Absence of an Essential Regulatory Influence of the Adenovirus E1B 19-Kilodalton Protein on Viral Growth and Early Gene Expression in Human Diploid WI38, HeLa, and A549 Cells

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Mutations in the gene encoding the adenovirus (Ad) early region 1B 19-kDa protein (the 19K gene) result in multiple phenotypic effects upon infection of permissive human cells. It has been reported, for example, that Ad type 2 (Ad2) and Ad5 with mutations in the 19K gene (19K-defective mutants) have a marked growth advantage compared with wild-type virus in human diploid WI38 cells (E. White, B. Faha, and B. Stillman, *Mol. Cell. Biol.* 6:3763–3773, 1986), and it was proposed that this host range phenotype stems from the large increase in viral early gene expression reported to occur in the mutant-infected cells. These observations gave rise to the hypothesis that the 19-kDa protein (the 19K protein) normally functions as a negative regulator of Ad early gene expression and growth. We have tested this hypothesis and find that Ad5 and Ad12 wild-type viruses grow as efficiently as their respective 19K-defective mutants, *in1* and *dl337* and *pm700* and *in700*, in WI38 and other human cell types. Neither the accumulation of E1A cytoplasmic mRNAs nor the synthesis of E1A and other viral early proteins in these cells is altered as a result of these mutations in the 19K gene, and we conclude that the 19K protein does not play an essential role in regulating viral early gene expression or viral growth in human cells.

Adenovirus (Ad) early region 1 (E1) is located at the left end of the viral genome and comprises two discrete transcriptional units, E1A (map units 1.3 to 4.5) and E1B (map units 4.6 to 11.2). Some of the gene products of this complex region function to regulate the expression of other viral genes during the productive infection of human cells, and some of them are necessary and perhaps sufficient for the transformation of rodent cells in vitro (for reviews, see references 12 and 46). Transcripts made from the E1B gene are differentially spliced to generate two major mRNA species of 22S and 13S and two minor species of 14.5S and 14S, all of which have identical 5' and 3' termini (3, 38, 39). The E1B 22S mRNA encodes two proteins from distinct but overlapping reading frames (5). The first frame commences at the 5'-proximal AUG to generate the 19,000-molecular-weight protein (the 19K protein), which comprises 176 amino acid residues (176R) in Ad type 5 (Ad5) and 163R in Ad12. The second frame initiates at the next AUG and generates the 55K protein, which is 496R in Ad5 and 482R in Ad12. The E1B 13S mRNA also encodes the 19K protein (in both Ad5 and Ad12) and a smaller species related to the 55K protein. The roles of the E1B 19K protein in viral replication and oncogenic transformation have been probed by genetic analyses of a variety of type 5, type 2 (group C), and type 12 (group A) mutants. These investigations reveal that the gene encoding the 19K protein (the 19K gene) may be pleiotropic, for many of the mutant alleles appear to affect multiple and potentially unrelated phenotypic traits. Group A and group C Ads with mutations in the 19K gene (19K-defective mutants) display, for example, increased cytopathogenicity in human cells (*cyr* phenotype) and form large plaques (*lp* phenotype) in some cell types (2, 9, 29, 33, 45). Some of the 19K-defective

mutants also elicit a cell-dependent DNA degradation (*deg*) phenotype, with which extensive breakdown of cellular and viral DNA occurs (2, 9, 22, 29, 33, 45). Many 19K-defective mutants have also been shown to be defective for complete transformation of rodent cells in vitro, and it has been widely reported that the 19K protein is required for oncogenic transformation (1, 2, 4, 7, 29, 33, 35). However, we have constructed new null mutants of both Ad12 and Ad5 and have used these to demonstrate that the E1B 19K proteins of both serotypes are not required for the transformation of rat and mouse primary cells in vitro, or, in the case of Ad12, for the direct induction of tumors in rats (9, 36). Others have also used mutants to show that the Ad12 19K protein is not obligatory for transformation in vitro (52). A generally similar conclusion has been drawn from tests involving Ad5 E1 plasmid-induced transformation of baby rat kidney primary cells (4, 26, 40), in which each E1B protein is able to cooperate with E1A to induce transformation (at reduced levels) by independent and apparently additive means. It has been proposed that E1A products induce apoptosis which is in turn prevented by the 19K protein and that the p53 protein is in some way involved in this E1A-induced cell killing (8, 41).

In addition to these phenotypic properties of 19K-defective mutants, it has also been reported that the group C 19K protein actually has a negative effect on viral growth in certain human cell types in that Ad2 and Ad5 mutants were described to possess a distinct growth advantage over wild-type virus in human cells of the WI38 strain (43). The basis for this host range phenotype was reported to be an enhanced expression of viral early proteins in mutant-infected cells in comparison with cells infected with wild-type virus, and it was proposed that the 19K protein normally acts as a negative regulator of viral gene expression, acting at the level of transcription during infection (42, 43). This hypothesis was extended to include Ad-infected HeLa cells, and in these cells it was reported also that the E1B

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19K mutations present in a 12S E1A cDNA background elicit an even more profound effect on viral infectivity, viral growth rate, and production of the 12S E1A protein. An alternative view of the regulatory capacity of the 19K protein emerged from the results of plasmid-mediated transient-expression assays in HeLa and various rodent cells (51). These results suggested that the 19K protein might activate transcription of the viral E1A gene and certain cellular genes via an enhancer-dependent process and that it might also relieve E1A-mediated repression. A third view of the potential regulatory effects of the 19K protein has also been presented. In this case, a set of viruses bearing mutations in the 19K gene (and in some cases the E1A genes, too) was used to show that this protein does not alter viral early gene expression in either a positive or a negative way in both permissive HeLa cells and nonpermissive rat CRE6 cells (17). While characterizing the transforming properties of the Ad12 19K-defective mutants, we made the preliminary observation that they appeared to possess no growth advantage over Ad12 and gave no increase in viral early gene expression in WI38 cells, in direct contrast to the previously reported results with Ad2 and Ad5 mutants (43). In light of these Ad12 results and of the three apparently opposing views just mentioned, we made a more detailed examination of the growth and early gene expression patterns of these Ad12 viruses and of Ad5 and Ad5 19K-defective mutants in WI38 cells.

Growth characteristics of the *in1* virus in A549 and HeLa cells. Human A549 lung carcinoma cells (11) were used for growth and titration of Ad5 and *in1* and were derived from the stocks used in previous studies of E1 mutants (9, 23, 36). Human 293 cells (13) were used for growth of mutants defective in E1A and mutants defective in both E1A and 19K and for titration of Ad5 and its mutant viruses. The HeLa cells used in phenotypic characterization of mutants were from stocks previously used by us in genetic studies of Ad5 (14, 47). All cells were maintained in Dulbecco modified Eagle medium supplemented with 7.5% fetal calf serum. The Ad5 wild-type strain was derived from the original seed stock used in all of our prior genetic studies with this serotype (14, 46, 47). The construction of the Ad5 *in1* mutant was described previously (36). It possesses a single AT-base-pair insert immediately following nucleotide (nt) 1715 in the E1B sequence, which destroys the proximal AUG present in E1B mRNAs and blocks production of intact E1B 19K protein in infected cells. In some tests, Ad5 *dl337* was also used. This mutant, kindly provided by T. Shenk, Princeton University, carries a deletion of 146 bp commencing at nt 1770 in the 19K-gene reading frame (29). The *in1* mutant was originally isolated as a large plaque on A549 cells (36), and on these cells it generates markedly larger plaques (*lp*) than those formed by wild-type Ad5. In strong contrast, the mutant generates small and indistinct plaques on HeLa cells, particularly in the absence of $MgCl_2$. The efficiency of plaquing (infectivity) of *in1* on A549 is very similar to that of wild-type virus but is 10-fold lower than that of wild-type virus (or the A549 infectivity of *in1*) on HeLa cells. The mutant infectivity on 293 cells is almost identical to that obtained on A549, but plaque size on 293 is similar to that of Ad5 (*lp*⁺). We also find that mutant *dl337* forms large plaques on A549 cells and, as with *in1*, makes small, indistinct plaques on HeLa cells at reduced infectivity. The growth rates and final yields of *in1* and Ad5 are virtually identical on A549 cells (Fig. 1). The growth rates of *in1* and Ad5 are also identical on HeLa cells (and identical to those on A549), but the final yield of *in1* is markedly lower than that of Ad5; at 42 h postinfection it is 10-fold lower, and this difference gradually increases until at 60 h postinfection the differ-

FIG. 1. Growth of Ad5 and *in1* on A549 and HeLa cells. Cells were infected at an input multiplicity of 10 PFU per cell. Samples harvested at various times were frozen and thawed three times to release virus and assayed for infectivity by plaque assay (49) on 293 monolayers. Values represent the titers of cell-associated virus.

ence is >100-fold (Fig. 1). A cytopathic effect is more strongly and more rapidly expressed in HeLa and A549 cells infected with *in1* and *dl337* (the *cyt* phenotype of these 19K-defective mutants) than in Ad5-infected cells, although the time at which it occurs is related to the input multiplicity. At these later times, the HeLa cells are more noticeably damaged by *in1* than the A549 cells, and we surmised that the rate of virus release might be greater from HeLa than from A549, resulting in lower levels of cell-associated virus in the HeLa cultures, but measurements of the levels of cell-free virus at late times after infection showed that the rates of release from HeLa and A549 were similar. At present, we have no firm explanation for the reduced yields found in HeLa cells, but they may be related to the higher levels of DNA degradation (*deg*) found in HeLa in comparison with A549 cells. We find also that the final yield of *dl337* is reduced in HeLa cells (15 times lower than in A549; data not shown). These results are in complete agreement with those of Pilder et al. (29), who determined that *dl337* grows to roughly 10-fold-lower levels in HeLa than in A549 cells, but they differ from those of White et al. (42), who reported that the growth pattern of *dl337* in HeLa is the same as that of wild-type virus and who also reported that this mutant produced large plaques (*lp*) on HeLa monolayers, with infectivity similar to that determined on 293. We have no explanation for these differences; potentially, the HeLa cells used by us and by Pilder et al. may differ from those used by White et al. It should be mentioned that we have previously reported that the growth rates of Ad12 wild type and mutants *pm700* and *in700* are similar on A549 cells, and as with *in1*, these Ad12 mutants show the *lp* phenotype on A549 but make very small plaques (as does the wild-type virus) at reduced efficiency on HeLa (9).

In view of the fact that White et al. (42) reported a much more pronounced effect of the 19K mutations on viral growth and infectivity (with increased levels of both) when the muta-

TABLE 1. Infectivity of E1A-*in1* double-mutant viruses^a in 293 and HeLa cells

Virus	Infectivity (PFU/ml) in cell line:		Infectivity ratio (293/HeLa)
	293	HeLa	
Ad5	8.0×10^{9b}	7.5×10^9	1.1
<i>in1</i>	1.3×10^{10}	1.8×10^9	7.2
<i>pm12</i>	4.0×10^{10}	2.0×10^{10}	2.0
<i>rec pm12 in1</i>	1.5×10^{10}	2.5×10^9	6.0
<i>pm13</i>	3.5×10^9	2.0×10^7	175.0
<i>rec pm13 in1</i>	9.5×10^9	3.5×10^7	271.4
<i>dl12</i>	1.8×10^{10}	9.5×10^9	1.9
<i>rec dl12 in1</i>	1.2×10^{11}	1.6×10^{10}	7.5
<i>dl13</i>	1.5×10^9	6.0×10^7	25.0
<i>rec dl13 in1</i>	1.6×10^{10}	4.5×10^7	355.5

^a Mutants *pm12* and *pm13* (37) were made by introduction of point mutations into the donor splice junctions of the E1A 12S and 13S mRNAs by oligonucleotide mutagenesis as previously described (23); they possess a T-G transversion at nt 976 and a T-C transition at nt 1114, respectively. Mutants *dl12* and *dl13* (37) were made by replacing the normal E1A gene with a cDNA copy of either the 12S or the 13S mRNA, as first described by Winberg and Shenk (50). Cloned segments corresponding to each of these mRNAs were derived from Ad2 cDNA plasmids p121 and p131 (28), respectively; these were built by Winberg and Shenk into the Ad5 plasmid pE1A-WT (nt 1339), replacing the wild-type segment between the *Clal* site (nt 918) and the *XbaI* site (nt 1339), to generate the 12S and 13S cDNA plasmids p133 and p131, respectively. The latter two plasmids were kindly given to us by C. Tibbetts, Vanderbilt University. Upon receipt, we rebuilt the deleted cDNA sequences corresponding to the *Clal* (nt 918)-*XbaI* (nt 1339) segment into the Ad5 plasmid pGT509 (nt 1 to 1833) (36) to generate plasmids pGT121 (12S cDNA) and pGT131 (13S cDNA). The resulting E1A genes are, in fact, chimeric, with an Ad2 sequence between nt 918 and 339 flanked by Ad5 sequences on either side. These mutant E1A genes were built into the complete viral genome of either *dl309* (19) to generate the E1A-defective mutants or *in1* to make the E1A-19K double mutants by a modification of the method of Stow (34). Plasmid DNA linearized by *XbaI* (site at nt 1339 in E1A) was ligated to the *XbaI* A fragment of viral DNA doubly digested with *Clal* and *XbaI* (each cut *dl309* and *in1* DNA once, at nt 918 and 1339, respectively), and ligated products were transfected into 293 cells as previously described (10). Eight mutants resulted from these constructions. *pm12* bears a mutation at nt 976 and makes no 12S mRNA. *pm13* has a mutation at nt 1114 and makes no 13S mRNA. *dl12* contains the 13S cDNA copy only and is the equivalent of *dl348* (50). *dl13* contains only the 12S cDNA and corresponds to *dl347* (50). The other four viruses are recombinants of these E1A defectives with *in1* and are designated *rec pm12 in1*, *rec pm13 in1*, *rec dl12 in1*, and *rec dl13 in1*, respectively. At each stage in the construction of the E1A mutant viruses, the locations of the mutations or deletions of the introns were verified by double-stranded DNA sequencing of either plasmids or purified viral DNA (15), and digestion of the viral DNA with *EcoNI* was used to confirm the presence of the *in1* allele in the double mutants because the *in1* mutation destroys the *EcoNI* site normally located at nt 1710 in the E1B genome (36).

^b Final plaque counts were made at 10 days postinfection; the overlay medium contained 25 mM MgCl₂.

tions were present in an E1A-12S cDNA (289R-deficient) background, we made a set of such double mutants and reexamined this notion. As noted above, the *in1* and *dl337* viruses displayed reduced infectivities (~10 times lower) and viral yields in HeLa cells compared with those of Ad5. The same is also true for the infectivities of all four of the *in1* double-mutant viruses when compared with the parental viruses, irrespective of which E1A product is deleted (Table 1). There is, then, clearly no evidence for a negative influence of the 19K protein on viral plaque-forming efficiency. Exactly the same is true for the growth of these various E1A mutants in HeLa cells (data not shown); in all cases, the viral yields of the E1A mutants are reduced in the absence of the 19K gene product.

The 19K protein does not influence the growth of either Ad5 or Ad12 in WI38 cells. The host range effect observed in HeLa cells is not particularly strong, and the host range phenotype that has been reported for the growth of *dl337* in WI38 cells

(43) is much more profound. In this case, the mutant (and Ad2 *cyl106*) grew more rapidly and attained significantly higher yields (as great as 1,000-fold higher) in these cells than Ad2, although this effect apparently diminished when very high input multiplicities (100 PFU per cell) of virus were used. We were puzzled by the fact that the wild-type virus used in these tests did not grow well in WI38 cells unless very high multiplicities of virus were used, particularly in light of the fact that others had shown that Ad2 grows well in them, even at extremely low input multiplicities (31), and we ourselves showed that Ad12 grew well in these cells (23). For this reason we decided to examine carefully the growth of Ad5, *in1*, and *dl337*, as well as Ad12 and the Ad12 19K-defective mutants *pm700* and *in700*, in WI38 cells infected at low input multiplicities. The Ad12 strain Huie used by us and the construction of the Ad12 mutants have been previously described (9). Neither mutant produces intact 19K protein in infected cells; in *pm700* the AUG of the 19K-gene reading frame was changed to ATC by substitution of the G at nt 1543, while *in700* carries an insertion of an AT base pair at nt 1595. Human diploid strain WI38 cells (16) were obtained from the American Type Culture Collection at an early passage, and we used them during their active growth phase, prior to their entry into the crisis phase typical of such cell strains. A number of preliminary tests with Ad12, *pm700*, and *in700* suggested that the wild type and the mutants grew equally well in growth-arrested (confluent) WI38 cells even at low input multiplicities. The results of a complete test carried out over a period of 4 days (Fig. 2) make it very clear that not only do Ad12, *pm700*, and *in700* grow at similar rates and attain similar yields in growth-arrested WI38 cells, but so also do Ad5, *in1*, and *dl337*. We therefore conclude that the absence of the 19K protein, even at low input multiplicities, does not provide a growth advantage to either Ad5 or Ad12 in these cells. Comparison with the growth curves shown in Fig. 1 (drawn to the same scale) indicates that the growth rates and yields in WI38 are comparable to those of Ad5 and *in1* on A549 cells and the growth rates of these viruses on HeLa cells, apart from a delay of about 8 h in commencement of the exponential phase of viral growth in WI38 cells in comparison with the start of exponential-phase growth in A549 and HeLa. Consistent with these viral-growth results, we could find few or no differences in the levels of viral DNA synthesized by Ad5 and the two mutant viruses in WI38, HeLa, and A549 cells (data not shown). Microscopic examination of WI38 cells infected by Ad5, *in1*, and *dl337* revealed the enhanced cytopathic effect (*cyl*) characteristic of these mutants in HeLa and A549 cells, but the degree of the effect was considerably less than that observed in the latter two cell types. Finally, it should be noted that the *deg* phenotype, which we observe in HeLa cells, does not develop in WI38 cells or in A549 cells infected with *in1* or *dl337* (data not shown). On this point we are in agreement with White and Stillman (45), but we clearly differ with White et al. (43) with respect to the nature of the growth patterns of these viruses in WI38 cells.

The 19K protein does not influence the levels of viral early gene products in WI38 cells. It was previously reported that the selective growth advantage of 19K-defective mutants in WI38 cells results from increased expression of viral early genes including E1A, that the 19K protein acts as a negative regulator of early gene expression in wild-type-infected cells, and that the regulation takes place at the transcriptional level (43). In view of the fact that we could detect absolutely no differences in the growth patterns of Ad5 and Ad12 mutants and their wild-type counterparts in WI38 cells, we considered it unlikely that significant differences in viral early gene expression would

FIG. 2. Growth of Ad5 and Ad12 and their respective 19K-defective mutants on WI38 cells. In all cases, cells were infected at an input multiplicity of 10 PFU per cell, and samples harvested at various times after infection were frozen and thawed three times to release virus. In the case of the Ad5 viruses, the samples were assayed for infectivity by plaque assay on 293 cells, and in the case of the Ad12 viruses, samples were assayed on HER3 (6) monolayers. Values represent the titers of cell-associated virus.

be revealed in these cells; but despite this doubt, we monitored the expression of some virus early genes in these cells infected with Ad5 and Ad5 *in1* and with Ad12 and Ad12 *pm700*.

First, using an S1 nuclease protection assay, we measured at various times after infection the levels of the E1A 12S and 13S mRNA species accumulating in the cytoplasm of infected cells. In a number of tests, samples were collected at times up to 18 h postinfection, when the levels of viral DNA synthesis were high and when the exponential phase of viral growth was

FIG. 3. Hybridization-S1 nuclease analysis of E1A mRNAs in WI38 cells infected with Ad5 and *in1* (upper panel) and Ad12 and *pm700* (lower panel). Cells were infected at an input multiplicity of 30 PFU per cell and harvested at the times indicated below. Total cytoplasmic RNA was prepared from the infected cells as previously described (20, 48). In all cases, 125 μ g of this RNA was hybridized at 53°C to 0.01 μ g of the 3'-end-labelled probe DNA and digested with S1 nuclease at a final concentration of 850 U/ml (3, 27). The DNA fragments protected from nuclease digestion were denatured and separated by electrophoresis on 5% acrylamide gels containing 8 M urea. For detection of Ad5 E1A mRNAs, a 488-nt *NarI* fragment (nt 814 to 1302) labelled at the 3' end with 32 P was isolated from E1 plasmid pGT509 (nt 1 to 1833) (44) and used in the hybridization assay. The probe used to detect Ad12 E1A mRNAs was the *Asp718-Acc1* fragment (nt 593 to 1596) cut from a pGEM *HindIII* B plasmid (nt 1 to 2318) (23). Probe fragments were end labelled to a specific activity of 1 to 1.3×10^7 cpm/ μ g with [α - 32 P]dGTP with the Klenow fragment of *Escherichia coli* DNA polymerase I (25), purified by electrophoresis on a 5% polyacrylamide gel, and recovered by electroelution prior to use. The autoradiogram of the gel shows the DNA fragments protected by RNA from nuclease digestion. The sizes of these protected fragments (in bases) and the corresponding E1A mRNAs they identify are shown to the right of each panel. (Upper panel) Lanes A, C, E, and G represent RNA from cells infected with Ad5 and harvested at 9, 12, 15, and 18 h postinfection, respectively; lanes B, D, F, and H represent RNA from *in1*-infected cells taken at the same times; and lane P represents the labelled probe used. (Lower panel) Lanes are exactly as described for the upper panel but contain RNA from cells infected with Ad12 and *pm700* (lanes A, C, E, and G and B, D, F, and H, respectively).

commencing (Fig. 2). In all cases, the rates of accumulation of the major mRNA species were very similar for each wild-type virus and its corresponding mutant over this time period, with gradual increases in the levels of the mRNAs as a function of increasing time (Fig. 3). In parallel with these tests, we determined the levels of the E1A proteins made in infected WI38 cells by immunoprecipitation of lysates with antibodies specific for the E1A proteins. Mutant- and wild-type-infected cells contained similar levels of newly synthesized E1A products, even as late as 24 h postinfection, as shown in Fig. 4. In the case of Ad5, *in1*, and *dl337*, we have also measured the

FIG. 4. Immunoprecipitation of E1A proteins from lysates of WI38 cells infected with 19K-defective mutants. Cells were infected at an input multiplicity of 30 PFU per cell and labelled with [35 S]methionine (50 μ Ci/ml in Dulbecco modified Eagle medium lacking methionine) for 2 h at 24 h after infection. Methods for extraction of cells and for immunoprecipitation were those previously described (30). Ad5 E1A proteins were detected with antiserum from rats immunized with an *E. coli trpE* Ad2 E1A fusion protein expressed from plasmid pKRS103 (32), a kind gift from A. Berk, University of California, Los Angeles. The Ad12 E1A proteins were detected with a rabbit antiserum generated against a 16R polypeptide corresponding to the common carboxyl terminus of the Ad12 E1A 266 and 235R proteins (24), generously provided by M. Green, St. Louis University. In all cases, the labelled samples were analyzed by electrophoresis on sodium dodecyl sulfate-10% polyacrylamide gels (21), which were subsequently impregnated with 1 M sodium salicylate, dried, and exposed to X-ray films. (Left panel) Lanes: M, 14 C-labelled protein size standards (in kilodaltons [K]); A, noninfected cell lysate; B, Ad5 lysate; C, *in1* lysate; D, *dl337* lysate. (Right panel) Lanes: M, size standards (in kilodaltons [K]); A, noninfected lysate; B, Ad12 lysate; C, *pm700* lysate; D, *in700* lysate.

levels of the 72K DNA binding protein and the E1B 55K protein (data not shown) in infected WI38 cells and find them both to be very similar in mutant- and wild-type-infected cells, although as found previously (36), the level of 55K protein in cells infected by *in1* is about twofold higher than in those infected by *dl337* and Ad5. Thus, in keeping with the fact that the mutant and wild-type growth patterns are identical in WI38 cells, we can find no evidence for any major regulatory effect of the 19K protein on early gene expression in these cells. Given that we found no difference in the rates of accumulation of E1A mRNAs and synthesis of E1A proteins in these cells, we did not assay for any potential effect on transcription rates per se, and should there be a mutant-specific difference at that level, it is clearly not reflected in the efficiency of expression of the E1A products and has no overt influence whatsoever on viral growth.

Likewise, the levels of E1A and E1B mRNAs in the cytoplasm of A549 and HeLa cells infected with Ad5 and *in1* at early times after infection are very similar (data not shown). We have measured the levels of newly synthesized E1A and E2A 72K proteins in Ad5-, *in1*-, and, in some cases, *dl337*-infected A549 and HeLa cells at times well into the exponential-growth phase in these cells (Fig. 2), and as with WI38 cells, we detected no major differences between the levels found in mutant- and wild-type-infected cells. Further, the levels of newly synthesized E1A proteins in HeLa cells infected with the E1A-defective mutants *pm12* and *pm13* and their respective 19K-defective recombinants *rec pm12 in1* and *rec pm13 in1*

were measured; these viruses make similar amounts of the larger and smaller subsets, respectively, of these E1A products and in all cases make somewhat less than the wild-type levels (data not shown). Similar overall results were also found in A549 cells infected with Ad5 and its various mutants. Clearly there is, therefore, no pronounced increase in the levels of the E1A proteins synthesized in HeLa (or A549) cells infected by the double-mutant 19K-defective viruses. Lastly, it should be mentioned that we have also previously found (for a discussion, see reference 9) that steady-state levels of E1A mRNAs in A549 cells infected with Ad12 mutants *pm700* and *in700* are similar or slightly lower than those found in cells infected with parental wild-type virus.

Our results leave little doubt that both Ad5 and Ad12 grow extremely well in WI38 cells and show no marked differences from their respective 19K-defective mutants in their growth patterns in these cells. In fact, in the case of the Ad5 viruses there is little difference in their growth patterns in WI38 and A549 cells, and the only obvious differences are that the commencement of the exponential phase is delayed by some 8 h and that the final yields are slightly lower in WI38 cells. Further, given that all of the Ad5 and Ad12 viruses grow equally well in WI38 cells, it was not really surprising to find that there are no explicit differences in the expression of viral early genes in cells infected by mutant and wild-type viruses. While we have no certain explanation for this clear disagreement between our results and those of White et al. (43), we can offer a possible reason. In their hands, the Ad2 wild type failed to grow well in WI38 cells at lower input multiplicities (for example, 5 PFU per cell). This lower multiplicity is comparable to those employed in our experiments, and in our hands Ad5 and Ad12 still grow well at considerably lower inputs in WI38. That the apparent block was overcome at increasingly higher multiplicities (for example, 100 PFU per cell) might indicate that the wild type used by White et al. was defective in some way, for it is typical of many Ad mutants to show a multiplicity-dependent leak. If the Ad2 was defective, it might then show delayed and/or reduced expression of viral early genes, but this cannot really be defined as a negative regulatory effect of the 19K protein. Further, it seems odd that a putative negative regulatory event would be overcome by increasing the copy number of the gene encoding the normal protein protagonist. Finally, we should point out that while we used Ad5 as opposed to Ad2 in our tests, we have also shown that Ad2 grows extremely well in WI38 cells (data not shown), so the marginal serotypic difference between these two viruses cannot explain the differences in our results.

White and coworkers also discovered an E1B 19K-protein negative regulatory effect on viral early gene expression in HeLa cells (42) and reported that the mutant E1B 19K gene product had a much greater effect in a 12S cDNA virus background, with greatly increased levels of the 12S E1A product and increased plaquing efficiency of the 12S virus in HeLa cells infected with double mutants. Thus, we are again in apparent disagreement with White et al. on all of these counts but are essentially in complete agreement with Herbst et al. (17), who could find no evidence for any regulatory effects on viral early gene expression in either HeLa cells or rat CREB cells. On the face of it, our results and those of Herbst et al. also appear to contradict those obtained from plasmid-mediated transient assays (51). However, these differences might simply reflect intrinsic differences in the conditions determining the expression of a viral gene (and its measurement) isolated in a plasmid vector system as opposed to the complete viral genome. It should also be mentioned that the 19K protein has been shown to markedly influence the stability of plasmid

DNA following transfection into target cells (18), and the apparent transcriptional effects reported in the plasmid-mediated tests could perhaps be explained partly or wholly in terms of relative effective plasmid DNA concentrations in the recipient cells.

In virally infected human cells, at least, we propose that the E1B 19K protein does not impose any significant regulatory effects on viral growth or on the expression of the E1A gene or any other viral early gene. Given that we made this observation with the widely divergent serotypes 5 and 12, we predict that this will be the normal scenario for Ads in general. The effects of this protein on the phenotypic properties of mutant Ads (e.g., the *cyt* phenotype and the *deg* phenotype, etc.) are presumably mediated directly via loss of its functions or indirectly through the loss of a normal effect that it has on some other viral product or a cellular product. In this context, it is of interest to note the proposal that the E1A proteins are responsible for cytotoxicity in rodent and perhaps human cells infected with Ads, that the p53 protein plays a role in this process, and that the E1B 19K protein acts to prevent it (8, 41, 45). While this pathway of events could also influence the *deg* and *cyt* phenotypes observed in productively infected human cells, it does not seem to alter or be necessary for normal viral early gene expression and viral growth.

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