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Induction of Susceptibility to Tumor Necrosis Factor by E1A Is Dependent on Binding to either p300 or p105-Rb and Induction of DNA Synthesis

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The introduction of the adenovirus early region 1A (E1A) gene products into normal cells sensitizes these cells to the cytotoxic effects of tumor necrosis factor (TNF). Previous studies have shown that the region of E1A responsible for susceptibility is CR1, a conserved region within E1A which binds the cellular proteins p300 and p105-Rb at nonoverlapping sites. Binding of these and other cellular proteins by E1A results in the induction of E1A-associated activities such as transformation, immortalization, DNA synthesis, and apoptosis. To investigate the mechanism by which E1A induces susceptibility to TNF, the NIH 3T3 mouse fibroblast cell line was infected with viruses containing mutations within E1A which abrogate binding of some or all of the cellular proteins to E1A. The results show that TNF susceptibility is induced by E1A binding to either p300 or p105-Rb. E1A mutants that bind neither p300 nor p105-Rb do not induce susceptibility to TNF. Experiments with stable cell lines created by transfection with either wild-type or mutant E1A lead to these same conclusions. In addition, a correlation between induction of DNA synthesis and induction of TNF sensitivity is seen. Only viruses which induce DNA synthesis can induce TNF sensitivity. Those viruses which do not induce DNA synthesis also do not induce TNF sensitivity. These data suggest that the mechanisms underlying induction of susceptibility to TNF by E1A are intimately connected to E1A's capacity to override cell cycle controls.

Tumor necrosis factor (TNF) is a multifunctional cytokine which is part of the innate host response (83). It is produced by activated macrophages in response to inflammatory agents (83), and, in appropriate and regulated levels, it acts as a protective agent of the immune system, fighting against viral, bacterial, and parasitic infections (26, 68). Counterproductively, increased or deregulated production of TNF can cause damage to the host and induce pathology in localized areas of infection or in systemic reactions (8, 82).

The effects of TNF can vary widely in response to a virus infection. TNF production is triggered during the course of infection (25, 30), and TNF can then aid the host directly by halting DNA and RNA virus replication (57, 85, 94) or indirectly by upregulating other immune cells (70). In addition, TNF can induce direct lysis of virus-infected cells (32, 34). Conversely, in some systems TNF can aid the virus in setting up an infection in the host by promoting virus replication (35).

The numerous strategies which viruses have evolved to avoid or combat the effects of TNF further indicate the central role of TNF as an antivirus defense. For example, poxviruses produce secreted proteins with similarity to the TNF receptor, thus binding to TNF and preventing signal transduction which may occur when TNF binds to the cellular TNF receptors on an infected cell (72). Adenovirus counters the cytolytic effects of TNF by use of the E3-encoded gene product of 14,700 Da (33) and the E3-encoded heterodimer of 10,400 Da-14,500 Da (34). These viral gene products act intracellularly to block the cytolytic effects of TNF on adenovirus-infected cells (reviewed in reference 34). Recent data suggest that these proteins prevent the activation of cytoplasmic phospholipase A_2 (49, 54), an essential step in TNF-induced killing (39).

The adenovirus gene responsible for the susceptibility of infected cells to TNF is E1A (18). Susceptibility is seen when E1A is expressed during acute infection (18) or by stable transfection (14). By using large deletions within E1A, the CR1 region was found to be necessary for induction of TNF susceptibility (2, 19). This region, from residues 40 to 80, is conserved among all serotypes of adenoviruses. E1A functions associated with the CR1 region include cellular transformation in cooperation with ras (69, 81), induction of mitosis (11, 96), induction of DNA synthesis (47, 87), apoptosis (63, 66, 91), suppression of differentiation (40, 51, 88), immortalization (7, 12, 45), and regulation of gene expression (for reviews, see reference 6 and references therein). E1A mediates these pleiotropic effects on the virus and host cell by binding to cellular proteins (6). Of the eight cellular proteins determined so far to bind to E1A, the best characterized are p300 (3, 22) and p105-Rb (4, 92). p300 and p105-Rb bind to unique, noncompeting sites within CR1 (5, 20, 23, 87, 93). In this study, finer E1A point and deletion mutant viruses and plasmids with defined cellular protein binding characteristics were employed to determine the relationship between E1A binding to these proteins and E1A induction of susceptibility to TNF cytolysis.

MATERIALS AND METHODS

Materials. Human recombinant TNF was purchased from Genzyme, Framingham, Mass. $Na^{51}CrO_4$ (1,000 Ci/g) was obtained from New England Nuclear/DuPont, Boston, Mass. The enhanced chemiluminescence Western blotting (immunoblotting) detection system was purchased from Amersham Corporation, Arlington Heights, Ill.

Cells and viruses. The NIH 3T3 cell line was derived from Swiss mouse

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embryo fibroblasts and was obtained from the American Type Cell Culture (Rockville, Md.) and maintained in Dulbecco modified Eagle medium (Gibco BRL, Grand Island, N.Y.) with 5% glucose supplemented with 10% fetal calf serum (HyClone, Logan, Utah).

The adenovirus *dl*309 (50) was obtained from T. Shenk, Princeton University, Princeton, N.J. *dl*309 is an adenovirus type 5 (Ad5) mutant that lacks the E3B region, including the genes for the 14,700-, 14,500-, and 10,400-Da proteins, but retains the wild-type E1A region. Mutants *dl*1101, *dl*1102, *dl*1103, *dl*1104, *dl*1105, *dl*1107, and *dl*1108 (48) and *dl*1143/520, 01/06/520, 01/07/520, 01/08/520, 02/06/520, 02/07/520, 02/08/520, 05/07/520, and 43/08/520 (47) produce 12S E1A mutants. These mutants are in the Ad5 *dl*309 background, *dl*520 (36). All of them were obtained from S. T. Bayley, McMaster University, Hamilton, Ontario, Canada. Virus mutants 12S.928 (60) and 12S.RG2, 12S.RG2/928, 12S.YH47, and 12S.YH47/928 (87) are also in the Ad5 *dl*309 background, express only 12S E1A, and were obtained from E. Moran, Temple University, Philadelphia, Pa. All of the above-described mutants were grown in monolayers of human 293 cells as described previously (18). Briefly, nearly confluent monolayers in 150-cm^3 plates were infected with 1.0 ml of virus in a 37°C incubator. After 2 days, when extensive cytopathic effects were apparent, cells were scraped into the medium, collected by centrifugation, resuspended in 5 ml of medium plus 10% fetal calf serum, freeze-thawed three times, sonicated, and centrifuged at 1,000 rpm for 10 min (centrifuge from Beckman, Arlington Heights, Ill.). The supernatants were frozen and stored at −70°C. The titers of all viruses were determined by plaque assay on 293 or A549 cells.

Plasmids. p12S.928 (60), p12S.RG2, p12S.RG2/928, p12S.YH47, and p12S.YH47/928 (87), PSdl (58), and CXdl (60) were obtained from E. Moran. These plasmids make the 12S gene product of E1A, except for PSdl and CXdl, which make the 13S gene product. pE1A (79) was obtained from G. Chinnaduria and makes both the 12S and 13S gene products. All stable transfectants were also transfected with pSV2-neo (74) and T24 ras (71).

Transfections. The wild-type and mutant E1A plasmids were cotransfected with both pSV2-neo and T24 ras plasmids into NIH 3T3 cells. Control cells were transfected with pSV2-neo and T24 ras alone. Lipofectin (BRL Life Technologies, Inc., Gaithersburg, Md.) was used as directed by the manufacturer to make permanent cell lines. Stable transfectants were selected with G418 (8 mg/liter) (Gibco BRL) for neomycin resistance.

Cytotoxicity assays. The procedure used for assaying the susceptibility of cells to TNF cytolysis has been described previously (19). Briefly, cells were plated at a density of 8.5×10^5/60-mm-diameter plate. For the infection assays, cells were infected with 100 to 300 PFU of the appropriate virus per cell after the cells had adhered to the plates, and the infection proceeded for 22 h prior to harvest. Both infected and transfected cells were labeled by adding 200 μl of $Na^{51}CrO_4$ (200 μCi) to each 60-mm-diameter plate at least 8 h prior to use. Infected or transfected cells were harvested, washed, and added to wells of standard 96-well microtiter plates (10^4 cells per well in a total volume of 200 μl) containing either medium alone (to determine spontaneous release) or the designated concentration of TNF. After 18 h of incubation at 37°C in 8% CO_2, 2 N HCl was added to some triplicate wells to determine the maximum releasable counts, and the plates were centrifuged at $180 \times g$ for 10 min. A 100-μl portion of supernatant was removed from each well, and the radioactivity was measured. The percent specific ^{51}Cr release was determined from the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. All determinations were done in triplicate. In infection experiments, immunofluorescence staining for adenovirus proteins was used to determine the extent of infection. Infected cells were plated onto glass plates, fixed, and incubated with Ad5 virion antiserum (American Type Culture Collection). The cells were washed and then treated with fluorescein-conjugated goat anti-rabbit 7S globulins (Cedarlane, Hornby, Ontario, Canada) to determine infection rates. In all experiments reported here, >90% of cells were infected.

Western blots. Cell lines were grown in 100-mm-diameter plates to confluency. Cells were removed from the plate and transferred to tubes containing phosphate-buffered saline (PBS). The cells were centrifuged, and the PBS was removed. Two hundred microliters of lysis buffer (1% Nonidet P-40, 20 mM Tris chloride [pH 8.0], 150 mM NaCl, 1% aprotinin [Sigma, St. Louis, Mo.]) was added to each sample. The lysates were vortexed, incubated for 20 min on ice, sonicated for 3 min, and microcentrifuged for 10 min. The supernatants were removed and stored at −70°C until used.

Protein concentrations were determined for each lysate by the bicinchoninic acid method (73). Equal amounts (20 μg) of each lysate were loaded onto lanes of a sodium dodecyl sulfate–12% polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose with a semidry blotter (Bio-Rad Laboratories, Richmond, Calif.).

The blotted nitrocellulose was blocked overnight in TBS (20 mM Tris chloride [pH 7.5], 500 mM NaCl) plus 5% nonfat milk. The blot was washed three times for 15 min each in TTBS (TBS plus 0.1% Tween 20). Monoclonal anti-E1A M73 antibody (38, 78) diluted with TTBS (200 μl of antibody per 10 ml of TTBS) was added to the nitrocellulose and incubated for 1 h at room temperature with rocking. The M73 antibody binds to exon 2 of E1A and recognizes each of the E1A constructs used in this study (78). The nitrocellulose was washed three times for 15 min each with fresh TTBS and incubated with horseradish peroxidase-linked sheep anti-mouse immunoglobulin (Amersham) in TTBS (20 μl in 12 ml) for 1 h with rocking. The nitrocellulose was washed once for 15 min and then four times for 5 min each in TTBS. The enhanced chemiluminescence procedure (Amersham) was used to detect E1A proteins for Western blotting. Blots were developed according to the protocol provided by Amersham. Briefly, TTBS was drained off the nitrocellulose, and reagents A and B (1:1) from the enhanced chemiluminescence kit were added to the nitrocellulose and incubated for 1 min. Excess reagent was drained, and the blot was covered with plastic wrap and was immediately exposed to X-ray film for 30 s to 3 min. The film was developed directly after the exposure period.

Dye exclusion viability assay. Cells were plated in duplicate at 10^4 per 30-mm-diameter plate. After incubation for 4 to 8 h, TNF was added to each 30-mm-diameter plate for a final concentration of 500 U/ml. Cells were incubated with TNF at 37°C in 8% CO_2 for various times. For each cell line, the cell culture medium and trypsinized cells were collected, pooled, and stained with 0.25% nigrosin. Viable and dead cells were counted with a hemocytometer. Results are expressed as percent viability, calculated as [number of live cells/(number of live cells + number of dead cells) × 100].

RESULTS

Minimal binding of p300 or p105-Rb to E1A is required for susceptibility to TNF cytolysis. Figure 1 shows the E1A deletion and point mutations that were used in the work described in this paper. NIH 3T3 is a mouse fibroblast cell line that is normally resistant to TNF cytolysis (34). If infected with *dl*309, an adenovirus which expresses both 13S and 12S E1A, the infected cells become sensitive to TNF (18). Expression of either 13S or 12S alone was sufficient to induce susceptibility (18). The parent viruses from which mutants in this study were derived, *dl*520 or 12SWT, produce only 12S E1A. All viruses were initially derived from *dl*309, an Ad5 virus that lacks the E3B region proteins that would otherwise suppress TNF sensitivity.

Previous work with acute virus infection of NIH 3T3 cells showed that residues within CR1 are responsible for induction of TNF sensitivity (19). Previous studies have shown that this region of E1A binds both p300 and p105-Rb in unique and nonoverlapping sites, although there are some discrepancies in the current literature as to exactly which residues are involved (discussed in reference 6). In addition, p300 binds to a second site in E1A within residues 1 to 25 (5, 23, 75, 87), and p105-Rb binds to a second site within CR2 residues 121 to 127 (20, 21). With either protein, the removal of only one binding site is sufficient to abrogate or greatly reduce binding of that cellular protein to E1A. NIH 3T3 cells were first infected with smaller deletion mutants that more precisely remove binding to both p300 and p105-Rb simultaneously. The *dl*1143/520 mutation (47) deletes residues 38 to 60, abrogating p300 binding and greatly reducing p105-Rb binding. Cells infected with this virus are resistant to TNF, indicating that residues 38 to 60 are required for susceptibility (Fig. 2). 43/08/520 (47) is the *dl*1143/520 construct with an additional 4-amino-acid deletion of residues 124 to 127 in CR2, thus deleting one p300 binding site and both p105-Rb binding sites (47). Cells infected with this virus are also resistant to TNF cytolysis (Fig. 3). The 01/07/520 mutation (47) deletes residues 4 to 25 and 111 to 123, removing the N-terminal binding site for p300 and the CR2 binding site for p105-Rb. This construct, which does not bind either p300 or p105-Rb, is resistant to TNF (Fig. 3). 01/08/520 (47) has an N-terminal deletion identical to that of 01/07/520 but has a different CR2 deletion of residues 124 to 127. As with 01/07/520, both p300 binding and p105-Rb binding are abrogated, and 01/08/520-infected cells are resistant to TNF cytolysis (Fig. 3). These data illustrate that either a large CR1 deletion, as seen with *dl*1143/520, or a combination of two smaller N-terminal and CR2 deletions, as seen with 01/07/520 and 01/08/520, causes the same cellular protein binding patterns and TNF phenotype. In addition to the deletion mutations described above, a point mutant construct, 12S.RG2/928, with substitutions of a glycine for arginine at amino acid 2 and a glycine for

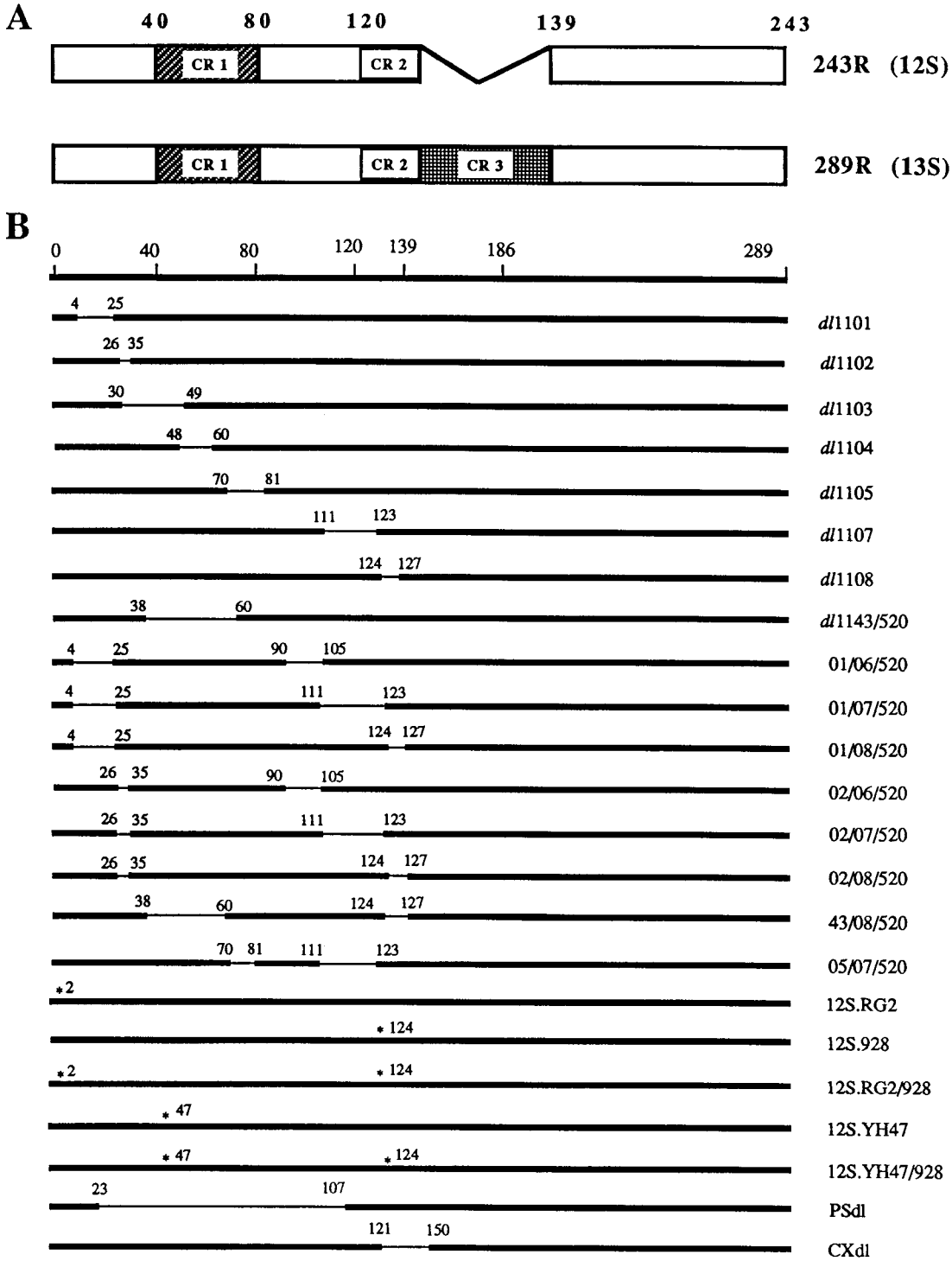

FIG. 1. Structures of adenovirus E1A mutations. (A) Schematic map of the 243R and 289R protein products of the E1A gene. (B) Protein-coding regions of the E1A gene of the *dl*309 Ad5 construct. The deletion and point mutation virus and plasmid constructs used in the work described in this paper are shown. The thin lines represent sequences deleted from the mutants. The E1A amino acids which constitute the deleted region are identified by the amino acid numbers above each construct. All constructs, with the exception of *dl*309, express 12S E1A only.

cysteine at amino acid 124 (87), was assayed. This construct loses p300 and p105-Rb binding, and infected cells are resistant to TNF cytolysis (Fig. 4). Because this particular mutant abrogates p300 and p105-Rb binding through single-amino-acid point mutations, the possibility of other, as yet unidentified, E1A-binding proteins being responsible for induction of TNF sensitivity is virtually eliminated. Thus, these results confirm our previous finding that deletion of both p300 and p105-Rb binding sites eliminates induction of TNF sensitivity (19).

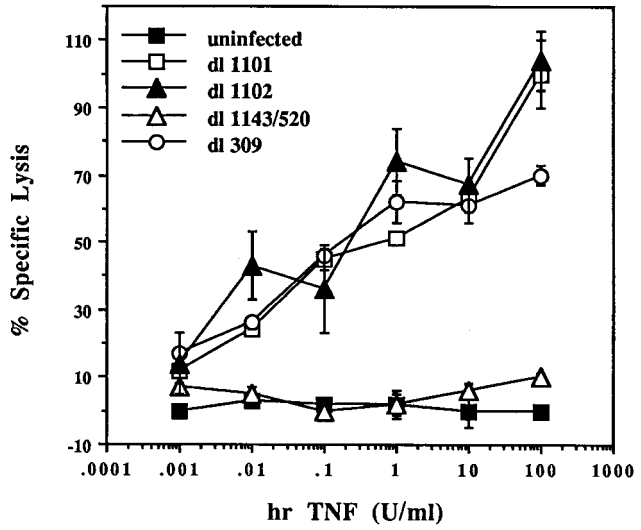

FIG. 2. Analysis of N-terminal E1A mutant proteins which abrogate binding of p300 and induce susceptibility to TNF cytolysis in acute virus infections. hr, human recombinant.

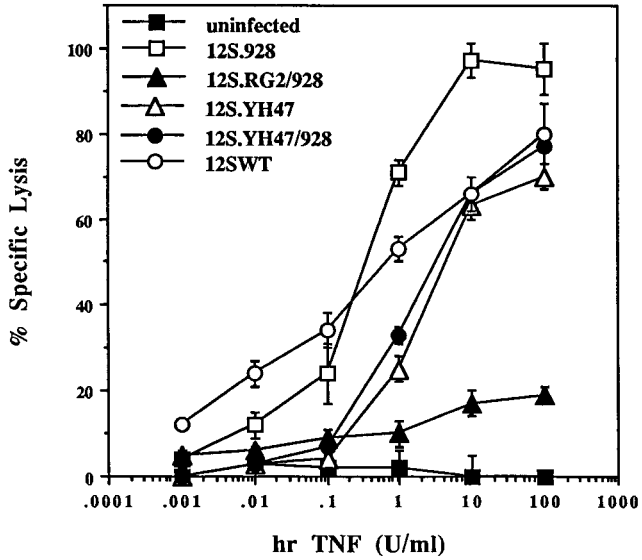

FIG. 4. Analysis of point mutant E1A proteins inducing susceptibility to TNF cytolysis in acute virus infections. hr, human recombinant.

The next set of experiments tested the TNF sensitivity of cells infected with E1A mutants that bind p105-Rb but not p300. NIH 3T3 cells were infected with *dl*1101 (48), whose mutation deletes the N-terminal p300 binding site while retaining p105-Rb binding. Cells infected with this virus are sensitive to TNF (Fig. 2). *dl*1104 (48), whose p300 CR1 binding site has a large deletion of amino acids 48 to 60, also induces susceptibility to TNF cytolysis (Fig. 5). The 01/06/520 mutation (47), which deletes both p300 binding sites while retaining p105-Rb binding, also induces TNF sensitivity (Fig. 6). The *dl*1103 mutation (48), which decreases but does not completely abrogate p300 binding through the deletion of residues 30 to 49 (5), induces susceptibility to TNF (Fig. 5), indicating that the relative levels of p300 bound to E1A are irrelevant when p105-Rb is bound by E1A at wild-type levels. Together, these data suggest that retention of p105-Rb binding and abrogation of p300 binding, through deletion of either one or both p300 binding sites, results in sensitivity to TNF cytolysis.

Next, NIH 3T3 cells were infected with viruses that bind p300 but not p105-Rb. The 02/07/520 mutation (47) deletes residues 26 to 35 and 111 to 123, abrogating p105-Rb binding at two sites while retaining p300 binding. 02/08/520 (47) is identical to 02/07/520 except that it has the smaller, 4-amino-acid deletion in CR2. Infection of NIH 3T3 cells with either virus induces sensitivity to TNF (Fig. 6), indicating that abrogation of p105-Rb binding alone does not alter TNF sensitivity. 05/07/520 (47), with a different CR1 deletion but the same CR2 deletion as 02/07/520, also abrogates p105-Rb binding while retaining p300 binding. It too induces sensitivity to TNF cytolysis (Fig. 6). Deletion of either CR2 p105-Rb binding site, e.g., as in *dl*1107 (48), which lacks amino acids 111 to 123, or *dl*1108

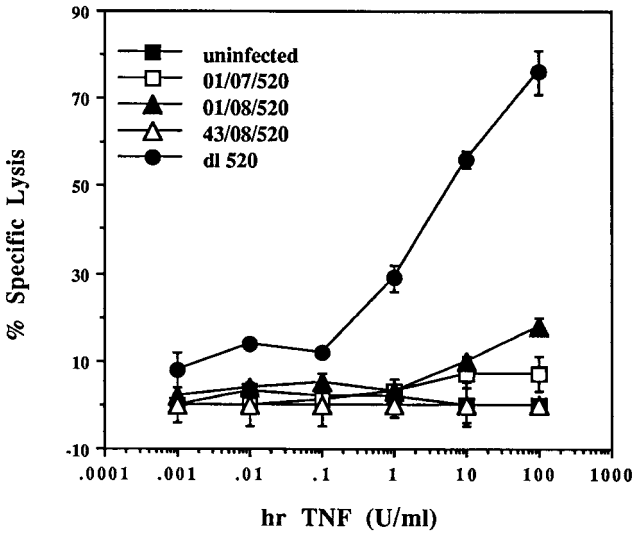

FIG. 3. Analysis of mutant E1A proteins which lack N-terminal and CR2 residues. These protein products do not bind p300 and p105-Rb, and infected cells are resistant to TNF cytolysis. hr, human recombinant.

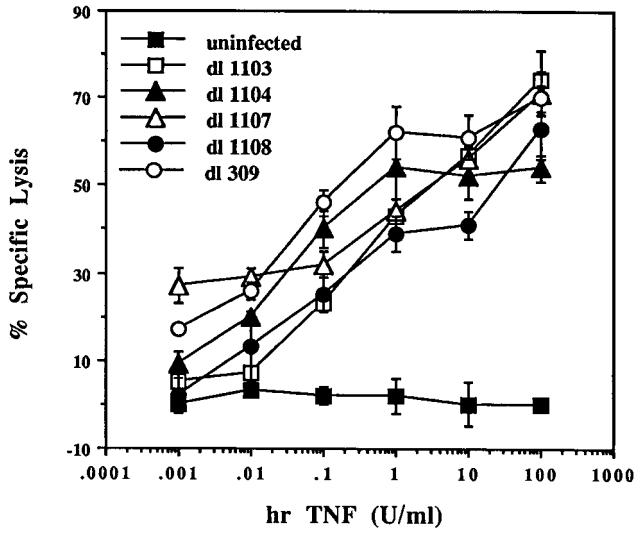

FIG. 5. Analysis of mutant E1A proteins which lack either N-terminal or CR2 residues and induce sensitivity to TNF cytolysis. hr, human recombinant.

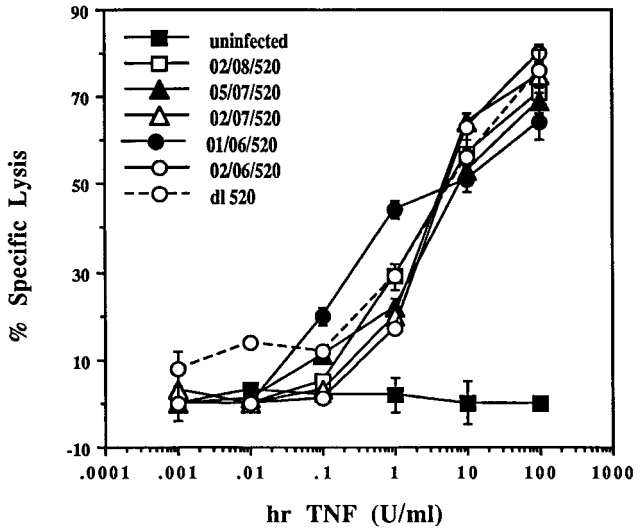

FIG. 6. Analysis of mutant E1A proteins inducing susceptibility to TNF cytolysis in acute virus infections. hr, human recombinant.

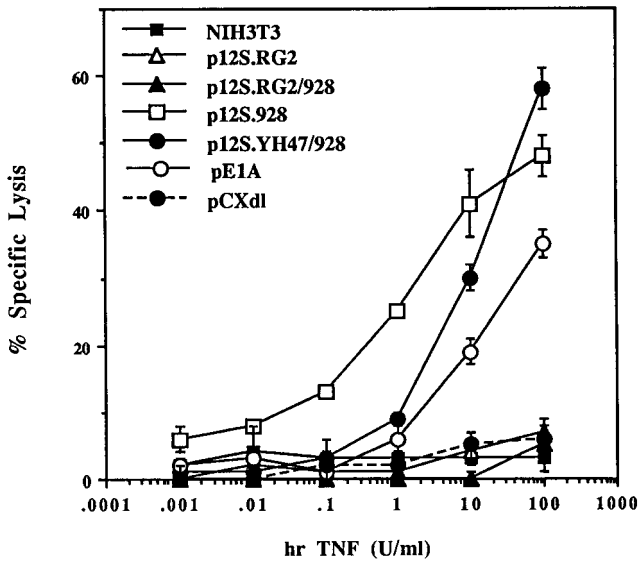

FIG. 7. Stable transfection of mutant E1A plasmids in NIH 3T3 cells shows that susceptibility to TNF is dependent upon the ability of E1A to bind to either p300 or p105-Rb. hr, human recombinant.

(48), which lacks residues 124 to 127, is sufficient to prevent p105-Rb binding but does not eliminate TNF sensitivity (Fig. 5).

Single-point-mutant viruses were also assayed for TNF sensitivity. The 12S.928 mutation changes residue 124 from cysteine to glycine, abrogating binding of p105-Rb at CR2 but retaining p300 binding (60). The 12S.YH47 mutation changes amino acid 47 from tyrosine to histidine, abrogating p105-Rb binding at CR1 while retaining p300 binding. 12S.YH47/928 contains substitutions at both residues 124 and 47 while still retaining p300 binding. All three viruses induce sensitivity to TNF cytolysis (Fig. 4). These results show again that mutation of only one p105-Rb binding site, as with 12S.YH47 and 12S.928, also fails to alter the sensitive phenotype of the infected cell.

Sensitivity to TNF occurs with either transient or stable E1A expression. The results described above were derived from acute virus infections. E1A also induces TNF sensitivity in cell lines that express the protein constitutively (2, 19). NIH 3T3 cells were stably transfected with the point mutant plasmids p12S.RG2/928, p12S.928, p12S.YH47, p12S.YH47/928, and p12S.RG2 (87) and the wild-type E1A plasmid, pE1A (79), and stable cell lines were produced. All negative controls were transfected with neo and ras. Cells transfected with wild-type E1A are susceptible to TNF cytolysis (Fig. 7) (18). The p12S.RG2/928 mutant, which does not bind either p300 or p105-Rb, causes resistance to TNF when expressed from a plasmid (Fig. 7), as was seen with acute infection (Fig. 4). The p12S.928, p12S.YH47, and p12S.YH47/928 constructs, all of which bind only p300, similarly render cells sensitive to TNF in stable transfections (Fig. 7), as well as in infections (Fig. 4).

Analysis of transfectants revealed two mutants whose behavior contradicts that seen in acute infection. p12S.RG2 (87) and CXdl (60) cell lines are resistant to TNF cytolysis (Fig. 7). p12S.RG2 has an arginine-to-glycine mutation at residue 2 and does not bind p300, but it still binds p105-Rb. CXdl has a deletion of amino acids 121 to 150 and does not bind p105-Rb but retains binding to p300. All of the stable transfectants produce roughly equal amounts of E1A as measured by Western blotting (Fig. 8), eliminating the possibility that TNF resistance is due to the expression of low levels of E1A.

Sensitivity to TNF cytolysis occurs at later time points with some mutants. Other studies with both CXdl and RG2 mutants have revealed a delayed time course of functioning compared with those of other mutants and the parent virus (86, 87). This prolonged time course of activity may explain the apparent paradoxical behavior of these mutants in induction of TNF sensitivity. The ^{51}Cr release assay used here has a practical limitation of 24 h because spontaneous release of the isotope exceeds 50% after this time. To determine TNF susceptibilities after longer incubation periods, dye uptake viability assays with nigrosin as a vital dye were done at various time points with the stable cell lines. The results of coincubation of E1A transfectants with TNF for 60 h are shown in Fig. 9. Control transfectants resist TNF cytolysis for up to 60 h, while transfectants expressing wild-type E1A are susceptible (Fig. 9). The p12S.RG2 and CXdl cell lines, which are not sensitive to TNF after 18 h of incubation, do become sensitive in a 60-h incubation (Fig. 9). In contrast, PSdl transfectants, which lack both p105-Rb and p300 binding sites, remain resistant at 60 h.

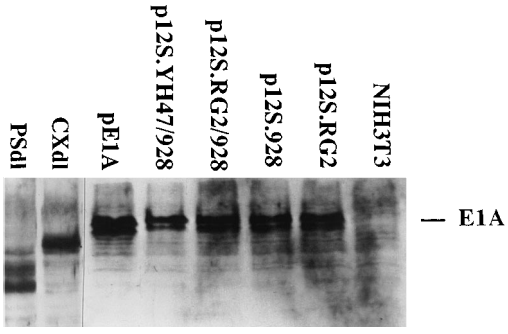

FIG. 8. Western blot analysis with the M73 antibody against proteins produced in cells stably transfected with different E1A plasmids. Lysates were prepared from transfected cells as described in Materials and Methods, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, blotted with anti-E1A and anti-mouse horseradish peroxidase-conjugated antibody, and exposed to X-ray film.

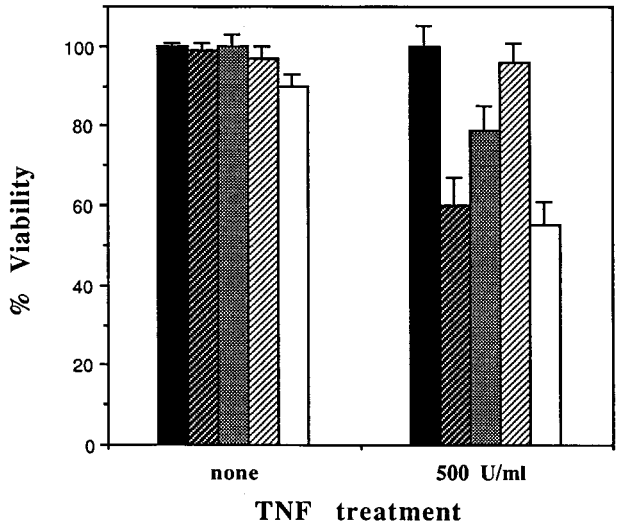

FIG. 9. Sensitivity of transfected NIH 3T3 cells to TNF cytolysis at longer incubation periods. TNF cytotoxicity was assayed by staining cells with nigrosin dye as described in Materials and Methods. Cells were either untreated or treated with 500 U of TNF per ml for 60 h. Symbols: ■, NIH 3T3; ▨, pE1A; ▩, CXdl; ▨, PSdl; □, p12S.RG2.

DISCUSSION

E1A exerts its pleiotropic effects by binding to cellular proteins, some of which are thought to be involved in the regulation of the cell cycle. Of the cellular proteins known to bind E1A, p105-Rb is the best characterized. p105-Rb binds to E1A via residues located within both CR1 and CR2 (20). p105-Rb is a known tumor suppressor gene product (for review articles on p105-Rb, see references 67 and 89). It prevents the progression of G_1 to S phase by binding to and repressing the E2F transcription factor (37, 42, 90). As a result, cellular genes involved in growth control and DNA replication (65) that contain E2F target sequences are not transcribed. In cells expressing the E1A gene product, p105-Rb is bound by E1A (4) and phosphorylated through E1A-mediated induction of $p34^{cdc2}$ (86), allowing E2F to act as a transcription factor. Once E2F activity is restored, the S phase competence of the host is enhanced through transcriptional upregulation. As a result, cells expressing E1A are forced out of G_1 and enter S phase (43).

p300 is thought to control the same part of the cell cycle as p105-Rb, albeit through different mechanisms. p300 has been proposed to be a regulator of transcription (22) given that (i) it associates with the TATA-binding protein (1), (ii) it contains a bromodomain (22) characteristic of some transcriptional activators, and (iii) it is homologous to CBP (3), a transcriptional coactivator which may deliver cyclic AMP-dependent cell arrest and differentiation signals (3). Thus, p300 may prevent progression of G_0/G_1 to S phase by activating transcription of differentiation genes. Expression of E1A in normal cells may result in the inhibition of differentiation genes or may abrogate arrest controlled by p300, causing cells to exit from G_0/G_1 into S phase (59). Additionally, p300 may drive cells into S phase by inducing synthesis of $p34^{cdc2}$ kinase (17, 86), thus phosphorylating p105-Rb.

The results reported here show that cellular sensitivity to TNF lies in the ability of E1A to bind either p300 or p105-Rb. p105-Rb binding alone induces TNF sensitivity, as seen with infections with *dl*1101, *dl*1104, and 01/06/520. Binding of p300 alone can induce TNF sensitivity, as seen with *dl*1107, *dl*1108, 02/07/520, 05/07/520, 02/08/520, 12S.928, 12S.YH47, and 12S.YH47/928 virus infections. Simultaneous abrogation of p300 and p105-Rb binding to E1A, as seen with *dl*1143/520, 43/08/520, 01/07/520, 01/08/520, and 12S.RG2/928, results in resistance to TNF cytolysis. These data are summarized in Table 1. The mutant E1A virus and plasmid constructs used in these studies have been previously characterized by others for their abilities to bind cellular proteins in human cells (5, 23, 87, 95). In addition, the *dl*1101 to *dl*1108 and 01/06/520 to 05/07/520 series have been analyzed for binding of cellular proteins in rat and mouse cells (61–63), and we have confirmed the binding patterns of some of these mutants in the murine NIH 3T3 cell line (unpublished data).

Other cellular proteins known to bind E1A do not affect TNF sensitivity. Mutants with identical p300 and p105-Rb binding characteristics but differing in p107 binding were compared. For example, 01/07/520 does not bind either p300 or p105-Rb but does retain p107 binding, while 01/08/520 does not bind p300, p105-Rb, or p107 (Table 1). Yet, cells infected with either mutant are resistant to TNF (Fig. 3). Conversely, mutant 12S.YH47 binds p300 and p107, while mutant 12S.YH47/928 binds only p300 (Table 1), yet cells infected with either virus are susceptible to TNF cytolysis (Fig. 4). This indicates that binding of p107, a cellular protein similar to p105-Rb (24), does not interfere with or enhance the induction of susceptibility to TNF cytolysis. p400 (6) is known to bind to the N-terminal region of E1A. 02/06/520 binds p300 and p105-Rb but not p400 and induces sensitivity to TNF cytolysis (Table 1 and Fig. 6). 02/07/520 binds p300 but not p400 (Table 1), and 05/07/520 binds both p300 and p400 (Table 1). Both mutants induce sensitivity to TNF in infected cells, indicating that p400 is not necessary for TNF cytolysis (Fig. 6). p130 is similar to p105-Rb in that it binds to E1A in both CR1 and CR2 (21). At present, binding sites for p105-Rb and p130 are indistinguishable; therefore, we cannot exclude a role for p130, a cellular protein similar to p107 (31, 56), in TNF sensitivity. Both TATA-binding protein (29, 55), which binds CR3, and CtBP (10), which binds to the C terminus of exon 2, can be eliminated as binding proteins involved in TNF susceptibility because the residues of E1A involved in binding each of these cellular proteins were shown previously not to be involved in the induction of TNF sensitivity (19).

An examination of the results reported here shows a strong correlation between the abilities of E1A mutants to induce TNF sensitivity and the previously reported capacities of these mutants to induce DNA synthesis in nondividing cells (47, 86, 87). Cells infected with viruses which do not induce DNA synthesis are resistant to TNF cytolysis (Table 1). Those infected with viruses which induce DNA synthesis are sensitive to TNF cytolysis. The correlation between induction of DNA synthesis and sensitivity to TNF cytolysis is seen in cells expressing E1A plasmid constructs as well. The NIH 3T3 cells used here for infection and transfection assays are transformed and are thus continuously cycling and synthesizing DNA. Yet, these cells are resistant to TNF, indicating that it is not DNA synthesis itself which renders infected cells susceptible to TNF. These data suggest that the underlying mechanisms which drive quiescent cells into S phase are the same or similar ones which induce susceptibility to TNF cytolysis, on the basis of the mutational analyses performed here.

Previous studies with the 12S.RG2 and CXdl viruses have revealed that these two viruses induce DNA synthesis at a much lower rate than, and with a delayed time course compared with, their parent construct (86, 87). A delay in the induction of TNF sensitivity is seen as well (Fig. 9). RG2 is a point mutant virus (87) which induces DNA synthesis at least 4 h later than the 12S wild-type construct and reaches wild-type

TABLE 1. Representative viruses and their characteristics

Virus	Induction of DNA synthesis (reference)	Sensitivity of infected cells to TNF cytolysis[a]	E1A binding to (reference)[b]:			
			p400	p300	p105-Rb	p107
*dl*309	100% (95)	S	+ (95)	+ (95)	+ (95)	+ (95)
*dl*520	100% (47)	S	+ (47)	+ (47)	+ (47)	+ (47)
*dl*1143	38% (47)	R	− (63)	− (63)	↓ (63)	↓ (63)
*dl*1101	65% (47)	S	− (63)	− (63)	+ (63)	+ (63)
*dl*1102	90% (47)	S	− (63)	+ (63)	+ (63)	+ (63)
*dl*1103	80% (47)	S	− (63)	↓ (63)	+ (63)	+ (63)
*dl*1104	95% (47)	S	+ (63)	− (63)	+ (63)	+ (63)
*dl*1105	110% (47)	S	+ (63)	↓ (63)	+ (63)	+ (63)
*dl*1107	115% (47)	S	+ (63)	+ (63)	− (63)	+ (63)
*dl*1108	120% (47)	S	+ (63)	+ (63)	− (63)	↓ (63)
43/08/520	14% (47)	R	− (63)	− (63)	− (63)	− (63)
01/07/520	20% (47)	R	− (63)	− (63)	− (63)	+ (63)
01/08/520	29% (47)	R	− (63)	− (63)	− (63)	− (63)
01/06/520	69% (47)	S	− (63)	− (63)	+ (63)	+ (63)
02/07/520	80% (47)	S	− (63)	+ (63)	− (63)	+ (63)
05/07/520	92% (47)	S	+ (63)	+ (63)	− (63)	+ (63)
02/06/520	121% (47)	S	− (63)	+ (63)	+ (63)	+ (63)
02/08/520	89% (47)	S	− (63)	+ (63)	− (63)	↓ (63)
12S.928	+ (87)	S	ND[c]	+ (87)	− (87)	+ (87)
12S.RG2/928	− (87)	R	ND	− (87)	− (87)	− (87)
12S.YH47	ND	S	ND	+ (87)	− (87)	+ (87)
12S.YH47/928	+ (87)	S	ND	+ (87)	− (87)	− (87)
12S.RG2	+ (87)	S	ND	− (87)	+ (87)	+ (87)
E1A.CXdl	+ (86)	S	ND	+ (86)	+ (86)	+ (86)
E1A.PSdl	− (58)	R	ND	ND	ND	ND

[a] S, sensitive; R, resistant.
[b] ↓, decreased binding levels.
[c] ND, not determined.

levels of DNA synthesis 8 to 16 h later than the wild-type E1A (87). The p12S.RG2 cell line is sensitive to TNF (Fig. 9) but at a much later time point than the rest of the E1A-transfected cell lines (Fig. 7). The CXdl virus induces low levels of DNA synthesis 8 h later than its wild-type parent construct and induces DNA synthesis to wild-type levels 38 to 42 h postinfection (86). Studies by others found a CXdl-transfected cell line to be resistant to TNF after a 48-h incubation period (2). However, upon incubating the CXdl transfectant with TNF for longer time periods, we found that these transfectants are sensitive to TNF (Fig. 9). We have found that stable transfectants often express lower levels of E1A protein products than their virus counterparts. Thus, both later induction of DNA synthesis and lower levels of E1A gene products in transfections than in infections probably account for the necessity of longer incubations with TNF for sensitivity with these two cell lines.

Other E1A functions show the same requirements of binding to either p300 or p105-Rb for activity. For example, E1A suppresses differentiation of the neuron-like rat PC12 cells (9, 40) and mouse C2 myoblasts (13) by binding to either p300 or p105-Rb. Conversely, Kalman et al. (51) have found that E1A must bind both p300 and p105-Rb for inhibition of differentiation of PC12 cells. In contrast, only p300 binding is necessary for suppression of differentiation of mouse BC3H1 myoblasts (62). Also, the E1A regions required for induction of apoptosis (63) and expression of viral early genes (61) are the same as those responsible for induction of DNA synthesis, requiring either p300 or p105-Rb binding. However, not all E1A functions map to the same binding proteins as those for induction of the functions mentioned above. For E1A to induce mitosis, all three regions involved in p300, p105-Rb, and p107 binding are required (46, 58, 96). This is also true for induction of transformation in cooperation with ras (81). Expression of the proliferating cell nuclear antigen promoter requires binding of p300 and p107-cyclin A (52). Induction of expression of viral late genes (61), activation of the hsp70 promoter (53), and repression of the simian virus 40 enhancer (22) all are dependent upon E1A binding to p300 alone. These data indicate that E1A does not have a global effect on the cell which is inducing TNF sensitivity; rather, sensitivity is due to specific mechanisms or properties of E1A which can be defined on the basis of their requirement for interaction with cellular proteins.

It is interesting to note the few E1A constructs which have diminished, but not completely abrogated, binding capacities and how these affect TNF sensitivity. For example, *dl*1143/520 does not bind p300 and has decreased binding of p105-Rb, yet infected cells are still resistant to TNF (Fig. 2). This suggests that levels of E1A binding to p105-Rb are below the threshold needed for activation of E1A functions. Consistent with this, *dl*1143/520 also cannot induce DNA synthesis (Table 1).

Other studies have shown that a plasmid expressing the PSdl mutant, which binds neither p300 nor p105-Rb, induces sensitivity to TNF in a 48-h assay in stably transfected cells (84). This contradicts our finding that infection of NIH 3T3 cells with the PSdl virus does not induce sensitivity to TNF in an 18-h assay (19). The dye uptake assay used by Tsuji et al. (84), in which transfected cells were fixed and stained, measures

both cytolysis and cytostasis. The dye exclusion viability assay outlined here measures cell death only. Thus, the results obtained here and those of Tsuji et al. cannot be compared directly. Results from the viability assays reported here indicate that the PSdl transfectants are resistant to TNF even after 60 h of incubation (Fig. 9). E1A expression is known to disrupt the cellular microfilament lattice (unpublished observations). Thus, some viable cells, which may not be adherent because of E1A expression, may be excluded in the viability assay of Tsuji et al. (84) but included in the dye exclusion assays done here.

Previous studies have shown TNF-mediated cytotoxicity to be linked to the cell cycle (15). Specifically, cells incubated with TNF will advance through S and G_2 but will die as they exit G_2 into M phase (15). Additionally, to induce cytotoxicity, TNF must bind to its receptor between G_1/S and G_2/M for death to occur (15). From the data presented here, it is envisioned that E1A induces TNF sensitivity by binding to p300 and p105-Rb, which alters the cell cycle and induces S phase. Cells then progress into a phase of the cell cycle in which they are vulnerable to the cytotoxic signals given by TNF, resulting in cell death as mitotic events are initiated. It is not known whether E1A alters any of the parameters of DNA synthesis in NIH 3T3 cells which are actively dividing.

Why does adenovirus have E1A, a gene which would appear to decrease the chances of virus persistence by inducing sensitivity to TNF? Adenovirus infections occur in lung epithelial cells (44), which are differentiated and quiescent. To provide the cellular machinery necessary for DNA synthesis, adenovirus presumably must override cell cycle inhibition. E1A controls gene expression by simultaneously transactivating some cellular genes (28, 80) while repressing the enhancer sequences of other cellular genes (16, 27, 41, 76, 77), and these alterations in transcription result in the induction of cellular and concomitant viral DNA syntheses (64). Thus, it seems likely that E1A, while modifying host transcription machinery for the virus, inadvertently renders an infected cell susceptible to TNF. The virus must then encode other products (the 10,400-, 14,500-, and 14,700-Da proteins [33, 34]) to protect the infected cell from TNF lysis while apparently not interfering with E1A-modified gene regulation and S phase entry.

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