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Characterization of Mutants within the Gene for the Adenovirus E3 14.7-Kilodalton Protein Which Prevents Cytolysis by Tumor Necrosis Factor

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The 14,700-Da protein (14.7K protein) encoded by the E3 region of adenovirus has previously been shown to protect mouse cells from cytolysis by tumor necrosis factor (TNF). Delineating the sequences in the 14.7K protein that are required for this activity may provide insight into the mechanism of protection from TNF by 14.7K as well as the mechanism of TNF cytolysis. In the present study, we examined the ability of 14.7K mutants to protect cells from lysis by TNF. In-frame deletions as well as Cys-to-Ser mutations in the 14.7K gene were generated by site-directed mutagenesis and then built into the genome of a modified adenovirus type 5 (*dl*7001) that lacks all E3 genes. *dl*7001, which replicates to the same titers as does adenovirus type 5 in cultured cells, has the largest E3 deletion analyzed to date. ^{51}Cr release was used to assay TNF cytolysis. Our results indicate that most mutations in the 14.7K gene result in a loss of function, suggesting that nearly the entire protein rather than a specific domain functions to prevent TNF cytolysis.

Adenovirus early region E3 codes for at least seven proteins, including gp19K (29), 10.4K (36), 14.5K (35), 12.5K (21), 11.6K (37, 43), 6.7K (42), and 14.7K (39). The significance and function of region E3 are uncertain, but accumulating evidence suggests that several E3 proteins function to circumvent immune surveillance (46).

Cytotoxic T lymphocytes are a major defense against acute virus infections. One adenovirus E3 protein, a glycoprotein termed gp19K which is localized in the membrane of the endoplasmic reticulum, protects adenovirus-infected cells against lysis by adenovirus-specific cytotoxic T lymphocytes (reviewed in references 17, 28, 45, and 46). gp19K binds to major histocompatibility complex class I antigens and retains them in the endoplasmic reticulum, thereby preventing their transport to the cell surface.

Macrophages and cytokines secreted by activated macrophages appear to be another defense against virus infections. Tumor necrosis factor (TNF) is a potent cytokine secreted by activated macrophages in response to viruses, tissue damage, bacterial endotoxin, and other cytokines (reviewed in references 25 and 41). TNF is a 17K polypeptide that binds to specific receptors found on virtually all cells and leads to activation of signal transduction pathways, transcription factors, and protein kinases and the induction of a large number of cellular proteins. In addition, TNF can suppress the replication of both DNA and RNA viruses (26, 47) and is cytotoxic to many primary tumors and transformed cell lines (9, 33). The cellular events underlying TNF cytolysis remain obscure.

Previous work has shown that, during adenovirus infection of cultured cells, expression of the E1A proteins sensitizes cells to TNF cytolysis (6, 7) and that subsequent expression of three independent sets of adenovirus proteins inhibits TNF cytolysis (13–16). The E3 14.7K protein protects 13 of 15 mouse cell lines examined from lysis by TNF (15). A complex of the E3 10.4K and 14.5K proteins protects 11 of 15 mouse cell lines examined from lysis by TNF (15). The E1B 19K protein can protect infected human cells from TNF-mediated cytolysis (13). Human cells infected with virus mutants lacking the E1B 19K protein are not lysed by TNF, and this secondary protection effect has been mapped to the E3 region, presumably mediated by 14.7K and/or the combination of 10.4K and 14.5K (13). E1B 19K cannot protect mouse cells from TNF-mediated cytolysis. The E3 10.4K-14.5K complex of proteins also down-regulates the epidermal growth factor receptor (38).

The 14.7K protein prevents TNF lysis of cells that are spontaneously sensitive to TNF and cells that have been rendered sensitive by treatment with cycloheximide or cytochalasin E (15). Representative serotypes in adenovirus groups A, B, C, D, and E are protected from TNF cytolysis and produce the 14.7K protein, indicating that the protection from TNF afforded by 14.7K is common to most, if not all, adenovirus types (23). The 14.7K protein expressed in stably transfected cell lines can prevent TNF cytolysis in cells sensitized to TNF by treatment with cycloheximide, cytochalasin E, or expression of E1A, indicating that 14.7K can function independently of adenovirus infection (22). The 14.7K protein does not affect the number or affinity of these receptors as judged by binding parameters of ^{125}I-TNF (22).

Many DNA tumor virus proteins have a domain architecture, with individual domains performing specialized functions. For example, the adenovirus E1A 289-residue protein contains three domains that are highly conserved among serotypes and that allow the protein to bind tumor suppressor proteins, activate transcription, immortalize cells in culture (reviewed in reference 34), and induce susceptibility to TNF (1, 7). By analogy, identification of specific sequences in 14.7K that are involved in protection from lysis by TNF might provide further insight into the mechanism of action of 14.7K and the mechanism of TNF-mediated cytol-

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ysis. In this study, we examine a series of 14.7K mutant viruses in an attempt to define a sequence element in the 14.7K protein that is necessary for protection from TNF.

MATERIALS AND METHODS

Cell lines and viruses. C3HA is a mouse embryo fibroblast cell line (12) maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone, Logan, Utah). NIH 3T3 cells are mouse fibroblasts maintained in the same medium. KB is a human oral carcinoma cell line cultured in Joklik's modified minimal essential medium supplemented with 5% horse serum (Hazleton, Lenexa, Kans.). A549 is a human lung carcinoma cell line maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

The adenovirus mutants *dl*759 (36), *dl*706 (3), and *rec*700 (44) have been described elsewhere.

Plasmids. The plasmid pKA-14.7ΔE3 was constructed in several stages, starting with a plasmid (pED) consisting of the adenovirus type 2 *Eco*RI-D fragment cloned in pBR322. A *Bam*HI site was engineered into pED at nucleotide (nt) 362 in the E3 transcription unit of adenovirus type 2 or adenovirus type 5 (Ad5) (5). An *Eco*RI-*Bam*HI fragment, nt −236 to 362 of region E3, was excised and cloned into M13mp19 to produce M13EB. Site-directed mutagenesis following the Amersham (Arlington Heights, Ill.) oligonucleotide-directed mutagenesis system was performed on M13EB to remove the ATG at nt 291 in E3. Oligonucleotides were purchased from Operon Technologies (San Pablo, Calif.). The *Kpn*I-A fragment, corresponding to map positions 71 to 94 in the genome of *dl*759 (36), was cloned into the *Kpn*I site of a modified pBR322 to produce pKA (*dl*759); this plasmid has an *Eco*RI site at nt −236 and a *Bam*HI site at nt 2801 in region E3 of Ad5. The *Eco*RI-*Bam*HI fragment from pKA (*dl*759) was excised and replaced by the *Eco*RI-*Bam*HI fragment lacking ATG_{291} in M13EB; the resulting plasmid, termed pKA-ΔE3X, has a deletion in region E3 from nt 362 to 2801. Subsequently, a *Bam*HI-*Hin*dIII fragment (nt 2801 to 4273 in E3) was cloned from pKA-ΔE3X into M13mp19 to produce M13BH. Three rounds of site-directed mutagenesis were performed in M13BH: an *Eco*RI site was generated at nt 3424, a *Bam*HI site was generated at nt 2870, and *Xho*I sites were generated at nt 2880 and 3382. The mutated *Bam*HI-*Hin*dIII fragment from M13BH was then reinserted into pKA-ΔE3X to form the plasmid pKA-14.7ΔE3. Plasmid pKA-14.7ΔE3 contains the 14.7K gene, and it has nt 362 to 2870 in E3 deleted; important features of the E3 region in pKA-14.7ΔE3 are illustrated in Fig. 1B. Plasmid pKA-ΔE3, which is a derivative of pKA-14.7ΔE3, has the *Xho*I fragment (nt 2880 to 3382) containing the 14.7K gene excised. The *Eco*RI fragment (nt −236 to 3424, lacking nt 362 to 2870) from pKA-14.7ΔE3 was inserted into M13mp19 to produce M13EE. Mutagenesis of the 14.7K gene was performed in M13EE.

Virus mutants. The adenovirus mutants *dl*7000 ($14.7K^+$) and *dl*7001 ($14.7K^-$) were constructed by in vivo double-overlap homologous recombination (Fig. 1A) (24). Briefly, 1 μg of Ad5 terminal protein DNA was digested with *Eco*RI and combined with 5 μg of either pKA-14.7ΔE3 (*dl*7000) or pKA-ΔE3 (*dl*7001), 20 μg of salmon sperm DNA, 2× HBS (270 mM NaCl, 10 mM KCl, 1 mM Na_2HPO_4, 0.2% dextrose, 42 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid] [pH 7.1]), and 2 M $CaCl_2$. The mixture was transfected into A549 cells by calcium phosphate-mediated transfection using a glycerol shock at 5 h posttransfection. Plaques were picked and expanded, the Hirt supernatant of infected cells was screened for the correct construct, and the isolate was developed into virus stocks in human KB cells (44). Virus stocks were banded in CsCl, and their titers were determined on A549 cells as described previously (19).

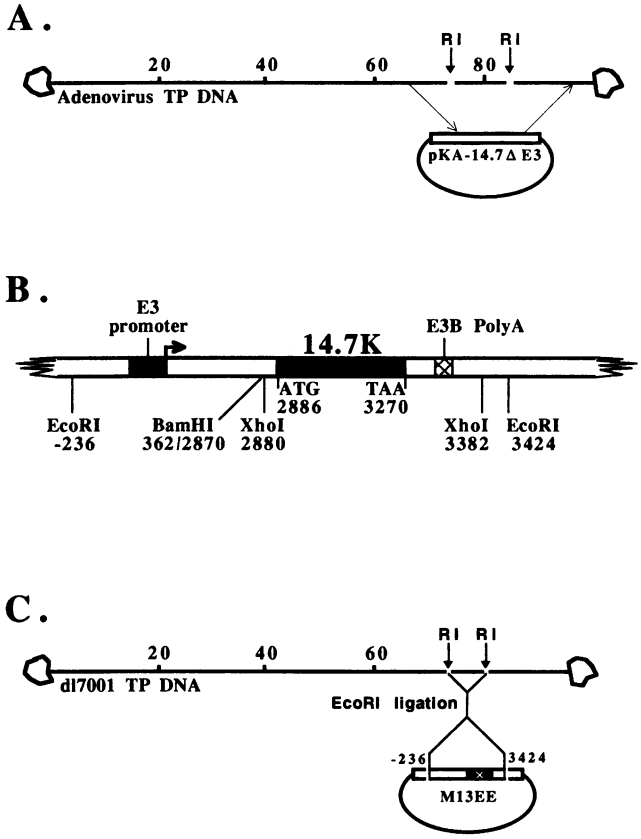

FIG. 1. Schematic representation of the method of construction of the 14.7K mutants. (A) The adenovirus deletion mutants *dl*7000 ($14.7K^+$) and *dl*7001 ($14.7K^-$) were constructed by in vivo homologous recombination using plasmids pKA-14.7ΔE3 and pKA-ΔE3, respectively (see Materials and Methods). *dl*7001 deletes all E3 proteins, and *dl*7000 removes all E3 proteins except 14.7K. Plasmids pKA-14.7ΔE3 and pKA-ΔE3 were derived from the cloned *Kpn*I-A fragment of *rec*700. The *Kpn*I-A fragment is derived from map positions 71.4 to 93.5 in the adenovirus genome. RI, *Eco*RI; TP DNA, terminal protein DNA. (B) Schematic representation of the E3 transcription unit in *dl*7000. Both *dl*7000 and *dl*7001 share a large deletion between nt 362 and 2870 and point mutations at nt 291 (changes ATG to CGG), 2870 (*Bam*HI), 2880 (*Xho*I), 3382 (*Xho*I), and 3424 (*Eco*RI). Plasmid pKA-ΔE3 contains an additional deletion between nt 2880 and 3382. (C) Method used to insert the 14.7K mutations into the *dl*7001 genome. *dl*7001 terminal protein DNA (TP DNA) was cleaved with *Eco*RI (RI), as was the plasmid M13EE containing the mutation (X) in 14.7K. The DNAs were mixed, ligated, and transfected into A549 cells, and plaques were screened for the 14.7K insert.

All 14.7K mutations were created in M13EE by oligonucleotide mutagenesis. Following isolation and sequencing of a mutant M13EE clone, the *Eco*RI fragment containing the mutation was gel purified, ligated to *Eco*RI-cleaved *dl*7001 terminal protein DNA, transfected into A549 cells (Fig. 1C), screened, and expanded into virus stocks as described above. For mutants *dl*7024, *dl*7026, *dl*7028, *dl*7031, and *dl*7032, the viral DNA was sequenced to confirm the presence of the mutation.

Northern (RNA) blot. Cytoplasmic RNA was isolated at 12 h postinfection (p.i.) from *dl*759-, *dl*7000-, or *dl*7001-infected KB cells as described previously (4). Polyadenylated [poly(A)$^+$] RNA was isolated from total cytoplasmic RNA with mRNA purification columns (Pharmacia, Piscataway, N.J.). Northern blots were prepared as previously described (3). Antisense RNA corresponding to nt 26 to 566, a region common to all E3 mRNAs, was labeled with [α-^{32}P]UTP and used to probe the blots. This probe was prepared with SP6 RNA polymerase and a p64-based plasmid containing an *Sst*I-*Bgl*II fragment (nt 26 to 566 in E3) that had been digested with *Eco*RI.

Immunoprecipitation analysis. C3HA cells at a density of 2.0×10^6 per 100-mm dish were infected with 150 PFU of virus per cell, and proteins were labeled from 17.5 to 18 h p.i. with Trans^{35}S-label (1,133 Ci/mmol; ICN Radiochemicals, Irvine, Calif.). Cells were pelleted, rinsed three times with 34 μg of phenylmethylsulfonyl fluoride per ml (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS), detached with 1 ml of trypsin, and collected. The cells were resuspended and lysed for 30 min on ice with 500 μl of lysis buffer (250 mM NaCl, 50 mM HEPES [pH 7.4], and 0.1% Nonidet P-40) (8) containing 34 μg of phenylmethylsulfonyl fluoride per ml and 0.5 μg of leupeptin per ml (Boehringer Mannheim, Indianapolis, Ind.). Nuclei were pelleted, and 14.7K was immunoprecipitated from the cytosolic fraction with 5 μl of an antiserum to a TrpE-14.7K fusion protein (39). Immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 16% gels (29.2:0.8 acrylamide-*N*:*N*-methylene-bisacrylamide) (Bethesda Research Laboratories, Gaithersburg, Md.).

KB cells in suspension were infected with 25 PFU of virus per cell and labeled with Trans^{35}S-label from 7 to 7.5 h p.i. The cells were collected and lysed on ice for 30 min with a buffer containing 0.5% Nonidet P-40 (39), and 14.7K was analyzed as described above.

All gels were fluorographed. Autoradiograms were traced with an LKB Ultrascan XL laser densitometer.

Western blots (immunoblots). Cells (1.0×10^6 per 100-mm dish) were infected with 150 PFU of virus per cell and collected as described for immunoprecipitation, and then the cell pellets were resuspended in 100 μl of 2× Laemmli sample buffer. Samples were frozen and thawed twice and drawn seven times through an 18-gauge needle. The samples were boiled for 5 min, and 50 μl was loaded onto SDS–16% polyacrylamide gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.) in 25 mM Tris–192 mM glycine–20% methanol at 150 mA for 30 min in a semidry blotting apparatus (Bio-Rad, Richmond, Calif.). Membranes were blocked overnight in PBS containing 5% Carnation milk and 0.2% Tween 20 (Sigma). The membranes were probed with a 1:500 dilution of TrpE-14.7K antiserum, washed three times with PBS–0.2% Tween 20–5% milk, and incubated with a 1:2,000 dilution of goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad). Proteins were detected by chemiluminescence with the ECL Western blotting reagent (Amersham).

Assay of TNF cytotoxicity (^{51}Cr release). The ^{51}Cr release assay of TNF cytotoxicity was performed as described previously (14). Briefly, mouse fibroblasts (10^6 per 60-mm dish) were infected with adenovirus (or mock infected); 24 h later, 200 μCi of $Na_2\,^{51}CrO_4$ (DuPont, NEN) was added to each plate and the cells were incubated at 37°C for an additional 6 h prior to being washed and replated for assay. Labeled cells were aliquoted into microtiter plates (10^4 per well) and incubated with human recombinant TNF (13×10^6 U/mg of protein; the generous gift of Cyron/Cetus Corporation, Emeryville, Calif.). After 18 h of incubation at 37°C in 8% CO_2, aliquots of supernatant were removed and the released radioactivity was determined with a Packard Autogamma counter. Results are expressed as percent specific ^{51}Cr release, which equals 100 × (^{51}Cr counts per minute released from cells in the presence of TNF − ^{51}Cr counts per minute released in the presence of medium alone [spontaneous release])/(^{51}Cr counts per minute released in 1 N HCl [maximum release] − ^{51}Cr counts per minute released in medium alone). Spontaneous release averaged about 30%. All determinations were done in triplicate. In all experiments, infected cells were plated onto glass slides, fixed, and stained with anti-Ad5 serum (American Type Culture Collection, Rockville, Md.) to confirm the percentage of cells infected. Cells were at least 95% infected in all experiments shown here.

RESULTS

Characterization of the *dl*7001 vector which was designed to express the 14.7K mutant proteins. Region E3 encodes about nine alternatively spliced mRNAs (4) and seven known proteins (46). We considered that it was possible that some of these E3 proteins could affect or obscure the ability of 14.7K to prevent TNF cytolysis. For example, the E3 10.4K-14.5K complex of proteins prevents TNF cytolysis in most mouse cell lines (15). Therefore, we designed an adenovirus vector, *dl*7001, that lacks all the E3 genes and another vector, *dl*7000, that lacks all E3 genes except that for 14.7K. These vectors were constructed by double-overlap homologous recombination between Ad5 terminal protein DNA and plasmid pKA-ΔE3 (*dl*7000) or pKA-14.7ΔE3 (*dl*7001), as described in Materials and Methods and illustrated in Fig. 1A. Both *dl*7000 and *dl*7001 replicated as well as wild-type Ad5 in KB cells, as determined by the final titer of the virus stocks. The deletion in *dl*7001, nt 362 to 3382 in the E3 transcription unit of Ad5, is the largest described to date for an E3 deletion mutant.

The structure of the modified E3 transcription unit in *dl*7000 is shown in Fig. 1B. The E3 promoter and E3B polyadenylation site are intact, all known E3 splice sites are deleted or mutated, and there are no ATGs between the transcription start site (nt +1) and the 14.7K gene. A single unspliced mRNA which is polyadenylated at the E3B site and which has a 376-nt 5′ untranslated region consisting of nt 1 to 362 of the first exon in wild-type E3 mRNAs (the wild-type first exon consists of nt 1 to 372) plus 14 nt of the sequence immediately upstream of the 14.7K gene should be produced. The only protein synthesized should be authentic 14.7K.

A Northern blot was performed to characterize the E3 mRNA synthesized by *dl*7000 and *dl*7001. An antisense RNA probe, specific to exon 1, hybridized to the four predominant E3 transcripts from *dl*759, mRNAs *a*, *c*, *f*, and *h* (Fig. 2A, lane 1). *dl*759 was analyzed instead of Ad5 because the deletion in *dl*759 (Δ2488-2803), which reduces the sizes of mRNAs *c* and *f* (36), allows the four E3 mRNAs to be detected more clearly (in Ad5, mRNAs *a* and *f* nearly comigrate). No mRNAs were detected in either total cytoplasmic (Fig. 2A, lane 3) or poly(A)$^+$ (lane 2) RNA prepared from *dl*7001-infected cells, as expected. A single transcript somewhat smaller than mRNA *h* was detected in *dl*7000 RNA (Fig. 2A, lanes 4 and 5). mRNA *h* from *dl*759 is 985 nt (4, 5), and the single mRNA from *dl*7000 is predicted to be 803 nt [not including the poly(A) sequence]. Therefore, the mRNA from *dl*7000 has the expected size. The identity of the faint band migrating between mRNAs *a* and *c* in Fig. 2A,

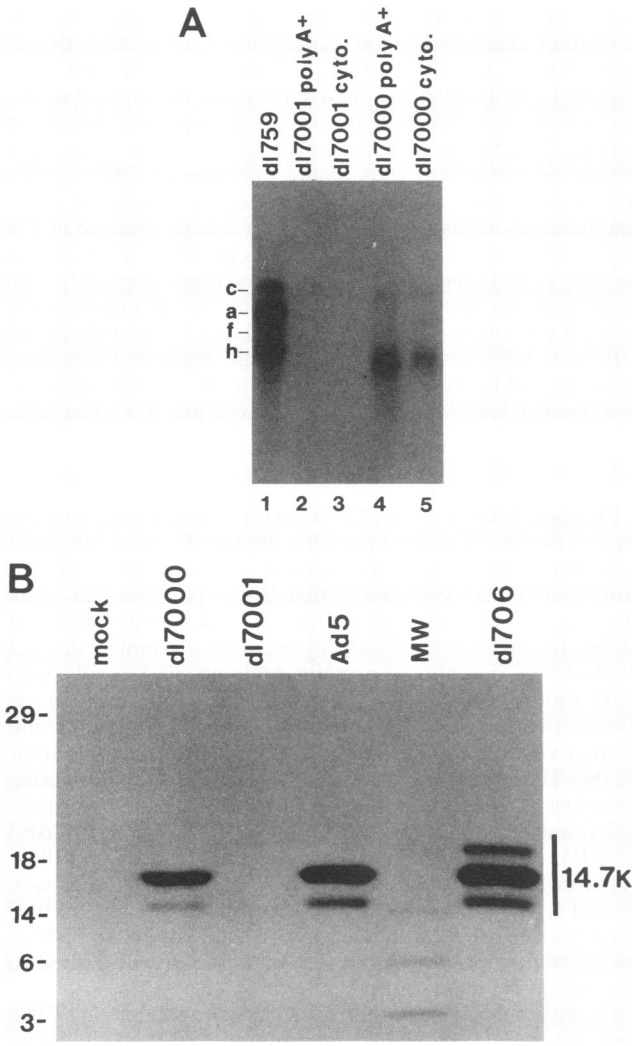

FIG. 2. Expression of E3 mRNA and the 14.7K protein from cells infected with *dl*7000 or *dl*7001. (A) Poly(A)$^+$ (1.8 μg per lane) and cytoplasmic (2 μg per lane) RNA prepared from infected KB cells. An antisense RNA probe specific for all E3 transcripts was used. Virus *dl*759 produces the four major E3 mRNAs, *a*, *c*, *f*, and *h*, whereas *dl*7000 produces the novel 14.7K mRNA. (B) Immunoprecipitation of 14.7K from KB cells infected with Ad5, *dl*706 (a mutant that overproduces 14.7K), or *dl*7000, using an antiserum to a TrpE-14.7K fusion protein. There is no protein detected in extracts from cells infected with *dl*7001. The two bands observed with *dl*7000 and Ad5 are forms of 14.7K. The extra 14.7K band in *dl*706 extracts is typically observed in mutants derived from *rec*700 (39). *dl*7000, *dl*706, and all mutants described in this paper have the Ad5 version of 14.7K. mock, mock-infected cells; MW, molecular weight standards (molecular weights, in thousands, appear at left).

lanes 4 and 5, is unknown; it may be a nuclear RNA species, or it may be an mRNA terminating downstream from the normal polyadenylation site.

The ability of *dl*7000 and *dl*7001 to produce 14.7K was assayed by immunoprecipitation with an antiserum to a TrpE-14.7K fusion protein. Two bands of 14.7K were obtained from cells infected with *dl*7000, while no precipitating protein could be detected in extracts from *dl*7001-infected cells (Fig. 2B). The amount of 14.7K immunoprecipitated from *dl*7000 was roughly equivalent to the level found with Ad5 but was less than that obtained with *dl*706. *dl*706 is a mutant that overproduces mRNA *h* and therefore overproduces the 14.7K protein (22). For reasons that are unknown, 14.7K from Ad5 and *dl*7000 migrates as two bands on SDS-PAGE whereas 14.7K from *dl*706 and related virus constructs migrates as three bands (14, 22, 39).

The amount of E3-specific mRNA obtained from *dl*7000 was roughly equivalent to that of mRNA *h* from *dl*759 (Fig. 2A) or Ad5 (data not shown). Since 14.7K is translated from mRNA *h* (29, 39), the mRNA result is in accord with the equivalent amount of 14.7K protein obtained from *dl*7000 and Ad5 (Fig. 2). We had expected that *dl*7000 would express much more 14.7K than Ad5, because only a single E3 mRNA would be synthesized in *dl*7000. Most likely, the single unspliced E3 mRNA in *dl*7000 is not stable or is not transported efficiently from the nucleus. In any event, *dl*7000 synthesized sufficient 14.7K such that the effects of mutations in the 14.7K gene could be analyzed in a *dl*7000 format.

Synthesis and steady-state levels of the mutant 14.7K proteins in infected cells. Mutations in the 14.7K gene were constructed in M13EE as described in Materials and Methods. These mutations were built into the genome of *dl*7001 by ligation of the *Eco*RI fragment from mutated M13EE (Fig. 1C). *dl*7000 is the wild-type positive control for these mutants, and *dl*7001 is the negative control.

A schematic representation of all the 14.7K mutants is presented in Fig. 3. The mutations consist of in-frame deletions and point mutations that converted all the Cys residues to Ser. The deletions were designed to coincide as much as possible with regions in the 14.7K protein that are conserved or diverged among the adenovirus serotypes (39).

Immunoprecipitation analysis was performed to determine whether the 14.7K protein was synthesized in human KB cells infected with the deletion mutants. Cells were labeled with Trans^{35}S-label for 30 min, and then 14.7K was extracted, immunoprecipitated, and analyzed by SDS-PAGE. As shown in Fig. 4B, all mutants synthesized 14.7K at levels similar to those in wild-type *dl*7000. For the most part, the mobilities of the two 14.7K bands increased in a manner consistent with the size of the deletion. Notable exceptions are *dl*7021, which migrated farther than expected; *dl*7022, which did not migrate far enough; and *dl*7031, which showed decreased mobility compared with wild-type 14.7K. We sequenced the entire 14.7K gene from the *dl*7021, *dl*7022, and *dl*7031 viruses, and no deletions or insertions were observed; thus, we cannot explain the aberrant mobility of 14.7K from these mutants at the present time. However, we note that the amino acid composition of 14.7K appears to affect its mobility in SDS-PAGE, because the Ad5 and adenovirus type 2 versions of 14.7K, which have the same number of amino acids and which are 78% identical, migrate differently on SDS-PAGE (14, 39). Only one band of 14.7K was obtained with *dl*7032; this will be discussed later.

The synthesis of the mutant 14.7K proteins also was analyzed by immunoprecipitation after 30 min of labeling in mouse fibroblasts, because the TNF cytolysis experiments were carried out in mouse cells (see below). Less 14.7K was synthesized in mouse cells (Fig. 4A) than in KB cells (Fig. 4B), but distinct bands could be seen for each deletion mutant. Most mutants yielded 14.7K bands that were roughly equivalent (within a factor of 2, as determined by densitometric tracing) to those for wild-type *dl*7000. The 14.7K bands from *dl*7021 varied between one-third and one-half the intensity of the wild-type bands, and the bands from *dl*7028 were only one-fourth as intense as the wild-type bands. The mobility of the bands was similar to that for KB cells.

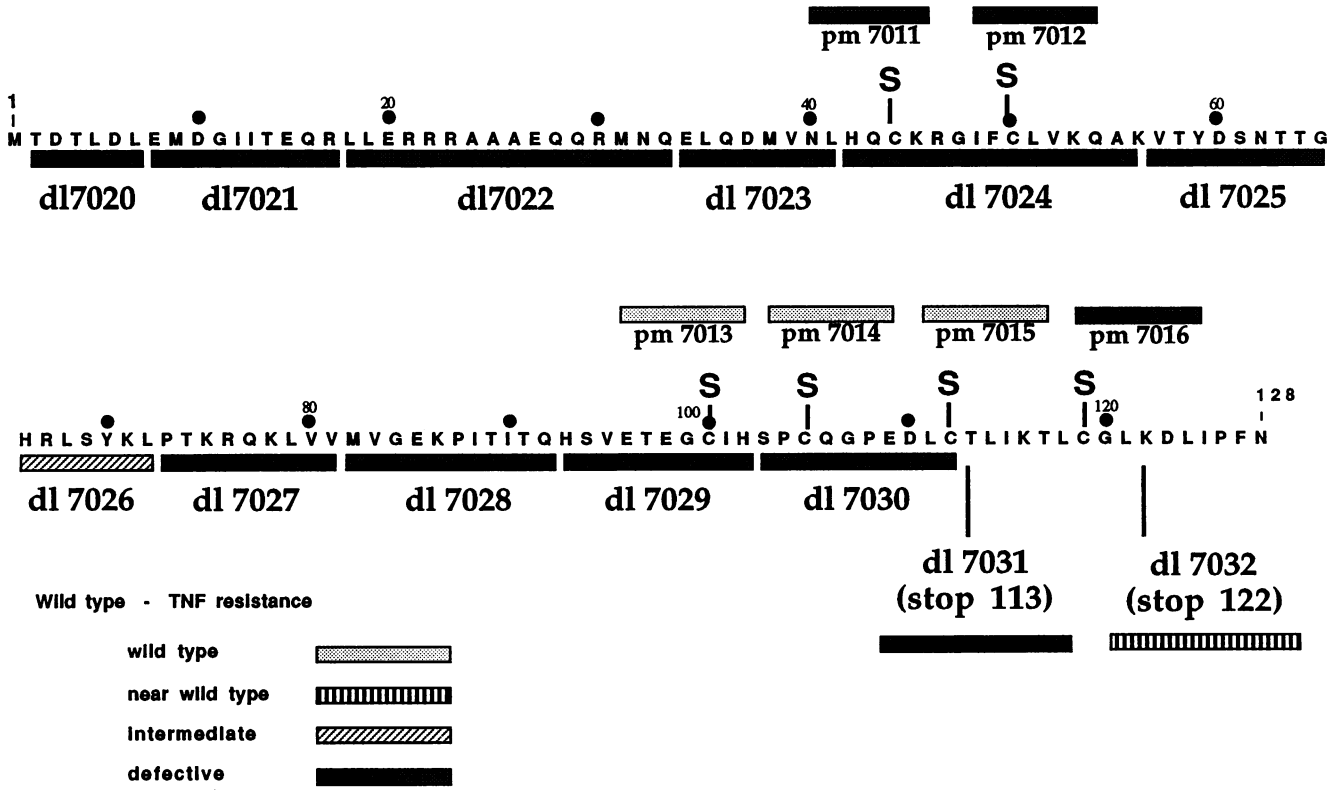

FIG. 3. Sequence of the Ad5 14.7K protein and representation of the 14.7K mutations. The regions deleted were chosen on the basis of sequence conservation in the 14.7K gene among adenovirus serotypes 2, 3, and 5 (39). The point mutations are conservative substitutions that replace the six Cys residues with Ser residues. Mutants that have lost the ability to prevent TNF cytolysis (defective) are indicated, as are mutants that retain this ability (wild type). *dl*7026 (intermediate) provides about one-half as much protection from TNF cytolysis as does the wild type. *dl*7032 (near wild type) is only marginally defective.

The 14.7K protein also was immunoprecipitated from mouse cells infected with viruses containing the Cys-to-Ser point mutations. Large quantities of 14.7K were obtained from *pm*7013, *pm*7014, and *pm*7015, while small amounts were detected in *pm*7011, *pm*7012, and *pm*7016 (Fig. 5).

To examine the stability of the mutant proteins, whole-cell extracts from infected mouse cells were subjected to Western analysis (Fig. 6). The mutant proteins tended to fall into three groups in terms of their stability. The first group, which was similar (within a factor of 2) to wild-type *dl*7000, included *pm*7013, *pm*7014, *pm*7015, *dl*7020, *dl*7021, *dl*7022, *dl*7027, *dl*7029, *dl*7030, *dl*7031, and *dl*7032. A second group, which produced less 14.7K, included *dl*7024, *dl*7025 (not shown), *dl*7026, and *dl*7028. A third group, which produced still less 14.7K, included *pm*7011, *pm*7012, *pm*7016, and *dl*7023. Curiously, the mutated 14.7K protein from *dl*7021 and *dl*7022, which was not immunoprecipitated well from mouse cells (Fig. 4A), showed a good signal in the Western blot (Fig. 6). Conversely, *dl*7023 showed a strong immunoprecipitated band (Fig. 4A) and a weak band by Western blot (Fig. 6), suggesting that the protein may be synthesized at a normal rate but that it is unstable relative to the wild type. These results also may reflect a difference in the avidity of the antibody under different experimental conditions.

The blotting and immunoprecipitation data suggest that most of the mutant proteins have approximately the same level of synthesis and stability in mouse cells as does the wild-type 14.7K protein. We conclude that these mutants are useful reagents for examining the mechanism of 14.7K suppression of TNF-mediated cytolysis.

With all mutations in the region from residues 34 to 72 of 14.7K, namely, *dl*7023, *dl*7024, *dl*7025, *dl*7026, *pm*7011, and *pm*7012 (Fig. 3), reduced amounts of 14.7K were detected. This was also true for *dl*7028, which deletes residues 82 to 92 in 14.7K. Thus, these regions appear to be important for the stability of 14.7K in mouse cells. The Cys at residue 119 (*pm*7016) also is important for the stability of 14.7K. Because 14.7K was produced in smaller amounts by these mutants, we cannot be sure whether the inability of some of these mutants to prevent TNF cytolysis is due to a loss of that function or to the low amount of 14.7K present.

We have examined the intracellular localization of 14.7K by immunofluorescence in C3HA and NIH 3T3 cells at 24 and 42 h p.i. with *dl*7000 and with all the mutants (40). In *dl*7000, 14.7K was detected in both the nucleus (excluding the nucleoli) and the cytoplasm extending to the periphery of the cells. The 14.7K staining pattern in the mutants did not differ markedly from that in the wild type or among the mutants, indicating that the mutants do not dramatically affect the intracellular location of 14.7K. The mutants that underproduced 14.7K as indicated by Western analysis also displayed reduced immunofluorescence staining for 14.7K; however, even in these mutants, 14.7K was readily detected.

Deletion of the C-terminal seven amino acids in 14.7K abrogates formation of the bottom band of the 14.7K doublet. With *dl*7000 and all the 14.7K mutants except *dl*7032, 14.7K migrates on SDS-PAGE as a doublet and with the upper band of the doublet more abundant than the lower band (Fig. 4B). Understanding how and why this doublet arises may be important in understanding the function of 14.7K. With *dl*7032, only a single

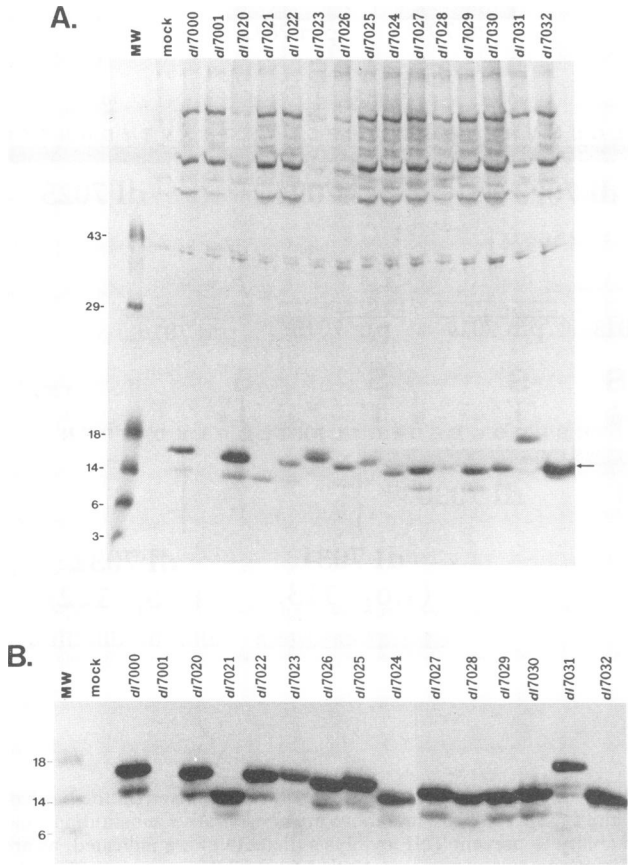

FIG. 4. Immunoprecipitation of 14.7K protein from mouse C3HA and human KB cells infected with mutant viruses. (A) C3HA mouse fibroblasts. (B) Human KB cells. MW, molecular weight standards (molecular weights, in thousands, appear at left); mock, mock-infected cells.

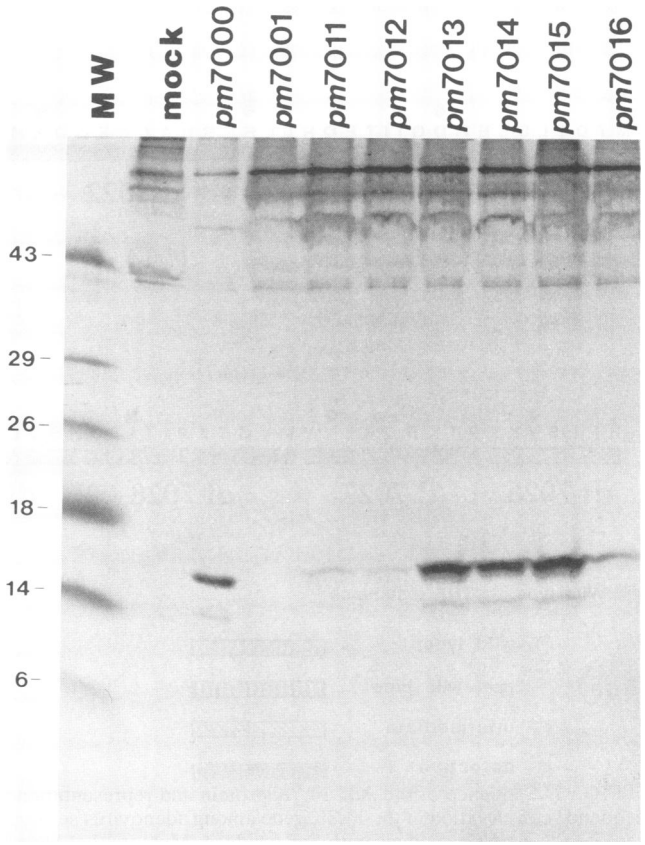

FIG. 5. Immunoprecipitation of 14.7K protein from C3HA cells infected with Cys-to-Ser mutant viruses. MW, molecular weight standards (molecular weights, in thousands, appear at left); mock, mock-infected cells.

band was seen by immunoprecipitation (Fig. 4A and B) and by Western blot (Fig. 6). As judged by its mobility, this band appears to be the upper band. Thus, the C-terminal seven amino acids which are deleted in *dl*7032 apparently are required to form the lower band. It is possible that the lower band arises from the upper band by proteolytic cleavage near the C terminus, but to date we have not detected a precursor-product relationship between the upper and lower bands in pulse-chase experiments (40). *dl*7031 deletes the C-terminal 16 amino acids in 14.7K (Fig. 3), so it should not display this putative processing at the C terminus. However, at least six 14.7K bands with aberrant mobility were obtained with *dl*7031 (Fig. 4A and B), and it is difficult to interpret this result in terms of our conclusions regarding *dl*7032.

Most mutations in the 14.7K protein abrogate the ability of 14.7K to prevent TNF cytolysis. The 14.7K mutants were tested for their ability to protect infected mouse fibroblasts from lysis by TNF in a ^{51}Cr-release assay. *dl*7000 expresses a wild-type 14.7K protein and no other E3 proteins, and it is the only adenovirus mutant of this type to be examined to date for prevention of TNF cytolysis. *dl*7000 exhibited the typical protection against TNF cytolysis (Fig. 7), which is similar to the response of wild-type adenovirus-infected cells and also uninfected cells (14–16). There was minimal lysis of *dl*7000-infected cells even with concentrations of TNF as high as 1,000 U/ml. This result confirms earlier conclusions (14–16) that 14.7K does not require other E3 proteins for it to prevent TNF cytolysis of adenovirus-infected cells. *dl*7001, which does not contain the 14.7K gene, was unable to protect cells from TNF cytolysis (Fig. 7).

Representative assays for the 14.7K deletion mutants are presented in Fig. 7. Most mutants were completely defective in their ability to protect infected cells from lysis by TNF. However, there were two mutants that partially prevented TNF cytolysis. *dl*7026, which deletes residues 66 to 72, typically gave about 50% protection against TNF cytolysis compared with the wild type and the mutants that were completely defective (Fig. 7B); therefore, we have designated *dl*7026 as having an intermediate phenotype. *dl*7032,

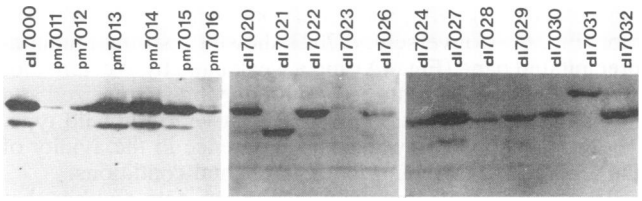

FIG. 6. Western blots of proteins extracted from C3HA cells infected with the 14.7K mutant viruses. Cells were harvested at 18 h p.i., and proteins were extracted, subjected to SDS-PAGE, and blotted. The blots were probed with a TrpE-14.7K fusion protein antibody. The second antibody was a goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase.

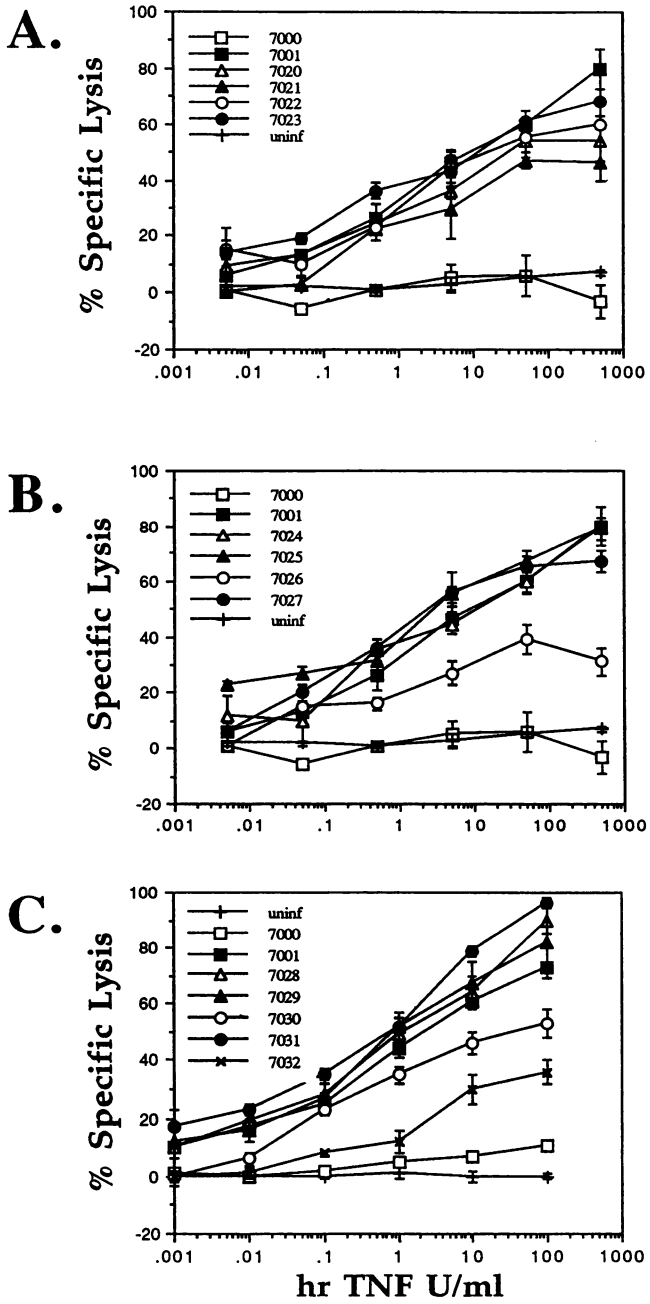

FIG. 7. Analysis of the 14.7K deletion mutants for prevention of TNF cytolysis. TNF-mediated cytolysis was measured by a ^{51}Cr-release assay. NIH 3T3 cells were infected (150 to 200 PFU per cell) with the indicated virus and labeled with $Na_2{}^{51}CrO_4$ at 24 h p.i., and human recombinant (hr) TNF was added 6 h later. Each point is the average of triplicates. Cytolysis was measured as ^{51}Cr release after an 18-h incubation.

which deletes the C-terminal seven residues, typically prevented TNF lysis nearly as well as the wild type (Fig. 7C) (the amount of cytolysis seen with *dl*7032 in Fig. 7C is somewhat higher than usual). Although *dl*7030 appeared to provide marginal protection against TNF cytolysis in the experiment shown in Fig. 7C, this protection was not observed in other experiments.

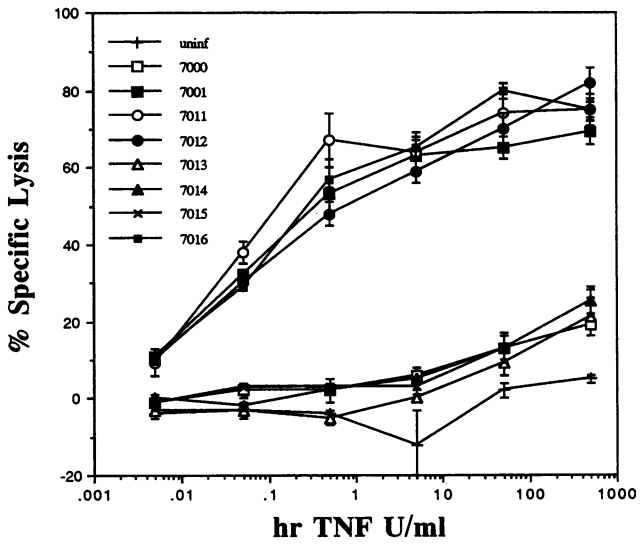

FIG. 8. Analysis of 14.7K point mutants for prevention of TNF cytolysis. TNF-mediated cytolysis of mutant virus-infected NIH 3T3 cells was measured as described for Fig. 7. hr, human recombinant.

Figure 8 shows that point mutants *pm*7011, *pm*7012, and *pm*7016 have lost the ability to prevent TNF cytolysis, whereas *pm*7013, *pm*7014, and *pm*7015 have the same abilities as the wild type. This cytolysis phenotype correlates with the 14.7K stability data from the Western analysis, i.e., mutants that make 14.7K abundantly prevent TNF cytolysis and vice versa.

The TNF cytolysis experiments shown in Fig. 7 and 8 were done with mouse NIH 3T3 cells. Similar results were found for mouse C3HA cells (data not shown). All experiments were repeated multiple times.

DISCUSSION

We have presented the results of a mutational analysis of the adenovirus E3 14.7K protein. Thirteen in-frame deletion mutants and six point mutants with Cys-to-Ser conversions were constructed and assayed for their ability to synthesize 14.7K and protect mouse cells from lysis by TNF. It was necessary to use mouse rather than human cells for these experiments because the E1B 19K protein, which is expressed by the mutants, prevents TNF cytolysis of human cells (13). An E3 function can also prevent cytolysis of human cells (13), so it is likely that the results obtained here with mouse cells are applicable to human cells.

When analyzed in human KB cells after a 30-min label, the mutants synthesized equivalent amounts of the 14.7K protein. When analyzed in mouse fibroblasts by immunoprecipitation after a 30-min label and when the steady-state level of the protein was examined by Western blot, most of the mutants synthesized stable 14.7K protein. However, all deletions (*dl*7023, *dl*7024, *dl*7025, and *dl*7026) as well as the two Cys mutants (*pm*7011 and *pm*7012) in the region encompassing residues 34 to 72 in the 14.7K protein synthesized reduced amounts of 14.7K. *dl*7028, which deletes residues 82 to 92, and *pm*7016, which alters the Cys at residue 119, also synthesized reduced quantities of 14.7K. Thus, these regions appear to be important for the stability of 14.7K in C3HA cells.

The mutants with Cys mutations at residues 100 (*pm*7013), 105 (*pm*7014), and 112 (*pm*7015) synthesized wild-type levels

of protein and were able to provide wild-type protection of cells from TNF lysis; thus, the Cys residues at these positions are not required to prevent TNF cytolysis. The three remaining mutants with Cys mutations, at residues 44 (*pm*7011), 50 (*pm*7012), and 119 (*pm*7016), which synthesized 14.7K at greatly reduced levels, were unable to protect cells from cytolysis by TNF. One interpretation of the latter results is that the lethal point mutations may have disrupted the folded structure of 14.7K such that it cannot function correctly. Analysis of wild-type 14.7K and the 14.7K Cys mutants on nonreducing gels indicated that 14.7K does not contain intermolecular disulfide bonds, but it is not yet clear whether 14.7K contains intramolecular disulfide bonds (32). Alternative explanations for the TNF-defective phenotype of *pm*7011, *pm*7012, and *pm*7016 are that the Cys residues are essential even if they do not participate in disulfide bonds or that these mutants do not synthesize sufficient quantities of 14.7K to prevent TNF cytolysis.

Most deletion mutants were unable to provide any protection of cells from TNF cytolysis. Again, for the mutants that synthesized unstable 14.7K, we do not know whether their inability to prevent TNF cytolysis is due to a loss of function for the protein or to insufficient amounts of the protein. On the other hand, for the other deletion mutants that synthesize approximately wild-type levels of 14.7K, we can conclude that residues 2 to 33, 73 to 81, and 93 to 121 appear to be essential for preventing TNF cytolysis, because deletions in these regions abrogate protection against TNF cytolysis. Both *dl*7026, which deletes residues 66 to 72 in 14.7K, and *dl*7032, which deletes the C-terminal seven residues in 14.7K, provided significant but not wild-type levels of protection against TNF cytolysis. Thus, residues 66 to 72 and 122 to 128 are not essential for prevention of TNF cytolysis. We note that *dl*7026 underproduces 14.7K, so this could explain why *dl*7026 has an intermediate phenotype.

We interpret these results to imply that the overall tertiary structure of the protein and not a specific domain(s) is required for prevention of TNF cytolysis. However, further analyses of the properties of 14.7K and how these mutations affect these properties are required before the phenotypes of these mutants can be fully understood. Immunofluorescence studies indicate that none of the mutations have a remarkable effect on the ability of 14.7K to localize to the nucleus and to the cytoplasm.

The 14.7K protein expressed from *dl*7000 and most of the mutants migrates as two bands on SDS-PAGE. However, only one band, very likely the upper band, was observed with *dl*7032, which lacks seven amino acids at the C terminus. This suggests that the lower band arises from the upper band by proteolytic cleavage near the C terminus. If so, this is not essential for prevention of TNF cytolysis, because *dl*7032 is nearly wild type for that property.

*dl*7001 and Ad5 virus stocks prepared in KB cells had titers as high as that of Ad5 on A549 cells. This is of interest because *dl*7001 has the largest E3 deletion of any mutant constructed to date and it is the only mutant that does not synthesize any E3 proteins (there are no ATGs in the remaining E3 sequences). All other E3 mutants synthesize at least one of the seven known E3 proteins, and it remained formally possible that some of the E3 proteins had redundant functions that permitted deletion mutants to replicate in culture cells; *dl*7001 proves that this is not the case.

There are 164 bp between the site of 3′ end formation at nt 3308 for the E3B mRNAs and the 3′ splice site at nt 3473 for fiber mRNA (4, 5). It is to be expected that the region from nt 3308 to at most about nt 3350 would be essential for 3′ end formation at the E3B site (31) and that the sequences from about nt 3435 to nt 3473 would be essential for splicing of fiber mRNA (20). However, there is no known function for the remaining sequences in the E3-fiber intergenic region. The deletion in *dl*7001 extends to nt 3381 (the *Xho*I site in Fig. 1B), and *dl*7001 has three point mutations at nt 3423, 3424, and 3425 (the *Eco*RI site in Fig. 1B); thus, the intergenic sequences deleted and mutated in *dl*7001 are not required for adenovirus multiplication in cultured cells.

*dl*7001 also deletes the polyadenylation signal for the L4 family of late mRNAs which is located at ca. nt 597 to 640 in region E3 (5). Since *dl*7001 lacks the E3 polyadenylation signals, the 3′ ends of the L4 mRNAs must form at the fiber site or at a cryptic site.

Since E3 is nonessential for replication in cultured cells (e.g., this paper) or in hamsters (27) or cotton rats (10), adenovirus is being used as an expression vector with foreign genes inserted into E3 (reviewed in references 2 and 18). One requirement for such vectors is that the E3 deletion be large so that large foreign genes can be inserted without exceeding the virion packaging limitation. The *dl*7000/*dl*7001 format should be suitable for the expression of foreign genes because the E3 deletion is large and 14.7K is expressed quite well. However, it might be advisable to introduce a spliceable intron into these constructs, as this might increase the amount of mRNA transported to the cytoplasm.

It should be noted that the TNF cytolysis results described in the introduction and presented here were obtained in cultured cells. Human Ad5 has been shown to infect bronchiolar epithelial cells in a cotton rat model (30) and to elicit the synthesis of TNF in a mouse model (11). Although the epithelial cells in the cotton rat are damaged during infection when TNF is presumably synthesized, cell lysis has not been observed (30). Therefore, it has not been shown that TNF lyses adenovirus-infected cells in animals or that the adenovirus 14.7K, 10.4K-14.5K, and E1B 19K proteins prevent TNF cytolysis in animals. Nevertheless, the results from studies with cultured cells are reproducible and dramatic and are likely to be relevant to adenovirus infection in humans.

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