctional proteins in eucaryotes have been defined by a combination of genetic and biochemical studies. One of the best characterized of these is the simian virus 40 (SV40) large T antigen, which exhibits separate functional domains as discerned by mapping of sets of mutations which alter one but not another of the various protein functions.

The human adenovirus 72-kilodalton DNA-binding protein (DBP) also plays several independent roles during the infectious cycle of this virus. Several mutants with temperature sensitive (*ts*) alterations in the DBP have been indispensable in defining several of the functions of this protein. The *ts* alterations at the nonpermissive temperature diminish the single-strand, DNA-binding activity of the protein in vitro (52) and inhibit DNA replication in vivo (13, 47, 53) and in vitro (20). Normal turn-off of viral early genes during the late phase of the lytic cycle is also disrupted at the nonpermissive temperature in cells infected with the *ts* mutant (6, 9, 10, 37, 38). The DBP directly affects early gene turn-off as well as DNA replication, since inhibition of viral DNA synthesis with either drugs or *ts* lesions in other viral early genes does not affect early gene expression (9, 10). In addition, at the nonpermissive temperature, the *ts* mutants transform rat cells in culture with an enhanced frequency compared with wild-type (*wt*) virus (16, 54).

A second class of mutants which greatly enhance the ability of this human virus to grow in monkey cells defines yet a second set of functions of DBP (1, 26, 29). In monkey cells infected with *wt* human adenovirus, the viral early genes are properly expressed (3), and viral DNA replication occurs normally (43). However, several of the late viral proteins, in particular the fiber protein, are synthesized at reduced levels (19, 27). The block to late gene expression appears to be due to reduced production of several viral late mRNAs (14, 27, 28) and poor translation in vivo, but not in vitro, of the fiber mRNAs which exhibit an altered pattern of splicing of their 5′ leader sequences (4; K. P. Anderson and D. F. Klessig, Proc. Natl. Acad. Sci. U.S.A., in press). The mutation that allows the virus to overcome these blocks is located in the DBP gene (29).

In addition to its roles in viral DNA replication, early and late gene expression, and cellular transformation, studies on a revertant [R(*ts*107)202] of one of the *ts* mutants suggest yet another function for this protein (39). R(*ts*107)202, although replicating its DNA and expressing its late genes, fails to assemble virion particles in human 293 cells at high temperatures.

Physical mapping of the *ts* and host range mutations by marker rescue experiments and DNA sequence determination places the 4 *ts* mutations in the 3′ half of the gene and the 10 host range mutations in the 5′ half (2, 29–32, 44). In addition, chymotryptic cleavage of *wt* DBP produces a 44- or 34-kilodalton carboxyl-terminal fragment, or both, which retains full activity in the in vitro elongation of adenovirus DNA (5, 15). Thus, like the SV40 T antigen, the DBP exhibits separate functional domains.

To better understand the interrelationships between the various functions of the DBP, we would like to obtain a more extensive collection of mutants. However, since this protein is involved in such essential viral processes as DNA replication and early and late gene expression, the lethal nature of many of the mutations would make isolation of viruses containing them infeasible. To circumvent this problem, we have tried to construct permissive human cell lines which contain and express the DBP gene for the propagation of these otherwise lethal, nonrecoverable DBP mutants.

Previous attempts to construct cell lines which contain and express the adenovirus DBP under control of its own promoter by cotransformation with the herpes simplex virus type 1 thymidine kinase gene were only partially successful (D. F. Klessig, T. Grodzicker, and V. Cleghon; Virus Res., in press). Although an expressing line (U13-2) was obtained, the amount of DBP produced was too low to efficiently complement *ts* mutants with altered DBP genes.

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In the course of those studies, evidence accumulated which suggested that the DBP gene might be toxic to the cells producing it (see below). To overcome this toxicity problem, yet at the same time construct a cell line which could produce high levels of DBP, we chose to place the DBP gene under the regulation of a promoter whose expression could be controlled.

Several such eucaryotic promoters have been described. These include the SV40 early promoter whose activity is modulated by large T antigen (23, 42, 50), the metallothionein gene promoter which responds to both glucocorticoid hormones and heavy metals (35, 41, 48), and the mouse mammary tumor virus (MMTV) promoter whose activity is also induced by glucocorticoid hormones (40, 46). Here we describe the construction and characterization of stable, viable human cell lines in which the production of the toxic adenovirus DBP is under the control of the MMTV promoter.

MATERIALS AND METHODS

Cells. All cell lines were cultivated in Dulbecco modified Eagle medium (Flow Laboratories, Inc.) 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. Transformants expressing the *Escherichia coli* xanthine-guanine phosphoribosyltransferase (*gpt*) gene were selected and grown in Dulbecco modified Eagle medium containing hypoxanthine (15 μg/ml), aminopterin (1 μg/ml), thymidine (10 μg/ml), mycophenolic acid (Eli Lilly & Co.; 25 μg/ml), xanthine (250 μg/ml), calf serum, streptomycin, penicillin, and glutamine. All medium supplements except serum and mycophenolic acid were purchased from Sigma Chemical Co.

Cell transformation. The transformation protocol was based on the $CaPO_4$ precipitation method of Graham and Van der Eb (18) as modified by Klessig and Grodzicker (29) for marker rescue studies. Each 60-cm dish of HeLa cells (90% confluent) was transfected with 0.1 to 5 μg of pMSG-DBP1 or pMSG-DBP2 mixed with 5 to 10 μg of calf thymus carrier DNA. Four hours after transfection, the cells were treated with 20% glycerol–Tris-buffered saline for 2 min (29) and were then incubated for 20 h in Dulbecco modified Eagle medium supplemented with 10% calf serum, streptomycin, penicillin, glutamine and mycostatin (20 μg/ml). At 24 h posttransfection, the cell monolayers were replated at 1 to 8 dilution, and at 72 h posttransfection, selection for *gpt* expression was applied. Selective media were changed after 24 h and then every 3 days. gpt^+ cell lines were isolated with cloning cylinders.

DNA analysis. DNA preparation and characterization was performed as described previously (Klessig et al., in press).

Protein synthesis. Viral late protein synthesis was analyzed by the methods of Klessig and Anderson (27). Synthesis and accumulation of DBP was determined by methods detailed previously (Klessig et al., in press).

RESULTS

Construction of *gpt*-DBP cell lines. The DBP gene is segmented, containing a main body which encodes the entire protein (coordinates 61.5 to 66 map units [m.u.] [31]) and two leaders (7, 12, 24). The second leader is located near the main body at coordinate 68, and the first leader, and presumably the promoter, is located either at coordinate 72 (late) or 75 (early), depending on the phase of the infectious cycle.

Two vectors (pMSG-DBP1 and pMSG-DBP2) were constructed in which the DBP gene of adenovirus type 5 (Ad5) minus its own promoter was ligated to the dexamethasone (DM)-inducible promoter carried on the long terminal repeat of the MMTV genome (Fig. 1; 21, 33, 34). Cleavage of Ad5 DNA with *Xho*I generates a fragment (28.1 to 68.5 m.u.) which contains the DBP gene minus its promoters. To attach the MMTV promoter directly to the second leader of the DBP gene, sequences upstream of the second leader were removed by *Bal* 31 treatment before addition of a *Hin*dIII linker. Digestion of this tailored segment of DNA with *Bam*HI and *Hin*dIII produced fragments (59.5 to 68 m.u.) which contained part of the second leader with attached linker, the main body of the DBP gene, and the intervening sequences between these two segments. These fragments were cloned into the *Bam*HI-*Hin*dIII cut pBR322. Plasmid DNAs from individual colonies were characterized first by restriction enzyme analyses and then by DNA sequence determinations. Two of these cloned *Bam*HI-*Hin*dIII fragments were placed behind the MMTV long terminal repeat by ligation to a *Hin*dIII-*Bgl*II partial cleavage fragment of pMDSG (33), which removes the dihydrofolate reductase (*dhfr*) cDNA gene. In the two resulting vectors, pMSG-DBP1 and pMSG-DBP2, the long terminal repeat was attached via the *Hin*dIII linker to the fragments which contained 58 and 45 base pairs, respectively, of the 76-base pair second leader as well as the main body of the DBP gene and the intervening sequences.

pMDSG as well as the two progeny vectors contain pBR322 sequences for propagation of *E. coli* and the *E. coli* xanthine-guanine phosphoribosyltransferase (*Eco-gpt*) gene under control of the SV40 early promoter. The *Eco-gpt* gene is a dominant selectable marker that allows cells which contain and express it to grow in the presence of mycophenolic acid (36).

HeLa cells were transfected with supercoiled or *Eco*RI-linearized pMSG-DBP1 or pMSG-DBP2 and selected for growth in the presence of mycophenolic acid. Large numbers of transformed colonies were visible after 2 weeks of selection. However, the majority (>80%) of these transient-

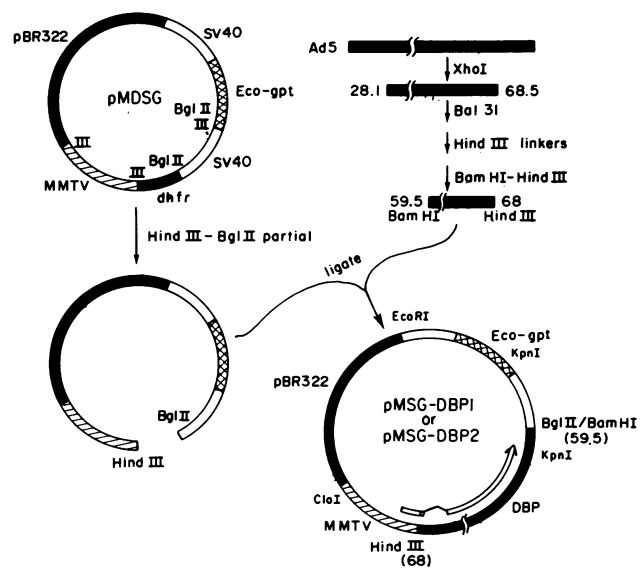

FIG. 1. Construction of pMSG-DBP vector. Numbers denote the m.u. (coordinates) on the AD5 genome. The open arrow in pMSG-DBP1 and pMSG-DBP2 show the presumed structure of the spliced, chimeric MMTV-DBP mRNA.

ly or abortively transformed colonies disappear between weeks 3 and 4. Stable gpt^+ colonies arose at a frequency of 5 to 50 colonies per μg of plasmid per 10^6 cells. Slightly higher numbers (~2×) of stable transformants were obtained with linearized rather than supercoiled plasmid DNA. Fifty-eight independent gpt^+ cell lines were isolated, grown up, and further characterized.

Amount and extent of adenovirus sequences in the *gpt*-DBP transformants. The adenovirus sequences in the gpt^+ cells lines were analyzed by restriction enzyme digestion and filter hybridization (49). Cellular DNAs from the gpt^+ lines were digested with *Eco*RI, an endonuclease which cuts once in the recombinant plasmids (Fig. 1). Analysis of the *Eco*RI digestion products by electrophoresis, transfer, and filter hybridization, using the nick-translated Ad5 *Bam*HI-*Eco*RI fragment (59.5 to 75.9 m.u.) as probe, showed that 38 of the 48 lines had one or more insertions of adenovirus sequences. Results from analysis of four of these lines, designated gmDBP1 through gmDBP4, are shown in Fig. 2A. With DNAs from gmDBP1 through gmDBP3, only a single band appears with each DNA, and their intensity compared with reconstruction indicates that the adenovirus sequences were present in ca. one copy per cell. In contrast, with gmDBP4 DNA, three very intense bands of hybridization are seen, suggesting that there are at least three insertions of DBP sequences. Because the intensity of each of these three bands equals that of a 5- to 15-copy reconstruction, we suspect that the sequences corresponding to each of these bands have been amplified during the establishment or propagation of this line.

The adenovirus sequences in the 38 gpt^+ cell lines were mapped by cleaving cellular DNA with combinations of restriction endonucleases that cut several times within the DBP recombination plasmids used for transformation and by determining which restriction fragments comigrated with the corresponding plasmid fragments. Cleavage of the plasmid DNA with *Cla*I and *Kpn*I generates two fragments (2 and 3.5 kilobases [kb]) with homology to the Ad5 *Bam*HI-*Eco*RI probe (Fig. 1). The 3.5-kb fragment contains most of the MMTV long terminal repeat, including the DM-inducible promoter and the entire DBP gene. The 2-kb fragment carries Ad5 sequences 3′ to the coding region of DBP and part of the *Eco-gpt* gene together with its controlling SV40 early promoter. Thus, the appearance of the 3.5-kb band after cleavage and Southern blot analysis of transformed cell DNA indicates that the MMTV-DBP chimeric gene is intact, and the presence of the 2-kb band suggests that the Ad5 and *Eco-gpt* sequences are maintained in the same arrangement in the transformed cell DNA as in the plasmid.

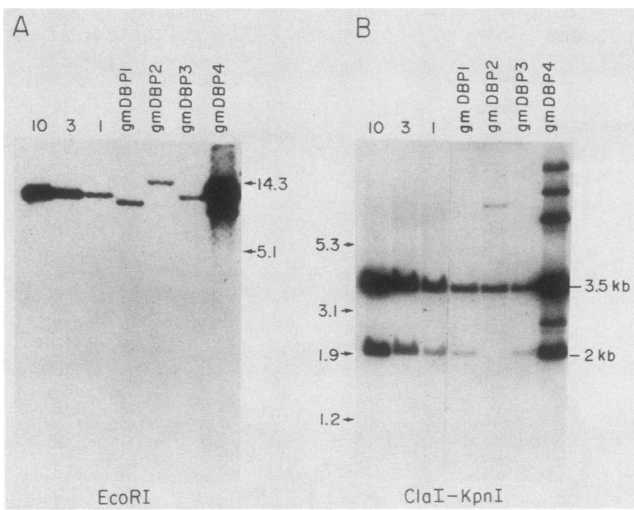

FIG. 2. Amount and structure of adenovirus insertions in *gpt*-DBP-transformed lines. High-molecular-weight DNAs from the cell lines indicated above each lane were digested with the restriction endonuclease denoted below each panel, fractionated on 1% agarose gels, transfected to nitrocellulose filters, and hybridized to Ad5 sequences containing the DBP gene (see the text). The left three lanes of each panel are reconstructions containing pMSG-DBP1 plasmid DNA equivalent to 10, 3, or 1 copy per diploid cell. The numbers between the two panels denote the positions on the autoradiogram of size markers in kilobases.

Both fragments are present in gmDBP1, gmDBP3, and gmDBP4 cellular DNA (Fig. 2B). Although the 3.5-kb fragment is seen with gmDBP2 DNA, the 2-kb fragment is missing. A new, larger but faint band appears, suggesting that the DBP and *Eco-gpt* sequences have an altered arrangement in this cell line compared with the transfecting plasmid. This rearragement has been confirmed by using the *Eco-gpt* sequences as a probe (data not shown). The intensity of the 3.5- and 2-kb bands with the gmDBP4 DNA is greater than that seen with the 10-copy reconstruction. In addition, four other bands are evident. This is consistent with one of the three amplified inserts maintaining the MMTV–DBP–SV40–*Eco-gpt* sequence arrangement of the plasmid, although this arrangement is altered in the other two amplified inserts.

Of the 38 lines carrying adenovirus sequences, 12 lines contained the intact 3.5-kb fragment.

Inducible expression of the DBP gene. The 12 lines which carried the intact chimeric gene were analyzed to determine whether the gene was functional. The cells were treated with DM for 24 h, and their proteins were labeled with [^{35}S]methionine and immunoprecipitated with anti-DBP serum. Although all of the lines produced little or no detectable levels of DBP in the absence of DM, five lines (gmDBP1 through gmDBP5) exhibited a significant increase in DBP synthesis after DM induction.

The kinetics of induction and the amount of DBP synthesis relative to that observed in infected HeLa cells was determined for the two highest expressing lines, gmDBP1 and gmDBP2. Results of such an experiment with gmDBP1 are shown in Fig. 3. A detectable increase in DBP synthesis is seen after 1 h of pretreatment with DM in these cells. In general, we have observed that DBP synthesis peaks at 6 to 8 h after DM addition and remains constant up to 72 h (the last time point taken); however, in the experiment shown in Fig. 3, a slight reduction was observed at 32 h. The level of induction of DBP synthesis with DM is 50- to 200-fold greater with gmDBP1 and gmDBP2.

This impressive rate of synthesis compares favorably with that observed in infected HeLa cells in which hundreds of gene copies are being used as templates. The level of synthesis in gmDBP1 and gmDBP2 is 5 to 15% of that seen at the peak time of DBP production in infected HeLa cells (24 h postinfection). Compared with gmDBP1 and gmDBP2, the rate of DBP synthesis was slightly lower in gmDBP3 and markedly reduced in gmDBP4 and gmDBP5 (data not shown).

Accumulation of DBP in these lines was followed by using Western blot analysis (Fig. 4). Accumulation peaked and then remained constant 16 h after induction. Although the rate of synthesis of DBP in these lines is 5 to 15% of that in infected HeLa cells, the level of accumulation is 2 to 5% for gmDBP1 and 5 to 10% for gmDBP2 (the rates of synthesis

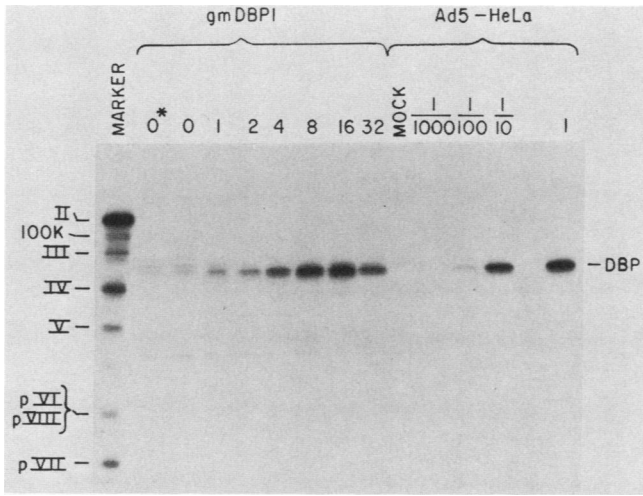

FIG. 3. Synthesis of DBP in the *gpt*-DBP-transformed cell lines after induction with DM. *gpt*-DBP cells were grown off selection and treated with 3×10^{-7} M DM for the time (in hours) shown above each lane before labeling for 1 h with [^{35}S]methionine. Labeling was done in the presence of DM, except at the 0 h sample indicated with an asterisk. Labeled proteins were immunoprecipitated with anti-DBP serum and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% gels. Various amounts of Ad5-infected HeLa cell extracts labeled in a parallel manner at 24 h postinfection were used as standards. The relative amount of infected cells versus *gpt*-DBP cells used for analysis is indicated by the fractional number above the infected HeLa cell lanes.

and accumulation were determined in several different experiments by using densitometry). The difference in the amount of accumulation of DBP between these two lines may simply reflect differences in the rate of synthesis. We have observed some variability in the rate of synthesis from experiment to experiment over the past six months, with gmDBP2 often exhibiting 2 to 3× higher rates than gmDBP1. The reason for this fluctuation is unclear.

The amount of DBP that accumulated in gmDBP3 compared with that seen in infected cells (1%) is also less than expected from the relative rates of synthesis (data not shown). This may suggest that the DBP in these three cell lines is less stable than that made in infected cells.

Propagation of DBP mutants. To determine whether the DBP made in these lines was functional, the lines were tested for their ability to complement the growth of mutants which carried *ts* alterations in the DBP gene (Ad5 *ts*125, Ad2 *ts*400, and Ad2$^+$ *ND*1 *ts*23). At the nonpermissive temperatures, the DBP *ts* mutants fail to replicate their DNA and express their late genes, since DNA replication is a prerequisite to late gene expression (51).

HeLa cells and the DBP-expressing lines were infected with various *wt* and *ts* mutants of adenovirus at the nonpermissive temperature, and the synthesis of the late viral proteins was analyzed (Fig. 5). The *ts* mutants fail to express their late genes in HeLa cells or in the *gpt*-DBP$^+$ lines not treated with DM. In contrast, when synthesis of *wt* DBP from the integrated gene copy is induced by addition of DM 6 to 24 h before infection, the DNAs of the mutant viruses are replicated (data not shown), and their late genes are expressed. In addition to gmDBP1 and gmDBP2 (Fig. 5A), gmDBP3 and gmDBP4 (but not gmDBP5) exhibited complementation of the *ts* mutants for viral late gene expression (data not shown). However, the slower rate of DBP synthesis in gmDBP4 compared with the other three lines is reflected in its poorer complementation of the *ts* mutants.

The impressive complementation of Ad5 *ts*125 and Ad2 *ts*400 for late gene expression (Fig. 5A) is not complete, however. A higher rate of viral late protein synthesis is seen with *wt* virus (Fig. 5B) or when the *ts* mutants are grown at the permissive temperature (data not shown).

The crucial test to evaluate the usefulness of these lines for propagation of DBP mutants was to determine whether the cell lines would support the growth of the *ts* mutants at the nonpermissive temperature. The four DBP-expressing lines were infected with *wt* and *ts* mutants of adenovirus at the permissive and nonpermissive temperature in the presence or absence of DM. The results from one of several of these experiments with gmDBP1 and gmDBP2 are shown in Table 1. Studies with gmDBP3 gave similar results, although complementation with the low expressing line, gmDBP4, was less complete than that observed with the other three lines.

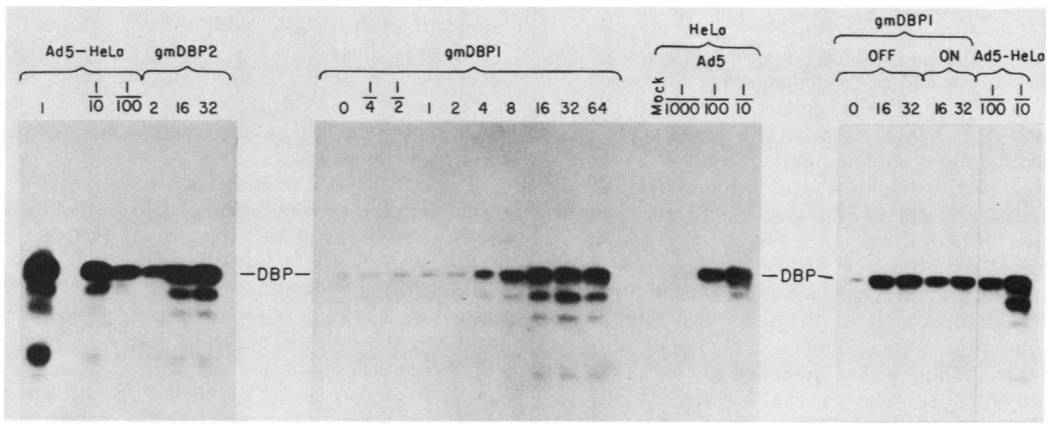

FIG. 4. Accumulation of DBP in the *gpt*-DBP-transformed cell lines after induction with DM. DBP from gmDBP1 and gmDBP2 was immunoprecipitated with anti-DBP serum, fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subjected to Western blot analysis (see the text). The numbers above each lane for gmDBP1 and gmDBP2 indicates the length of DM treatment (in hours) with 3×10^{-7} M DM before harvesting the cells for analysis. In these experiments, the *gpt*$^+$ cells were grown in absence of selection with mycophenolic acid except for the gmDBP1 cells shown in the right panel and indicated by ON. Various amounts of Ad5-infected HeLa cell extracts prepared 24 h postinfection were used as standards. The relative amounts of infected cells versus *gpt*-DBP cells used for analysis are indicated by the fractional number above the infected HeLa cell lanes.

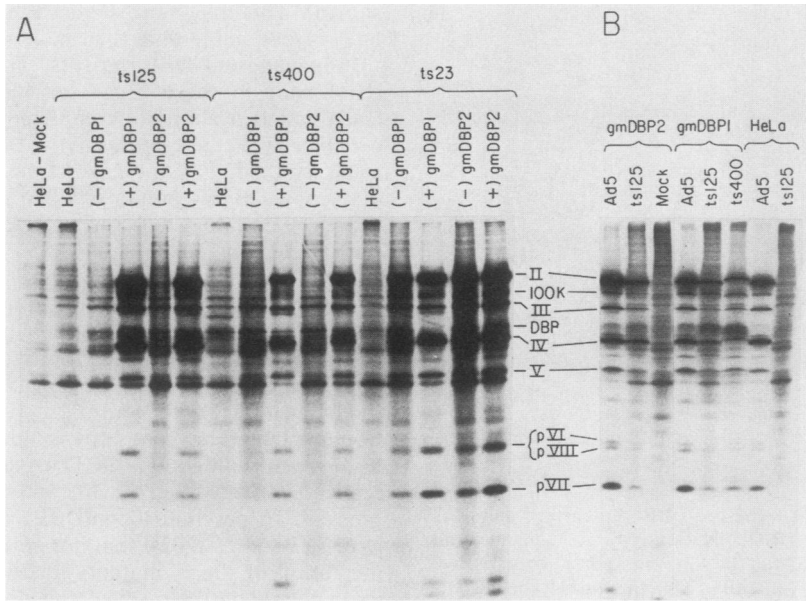

FIG. 5. Complementation of viral late gene expression in the *gpt*-DBP-transformed cell lines after infection with *ts* mutants at the nonpermissive temperature. The three cell lines were infected at 39.5°C with the viruses indicated. Thirty hours postinfection, the infected cells were labeled with [^{35}S]methionine for 1 h, and the newly synthesized proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The numbers between the two panels denote some of the viral late proteins. In A, the *gpt*-DBP cell lines were maintained on selection before and during the infection. Half of these (+) were treated with 3×10^{-7} M DM starting 4.5 h before infection. HeLa cells were not treated with DM. In B, all of the cells were grown in the absence of mycophenolic acid and induced with DM 8 h before infection. Treatment of HeLa cells with DM had little or no effect on the infection by *ts* or *wt* adenoviruses.

Like the parental HeLa cells, all four lines serve as excellent hosts for the propagation of *wt* adenoviruses. In contrast, Ad2$^+$ *ND*1 *ts*23 grows poorly on HeLa cells at 39.5°C, but in the presence of DM, the transformed lines support the replication of this *ts* mutant at either temperature. In fact, even in the absence of DM induction, Ad2$^+$ *ND*1 *ts*23 expresses its late genes (Fig. 5A) and replicates quite efficiently at the nonpermissive temperature (Table 1). This high level of complementation (50 to 100×) observed with Ad2$^+$ *ND*1 *ts*23 in the absence of DM is not seen with Ad2 *ts*400 or Ad5 *ts*125 and is consistent with the observation that this virus is less restricted for its growth than the other two mutants at the nonpermissive temperature, even on HeLa cells.

Growth of Ad2 *ts*400 and Ad5 *ts*125 is very restricted on HeLa cells at 39.5°C but can be enhanced several hundred- to several thousandfold on gmDBP1, gmDBP2, and gmDBP3 in the presence of DM. However, as with late gene expression, viral growth is not fully complemented.

wt Ad5, Ad2$^+$ *ND*1 *ts*23, and Ad2 *ts*400 grew well at 39.5°C on gmDBP1 and gmDBP2 regardless of whether the cells were grown on or off selection for *gpt* expression. A high level of complementation of Ad5 *ts*125 growth, however, was observed only when these two lines were maintained off selection for several generations (or for 6 months or more) before infection. The enhanced complementation is most probably due to the 2 to 4× increased rate of synthesis (data not shown) and accumulation (Fig. 4) of DBP when the cell lines are maintained off selection as compared with growth in the presence of mycophenolic acid. The increased production of DBP probably reflects the better state of health of the cells off selection, since they replicate twice as rapidly and form a much tighter monolayer than cells on selection.

Viral plaque formation on the *gpt*-DBP-transformed lines. Purification of viral mutants as well as quantitation of the infectious mutant viral particles is greatly facilitated by the availability of a plaquing assay. Thus, the cell lines were tested for their ability to support the formation of plaques with *wt* and *ts* mutants of adenovirus at various temperatures (Table 2). *wt* Ad5 formed plaques with equal efficiency on monolayers of HeLa, gmDBP1, and gmDBP2 cells at all temperatures tested. Formation of plaques by Ad2 *ts*400 and Ad5 *ts*125 on HeLa cells was dramatically reduced at 37.5, 38.5 and 39.5°C compared with formation at the permissive temperature of 32.5°C. In contrast, gmDBP1 and gmDBP2 monolayers supported plaque formation by these two *ts* mutants equally well at 32.5 and 37.5°C. At 38.5°C, an approximately 10-fold reduction in plaquing efficiency was seen with these two lines compared with 10^4- to 10^5-fold decrease on HeLa cells. Plaquing efficiency of the two mutants dropped 10- to 100-fold at 39.5°C compared with 38.5°C but was still several hundredfold above that seen on HeLa cell monolayers.

Toxicity of DBP to cells. If, as we suspected, the DBP is toxic to cells, then addition of DM to these DBP-expressing lines should result in reduced growth and possibly even death of the cells. Addition of DM at 3×10^{-7} M to HeLa cells or to a *gpt*$^+$ cell line, which does not contain the DBP gene but was maintained in the presence of mycophenolic acid to ensure *gpt* expression, had no effect on cell growth during 24 days that the cells were observed. In contrast, gmDBP1 cells maintained in the presence of mycophenolic acid went through only one or two generations after addition of DM before ceasing growth and detaching from the bottom of the culture dish. gmDBP2 was slightly more resilient. After DM addition, their generation time increased from 3 days to 5 to 8 days, the cells took on a ragged appearance, and growth ceased after 5 to 8 doublings when the cells began to detach from the dish.

TABLE 1. Ability of DBP-*gpt* cell lines to support the growth of *ts* mutants at the nonpermissive temperature[a]

Cell line	Virus	Virus yield (PFU/ml)			
		32.5°C (±DM)[e]	39.5°C (−DM)	39.5°C (+DM)[e]	32.5°/39.5°C (+DM)
HeLa	Ad5	8×10^8	2×10^9	—	
gmDBP1 on[b]	Ad5	—[f]	4×10^8	—	
gmDBP1 off[c]	Ad5	—	6×10^8	3×10^9	
gmDBP2 on	Ad5	—	3×10^8	8×10^8	
gmDBP2 off	Ad5	—	1×10^9	3×10^9	
HeLa[d]	Ad2$^+$ *ND1 ts23*	1.5×10^8	—	4×10^5	325
gmDBP1 on	Ad2$^+$ *ND1 ts23*	2×10^8	2×10^7	1.5×10^8	1
gmDBP2 on	Ad2$^+$ *ND1 ts23*	2×10^8	5×10^7	1.5×10^8	1
HeLa	Ad2 *ts400*	4×10^8	6×10^4	—	6,667
gmDBP1 on	Ad2 *ts400*	6×10^7	6×10^4	4×10^6	15
gmDBP1 off	Ad2 *ts400*	6×10^8	8×10^4	7×10^7	9
gmDBP2 on	Ad2 *ts400*	4×10^8	7×10^4	5×10^7	8
gmDBP2 off	Ad2 *ts400*	8×10^8	1×10^6	8×10^7	10
HeLa	Ad5 *ts125*	4×10^8	3×10^4	—	13,333
gmDBP1 on	Ad5 *ts125*	9×10^7	3×10^4	9×10^4	1,000
gmDBP1 off	Ad5 *ts125*	5×10^8	5×10^4	1×10^7	50
gmDBP2 on	Ad5 *ts125*	2.5×10^8	2×10^4	2×10^6	125
gmDBP2 off	Ad5 *ts125*	7×10^8	2.5×10^5	1.5×10^7	47

[a] Cells were infected at a multiplicity of 20 PFU per cell and incubated at 32.5°C for 5 days or 39.5°C for 3 days. Virus yield was determined by plaque titration of 32.5°C on monolayers of HeLa cells.

[b] Cells were grown in the presence of mycophenolic acid to maintain selection for *gpt* gene expression until the time of infection. After infection, the cells were maintained in selective or nonselective medium with little difference in results.

[c] Cells were grown and maintained in the absence of selection for *gpt* gene expression.

[d] As a second negative control, a *gpt*-DBP$^-$ cell line which fails to respond to DM induction was infected with the *ts* mutants at the various temperatures. Like HeLa cells, it failed to complement the growth of the *ts* mutants.

[e] DM was added to the medium 24 h before infection at a concentration of 3×10^{-7} M and maintained in the medium after infection.

[f] —, Not determined.

DISCUSSION

HeLa cells were transformed with plasmids containing the *Eco-gpt* gene as a dominant selectable marker and a chimeric gene in which the gene encoding DBP of Ad5 is under the control of the DM-inducible promoter of MMTV. Fifty-eight independent *gpt*$^+$ transformants were characterized. Two-thirds of these retained some or all of the Ad5 sequences present in the transfecting vector. Twelve of these lines contained an integrated copy of the intact MMTV-DBP chimeric gene, and approximately half (5 of 12) responded to DM induction. The two highest responding lines, gmDBP1 and gmDBP2, showed a detectable increase in DBP synthesis within 1 h of DM addition. DBP synthesis was enhanced 50- to 200-fold by DM and represented 5 to 15% the rate of peak DBP production in infected HeLa cells. This high level of induction is particularly impressive, since in these lines DBP is encoded by a single gene, although during an infectious cycle hundreds and perhaps even thousands of DBP genes may serve as templates.

The accumulation of DBP in these cells compared with infected cells (2 to 10%) is less than expected from its relative rate of synthesis (5 to 15%), which peaks and then remains constant between 6 and 72 h after addition of DM. This may suggest that the DBP made in these cell lines is less stable than that produced during infections. Perhaps the absence in these lines of replicating viral DNA to which DBP normally binds is responsible for its increased turnover if it is indeed less stable.

The DBP synthesized in these cells is functional, since it allows mutants with *ts* lesions in the DBP gene to replicate their DNA, express their late proteins, and produce progeny virus at the nonpermissive temperature. The several hundred- to several thousandfold enhancement of the growth of Ad5 *ts125* and Ad2 *ts400* in gmDBP1 and gmDBP2 is not fully complete, however. The reduced level of functional *wt* DBP in these cells compared with Ad5-infected HeLa cells appears to be partially responsible for lack of complete complementation. For example, when gmDBP1 and gmDBP2 are propagated in the absence of selection, they not only produce more DBP but also complement the growth of Ad5 *ts125* better than when they are maintained on selection. If functional DBP is indeed limiting in these cells, then the enormous quantity of DBP present in HeLa cells during the late phase of infection by *wt* adenovirus (10^6 to 10^7 molecules per cell) is probably required for maximum virus yield.

The lower DBP level in these cells is not the entire explanation for lack of full complementation, however. Increasing the amount of DBP in gmDBP1 and gmDBP2, by taking the cells off selection, does not appreciably increase the yield of Ad2 *ts400* at the nonpermissive temperature compared with its growth when these lines are maintained on selection. Furthermore, although gmDBP3 has a lower rate of DBP synthesis and accumulation than the above two lines, it complements the growth of Ad5 *ts125* and Ad2 *ts400* about as well as these two lines. Since the various functions of DBP might be carried out by various species of DBP which differ in their level of phosphorylation (25), the crucial factor may not be how much total DBP the cell is producing from its integrated gene but rather the amount of synthesis of a subspecies of the DBP whose function has been disrupted by the *ts* lesion in the infecting virus. Thus, although the amount of DBP produced may be a useful guideline in determining which cell line will serve as a good host for propagation of DBP mutants, it has limitations.

Lack of complete complementation might also be due to possible interference of *wt* DBP activity by the infecting virus *ts* DBP. Since mutant DBP is made in considerable excess over *wt* DBP during infection of these *gpt*-DBP$^+$ cells, then if DBP performs its functions as a multimer, negative complementation might be expected.

An alternative, but less likely possibility, to account for

TABLE 2. Ability of DBP-*gpt* cell lines to support plaque formation by *ts* mutants at various temperatures[a]

Cell line	Virus	Virus yield (PFU/ml) at[b]:							
		32.5°C		37.5°C		38.5°C		39.5°C	
		−DM	+DM	−DM	+DM	−DM	+DM	−DM	+DM
HeLa[c]	Ad5	1×10^{10}	—[d]	1.5×10^{10}	–	1×10^{10}	—	2×10^{10}	—
gmDBP1	Ad5	—	5×10^{9}	—	1.7×10^{10}	—	1×10^{10}	—	4×10^{9}
gmDBP2	Ad5	—	4×10^{9}	—	1.7×10^{10}	—	7×10^{9}	—	3×10^{9}
HeLa	Ad2 *ts*400	4×10^{9}	—	1×10^{6}	—	4×10^{5}	—	1×10^{5}	—
gmDBP1	Ad2 *ts*400	4×10^{8}	4×10^{9}	1×10^{7}	4×10^{9}	1×10^{4}	2×10^{8}	4×10^{4}	1×10^{7}
gmDBP2	Ad2 *ts*400	8×10^{8}	2×10^{9}	8×10^{6}	3.5×10^{9}	1×10^{4}	1×10^{8}	1×10^{5}	1×10^{7}
HeLa	Ad5 *ts*125	2×10^{9}	—	2×10^{6}	—	2.5×10^{4}	—	5×10^{3}	—
gmDBP1	Ad5 *ts*125	1.4×10^{9}	1×10^{9}	2×10^{7}	3×10^{9}	3×10^{4}	6×10^{8}	2×10^{3}	7×10^{5}
gmDBP2	Ad5 *ts*125	2×10^{8}	2×10^{9}	1×10^{7}	2.5×10^{9}	3×10^{4}	3×10^{8}	1×10^{5}	3×10^{6}

[a] Cell lines were grown in normal medium without selection before the time of infection. After viral adsorption at 37.5°C for 1 h, the cells were overlayed with the standard medium-agar mixture as described by Grodzicker et al. (19) plus or minus 6×10^{-7} M DM.

[b] Data shown are the number of plaques formed on the various cell lines under the various incubation conditions.

[c] In other experiments not shown here, addition of DM to HeLa cells did not affect plaque formation by *wt* or mutant virus. DM also did not affect plaque formation by *wt* virus on gmDBP1 or gmDBP2.

[d] —, Not determined.

the lack of full complementation concerns the autoregulatory function of the DBP. DBP turns down the expression of its own gene as well as the other viral early genes during the transition from the early to late phase of a productive infection (6, 9, 10, 37, 38). The endogenous DBP made in the transformants might prematurely turn down the expression of the early genes of the infecting virus and thereby prevent efficient viral multiplication. However, if this were the case, growth of *wt* adenovirus at either temperature or *ts* mutants at the permissive temperature should be reduced when DBP synthesis is induced in the transformed lines with DM. Such a reduction does not occur.

In contrast to the result with Ad5 *ts*125 and Ad2 *ts*400, the growth of Ad2$^+$ *ND*1 *ts*23 is fully complemented in gmDBP1-gmDBP3. Ad2$^+$ *ND*1 *ts*23 replication is less restricted than the other *ts* mutants at the nonpermissive temperature, even on HeLa cells, presumably because the mutant DBP retains more activity. These observations raised the disturbing possibility that although the DBP-expressing cells support the growth of *ts* mutants, which may retain some of the DBP functions, deletion mutants, in which all of the DBP function are lost, may not be viable in these cells. This is particularly relevant, since we have shown that DBP contains at least two independent functional domains, only one of which is destroyed by the *ts* lesions in Ad2 *ts*400 and Ad5 *ts*125 (44). Fortunately, this possibility has been ruled out. We have constructed two deletion mutants in which only sequences coding for a few amino acids at the N terminus of the protein remains. These mutants have been grown to high titers in gmDBP1 and gmDBP2 and are now being characterized (S. Rice and D. Klessig, unpublished data). Thus, these lines should allow the isolation and propagation of any DBP mutants and in so doing provide a powerful tool in dissecting the activities of this multifaceted protein.

The integrated genes appear to be very stable. The cells have been maintained in the absence of mycophenolic acid for 9 months without any detectable loss of their ability to grow under selective pressure or synthesize DBP after DM induction. Furthermore, in the absence of selection, they produced more DBP. Since these cells grow twice as rapidly and form better monolayers in the absence of selection than in its presence, the increased DBP synthesis probably reflects the better state of health of the cells.

Our earlier work (Klessig et al., in press), using cotransformation with the herpes simplex virus type 1 *tk* gene, suggested that DBP was toxic to mammalian cells, hence the new strategy outlined in this report for constructing DBP-expressing lines. This new approach proved successful for two reasons. First, DBP is indeed toxic, since induction of DBP synthesis with DM resulted in a reduced growth rate, ragged appearance, and finally death of the cells after two to eight generations. Fortunately, this toxicity does not affect the ability of the cell to support viral growth during the ensuing 3 to 5 days of infection. Indeed, the ability to form plaques on monolayers of these cells over a period of 10 to 15 days in the presence of DM suggests that either a very sick cell can still support viral growth or the cells are susceptible to the DBP toxin only when they are actively dividing. The latter explanation seems quite probable because the cells in a monolayer divide very little during a plaque assay and DBP preferentially binds to replicating single-strand DNA and in so doing may disrupt the metabolic activities of the cell.

Secondly, the approach has been successful because only a low, nontoxic level of DBP gene expression occurs in the absence of DM, whereas in its presence the activity of the MMTV promoter controlling DBP synthesis can be enhanced several hundredfold. The high level of expression from this single chimeric gene provides the large quantity of DBP required for growth of mutant adenovirus.

This approach should find general utility for introducing and expressing at high levels under controlled conditions other genes whose products are toxic to the recipient cell. Indeed Chapman and colleagues (11) have recently reported that although the high levels of xanthine-guanine phosphoribosyltransferase produced from the amplified MMTV–*Eco-gpt* chimeric gene is toxic to the recipient cells, these cells can be propagated, provided that the level of glucocorticoid hormone is carefully controlled.

The level of induction by DM of the MMTV promoter in gmDBP1 through gmDBP3 is considerably higher than the 5- to 15-fold increase generally reported for mouse or hamster cells, which contain integrated copies of DM-inducible genes (8, 11, 22, 33). At least two factors control the level of induction in human cell lines: the site of integration and cell type. Most of the 12 *gpt*-DBP$^+$ lines contained a single copy of the intact MMTV-DBP chimeric gene, which is integrated at different sites in the various lines. Of these 12 lines, 3 expressed DBP at very high levels, 2 produced only low amounts of DBP, and the remaining 7 were uninducible. Similar results have been obtained by others with different mammalian cells (21, 45).

Our attempts to construct similar lines, starting with the 293 cell line (a human embryonic kidney cell line morphologically transformed with *E1a* and *E1b* genes of Ad5 [17]), suggest that cell type is also important. Although 14 of 22 293 gpt^+ cell lines contained the intact chimeric gene, only 1 was inducible and only to a very low level. Perhaps the difference between poor and high responding cell types is the amount of receptors they have for this hormone.

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