

Reovirus-Induced Apoptosis of MDCK Cells Is Not Linked to Viral Yield and Is Blocked by Bcl-2

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In this study, we investigated the relationship between reovirus-induced apoptosis and viral growth. Madin-Darby canine kidney (MDCK) epithelial cells infected with prototype reovirus strains type 1 Lang (T1L) or type 3 Dearing (T3D) were found to undergo apoptosis, and T3D induced apoptosis of MDCK cells to a substantially greater extent than T1L. By using T1L × T3D reassortant viruses, we found that differences in the capacities of these strains to induce apoptosis are determined by the viral S1 and M2 gene segments. These genes encode viral outer-capsid proteins that play important roles in viral entry into cells. T1L grew significantly better in MDCK cells than T3D, and these differences in growth segregated with the viral L1 and M1 gene segments. The L1 and M1 genes encode viral core proteins involved in viral RNA synthesis. Bcl-2 overexpression in MDCK cells inhibited reovirus-induced apoptosis but did not substantially affect reovirus growth. These findings indicate that differences in the capacities of reovirus strains to induce apoptosis and grow in MDCK cells are determined by different viral genes and that premature cell death by apoptosis does not limit reovirus growth in MDCK cells.

Apoptosis is a process of physiologic cell death characterized by cell shrinkage, loss of intercellular contacts, chromatin condensation, and in some cases endogenous endonuclease activation, which results in cleavage of cellular DNA into oligonucleosome-length DNA fragments (reviewed in reference 45). Apoptosis plays a critical role in many biological processes, including developmental modeling of neural circuitry (29) and regulation of immune repertoires (17). Several viruses, including human immunodeficiency virus, influenza virus, measles virus, poliovirus, and Sindbis virus, induce apoptosis of their host cells (reviewed in references 30 and 34). It has been postulated that apoptosis serves as an antiviral defense mechanism to limit viral replication by either destruction of virus-infected cells (5, 21) or reduction of potentially harmful inflammatory responses elicited by viral infection (6).

Mammalian reoviruses are important models for studies of viral pathogenesis (reviewed in reference 39). Reoviruses are nonenveloped, icosahedral viruses that contain a genome consisting of 10 double-stranded RNA gene segments (reviewed in reference 27). Murine L929 (L) cells infected with reovirus exhibit many ultrastructural and biochemical features of apoptosis, including chromatin condensation, nuclear and cytoplasmic membrane blebbing, cytoplasmic vacuolization, and cleavage of genomic DNA into oligonucleosome-length fragments by an endogenous endonuclease (41). Prototype reovirus strain type 3 Dearing (T3D) induces apoptosis of L cells to a substantially greater extent than strain type 1 Lang (T1L) (41). However, strains T1L and T3D produce equivalent yields of viral progeny in L cells (41), suggesting that differences in the capacities of these strains to induce apoptosis of L cells do not influence their capacities to grow in these cells.

To better understand the role of apoptosis in the pathogenesis of viral infections, we investigated the relationship between apoptosis induction and viral growth, using Madin-Darby canine kidney (MDCK) cells, which are polarized cells of epithelial origin (4). MDCK cells were chosen for the current study because strains T1L and T3D differ in both apoptosis induction and growth in these cells. Therefore, we reasoned that genetic analyses using T1L × T3D reassortant viruses would provide a means to determine whether apoptosis and viral growth are linked. In addition, we investigated whether reovirus-induced apoptosis of MDCK cells is blocked by overexpression of Bcl-2, and we assessed the effect of Bcl-2 overexpression on viral yield. The results strongly suggest that apoptosis does not serve as a mechanism to limit reovirus growth in MDCK cells.

Reovirus induces apoptosis of MDCK cells. To determine whether reovirus induces apoptosis of cells of epithelial origin, MDCK cells were either mock infected or infected with reovirus strain T3D at a multiplicity of infection (MOI) of 40 PFU per cell and then analyzed for morphological hallmarks of apoptosis by electron microscopy (Fig. 1A and B). Infected cells showed nuclear condensation, blebbing of the plasma membrane, cytoplasmic vacuolization, and cell shrinkage, which are all characteristics of cells undergoing apoptosis (45). Infected cells also showed margination of the nuclear chromatin, nuclear collapse and breakage, and cellular fragmentation into apoptotic bodies (Fig. 1B and data not shown). In contrast, mock-infected cells showed no evidence of apoptosis (Fig. 1A). In multiple thin sections examined by electron microscopy, no inclusions of progeny virions were observed in MDCK cells infected with T3D (Fig. 1B and data not shown).

We next determined whether reovirus-induced apoptosis of MDCK cells is associated with activation of an endogenous endonuclease. MDCK cells were infected with either T1L or T3D at an MOI of 40 PFU per cell and incubated at 37°C for various intervals. Cells were then lysed, and cellular DNA was

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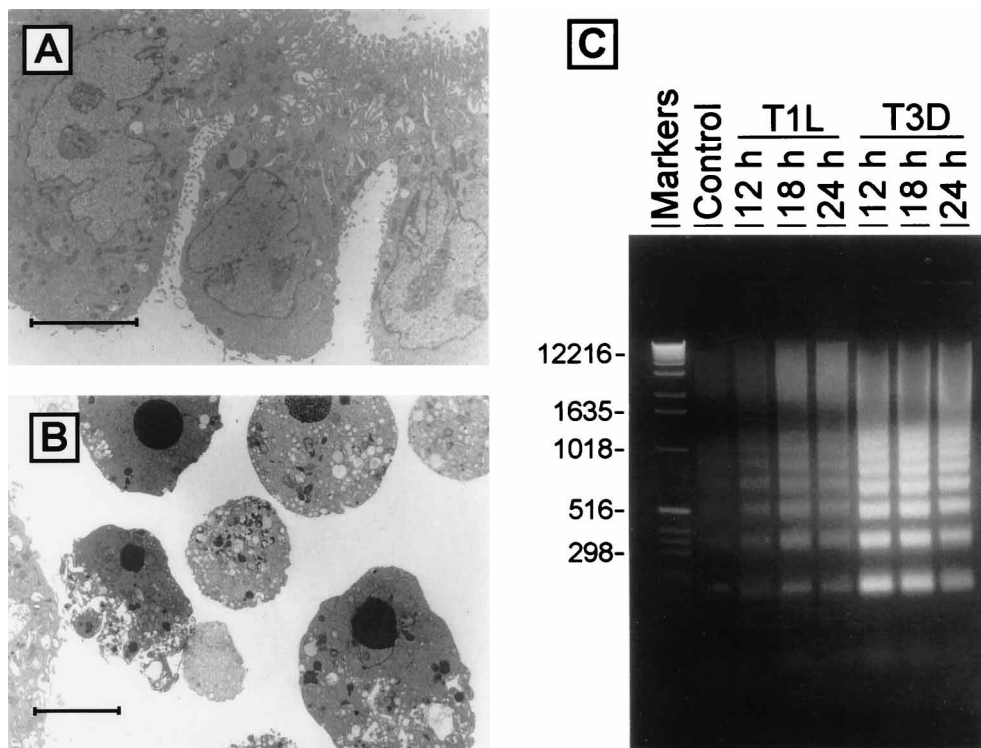


FIG. 1. Reovirus induces apoptosis of MDCK cells. (A and B) Electron microscopic appearance of uninfected (A) and T3D-infected (B) MDCK epithelial cells. MDCK cells were infected with reovirus strain T3D at an MOI of 40 PFU per cell and incubated at 37°C for 18 h. Cells were fixed in phosphate-buffered 2% glutaraldehyde, embedded in epoxy resin, sectioned, and stained with lead citrate-uranyl acetate. Bars, 5 μ m. (C) Agarose gel electrophoresis of total cellular DNA extracted from MDCK cells infected with T1L and T3D. MDCK cells were either mock infected (control) or infected with T1L or T3D at an MOI of 40 PFU per cell. After incubation at 37°C for the indicated intervals, purified cellular DNA was resolved by electrophoresis through 1.8% agarose and stained with ethidium bromide. Size markers (in base pairs) are indicated on the left.

extracted and subjected to agarose gel electrophoresis by previously described techniques (14) (Fig. 1C). Oligonucleosomal length ladders were clearly visible in samples of cellular DNA obtained 12 h after adsorption. Thus, both morphologic and biochemical assays indicate that reovirus induces apoptosis of MDCK epithelial cells.

Reovirus strains T1L and T3D differ in their capacities to induce apoptosis of MDCK cells. To determine whether T1L and T3D differ quantitatively in their capacities to induce apoptosis of MDCK cells, we used an acridine orange (AO) staining assay to assess the percentage of reovirus-infected cells undergoing apoptosis (11, 41). AO is a fluorescent dye that stains DNA and allows identification of apoptotic cells by detection of chromatin condensation. We have previously shown that quantitation of apoptosis by AO staining is associated with ultrastructural evidence of apoptosis, the presence of DNA fragmentation, and the appearance of oligonucleosomal laddering of host cell DNA following electrophoresis in agarose gels (41). MDCK cells were infected with either T1L or T3D at an MOI of 100 PFU per cell. At various intervals after adsorption, cells were trypsinized, stained with a dye solution containing AO (Sigma Chemical Co., St. Louis, Mo.) and ethidium bromide (Sigma) at a final concentration of 1 μ g of each per ml, and examined by epifluorescence microscopy (Nikon Labophot-2; B-2A filter; excitation, 450 to 490 nm; barrier, 520 nm; dichroic mirror, 505 nm). Apoptosis was quantitated by determining the percentage of cells containing condensed chromatin (Fig. 2). A greater percentage of cells infected with T3D (24.6%) than of those infected with T1L (12.6%) exhibited condensed chromatin when stained with AO. For both

strains, maximum apoptosis was detected 40 h after adsorption. Apoptosis of MDCK cells was also induced at considerably lower MOIs; however, an MOI of 100 was found to maximize AO staining differences between T1L and T3D (data not shown).

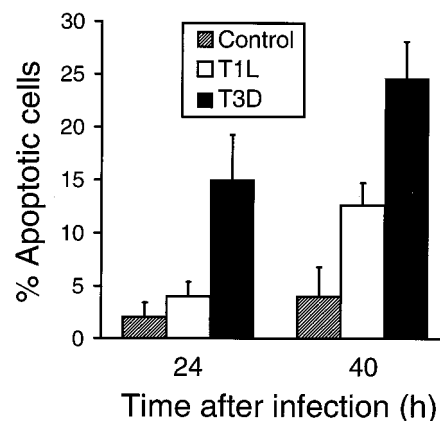


FIG. 2. Percentage of reovirus-infected MDCK cells undergoing apoptosis as determined by AO staining. MDCK cells were either mock infected (control) or infected with T1L or T3D at an MOI of 100 PFU per cell. After incubation at 37°C for the indicated intervals, cells were harvested and processed for AO staining. The results are expressed as the means of data obtained in two independent experiments. Error bars indicate standard deviations of the means.

TABLE 1. The capacities of T1L × T3D reassortants to induce apoptosis as detected by an AO staining assay

| Virus strain | Origin of gene segment ^a | | | | | | | | | | | | % of AO staining ^b | SEM | Rank ^c |
|--------------|-------------------------------------|----|----|----|----|----|----|----|----|----|------|-----|-------------------------------|-----|-------------------|
| | L1 | L2 | L3 | M1 | M2 | M3 | S1 | S2 | S3 | S4 | | | | | |
| Parental T1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 12.6 | 1.8 | |
| T3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 24.6 | 2.4 | |
| Reassortant | | | | | | | | | | | | | | | |
| KC150 | 3D | 1L | 1L | 1L | 3D | 1L | 3D | 3D | 1L | 3D | 33.3 | 1.7 | 1 | | |
| EB97 | 3D | 3D | 1L | 3D | 3D | 3D | 3D | 3D | 3D | 1L | 31.4 | 1.9 | 2 | | |
| KC19 | 1L | 1L | 1L | 1L | 3D | 1L | 3D | 1L | 3D | 1L | 31.0 | 1.0 | 3 | | |
| EB39 | 1L | 3D | 3D | 1L | 3D | 3D | 3D | 3D | 3D | 3D | 30.2 | 1.5 | 4 | | |
| EB138 | 3D | 1L | 1L | 3D | 3D | 1L | 3D | 3D | 1L | 1L | 27.0 | 1.7 | 5 | | |
| EB28 | 3D | 3D | 1L | 3D | 3D | 3D | 3D | 1L | 3D | 3D | 26.9 | 2.3 | 6 | | |
| H15 | 1L | 3D | 3D | 1L | 3D | 3D | 3D | 3D | 3D | 1L | 22.0 | 1.2 | 7 | | |
| EB1 | 1L | 3D | 1L | 1L | 3D | 1L | 1L | 1L | 3D | 1L | 20.6 | 1.5 | 8 | | |
| H9 | 3D | 3D | 1L | 3D | 1L | 1L | 3D | 3D | 3D | 3D | 20.2 | 1.8 | 9 | | |
| EB13 | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 1L | 19.2 | 1.5 | 10.5 | | |
| G2 | 1L | 3D | 1L | 1L | 1L | 1L | 3D | 1L | 1L | 1L | 19.2 | 1.9 | 10.5 | | |
| EB68 | 1L | 3D | 1L | 1L | 3D | 1L | 1L | 1L | 3D | 3D | 17.3 | 1.7 | 12 | | |
| EB120 | 3D | 3D | 3D | 1L | 1L | 3D | 3D | 3D | 1L | 1L | 15.4 | 1.0 | 13 | | |
| EB129 | 3D | 3D | 1L | 3D | 3D | 1L | 3D | 1L | 1L | 3D | 14.3 | 2.6 | 14 | | |
| EB144 | 1L | 1L | 1L | 1L | 3D | 3D | 1L | 1L | 3D | 1L | 14.1 | 1.2 | 15 | | |
| EB18 | 3D | 3D | 1L | 3D | 3D | 3D | 1L | 1L | 3D | 1L | 14.0 | 1.2 | 16.5 | | |
| EB98 | 1L | 3D | 1L | 1L | 1L | 1L | 1L | 3D | 1L | 3D | 14.0 | 1.0 | 16.5 | | |
| KC9 | 3D | 3D | 3D | 3D | 3D | 3D | 1L | 3D | 3D | 3D | 10.6 | 1.3 | 18 | | |
| EB145 | 3D | 3D | 3D | 3D | 3D | 1L | 1L | 3D | 3D | 3D | 8.8 | 1.5 | 19 | | |
| EB113 | 1L | 1L | 1L | 3D | 1L | 1L | 1L | 1L | 3D | 1L | 8.2 | 1.4 | 20 | | |
| EB143 | 3D | 1L | 1L | 1L | 1L | 1L | 3D | 1L | 1L | 1L | 7.8 | 1.3 | 21 | | |
| EB31 | 1L | 1L | 1L | 3D | 1L | 1L | 1L | 3D | 3D | 1L | 7.6 | 0.9 | 22 | | |
| H41 | 3D | 3D | 1L | 1L | 1L | 3D | 1L | 3D | 3D | 1L | 7.1 | 1.2 | 23 | | |
| EB121 | 3D | 3D | 1L | 3D | 1L | 3D | 1L | 3D | 3D | 3D | 5.4 | 1.6 | 24 | | |
| EB85 | 1L | 1L | 1L | 1L | 1L | 3D | 1L | 3D | 1L | 1L | 4.4 | 1.9 | 25 | | |

^a Parental origin of each gene segment: 1L, gene segment derived from T1L; 3D, gene segment derived from T3D.

^b MDCK cells were infected with viral strains at an MOI of 100 PFU per cell and stained with AO 40 h after adsorption. The percentage of AO-stained cells was determined by fluorescence microscopy. The results are expressed as the mean percentage of AO-stained cells from three to five independent wells.

^c Viruses are ranked from highest to lowest based on the percentage of AO-stained cells.

The S1 and M2 genes are the primary determinants of differences in apoptosis induction by reovirus strains T1L and T3D in MDCK cells. To identify viral genes associated with differences in the capacities of T1L and T3D to induce apoptosis of MDCK cells, we used 25 T1L × T3D reassortant viruses (2, 8, 44) and the AO staining assay (Table 1). MDCK cells were infected at an MOI of 100 PFU per cell and incubated at 37°C for 40 h. Cells were then stained with AO and ethidium bromide and examined for condensed chromatin. The results were analyzed with the Minitab (release 8) statistical software package (Addison-Wesley, Reading, Mass.). We found a highly significant association between the capacities of reassortant viruses to induce apoptosis and the T3D S1 gene (data analyzed by Mann-Whitney (MW) test, $P = 0.0007$; by t test, $P = 0.0002$) and a moderately significant association between induction of apoptosis and the T3D M2 gene (MW test, $P = 0.003$; t test, $P = 0.0011$). No other gene segments were significantly associated with differences in apoptosis induction ($P > 0.05$). By linear regression analysis, we obtained for the regression equation R^2 values of 86.4% ($P < 0.001$) for all 10 reovirus genes, 68.5% ($P < 0.001$) for the S1 and M2 genes, 47.0% ($P < 0.001$) for the S1 gene alone, and 34.7% ($P = 0.002$) for the M2 gene alone. These results indicate that the S1 gene is the principal determinant of differences in the capaci-

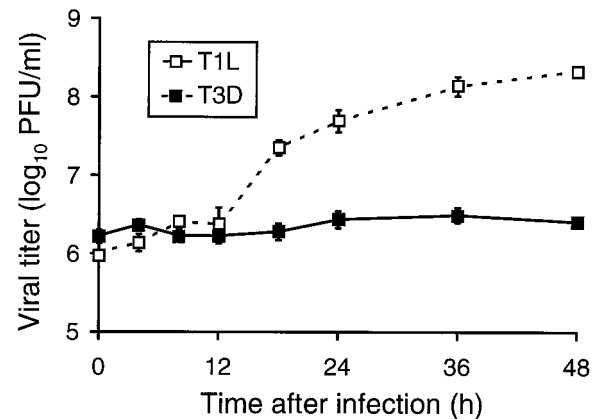


FIG. 3. Growth of T1L and T3D in MDCK cells. Monolayers of MDCK cells (2×10^5) were infected with either T1L or T3D at an MOI of 2 PFU per cell. After a 1-h adsorption period, cells were incubated at 37°C for the indicated intervals and virus in cell lysates was titrated on L-cell monolayers by plaque assay. The results are presented as the means of the viral titers from three independent experiments. Error bars indicate standard deviations of the means.

ties of T1L and T3D to induce apoptosis of MDCK cells; a smaller, independent contribution is made by the M2 gene segment.

The S1 gene encodes viral attachment protein $\sigma 1$ (18, 43) and nonstructural protein $\sigma 1s$ (12, 15, 31). The M2 gene encodes outer-capsid protein $\mu 1$ (24, 26). The $\sigma 1$ and $\mu 1$ proteins play important roles in viral entry into cells (27); therefore, segregation of the S1 and M2 genes with differences in the capacities of reovirus strains to induce apoptosis suggests that this cellular response is triggered by early steps in viral replication. These steps include binding of $\sigma 1$ to cellular receptors, endocytosis of the virus-receptor complex, proteolytic processing of viral outer-capsid proteins, and penetration of the processed form of the virus into the cytoplasm. Our data do not exclude the possibility that the $\sigma 1s$ protein influences the observed differences in apoptosis induction exhibited by T1L and T3D. However, UV-inactivated virions of both T1L and T3D, which do not contain $\sigma 1s$ (12, 15, 31) and are incapable of mediating viral protein synthesis (33), are capable of inducing apoptosis (41). This finding strongly suggests that the $\sigma 1$ protein is the S1-encoded determinant of strain-specific differences in reovirus-induced apoptosis. The S1 and M2 genes also determine differences in the capacities of strains T1L and T3D to induce apoptosis of L cells (41), which suggests that reoviruses use similar mechanisms to induce apoptosis of diverse cell types.

Reovirus strains T1L and T3D differ in their capacities to grow in MDCK cells. The absence of viral inclusions in MDCK cells infected with T3D prompted us to examine whether differences in the capacities of T1L and T3D to induce apoptosis of MDCK cells are associated with differences in their capacities to grow in these cells. MDCK cells were infected with each virus strain at an MOI of 2 PFU per cell, and viral titers in the cell lysates were determined by plaque assay at various intervals after adsorption (Fig. 3). A low MOI was chosen for these experiments in order to maximize detection of viral growth. After 24 h of incubation, there was no growth of T3D whereas T1L produced approximately 50 PFU per input PFU. These data indicate that reovirus strains T1L and T3D, which differ in their capacities to induce apoptosis of MDCK cells, also differ in their capacities to grow in these cells.

TABLE 2. The capacities of T1L × T3D reassortants to grow in MDCK cells

| Virus strain | Origin of gene segment ^a | | | | | | | | | | Viral yield (log ₁₀) ^b | SEM | Rank ^c |
|--------------|-------------------------------------|----|----|----|----|----|----|----|----|-----|---|------|-------------------|
| | L1 | L2 | L3 | M1 | M2 | M3 | S1 | S2 | S3 | S4 | | | |
| Parental | | | | | | | | | | | | | |
| T1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 1L | 2.1 | 0.5 | |
| T3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 0.5 | 0.2 | |
| Reassortant | | | | | | | | | | | | | |
| EB1 | 1L | 3D | 1L | 1L | 3D | 1L | 1L | 1L | 3D | 1L | 3.5 | 0.6 | 1.5 |
| EB39 | 1L | 3D | 3D | 1L | 3D | 3D | 3D | 3D | 3D | 3D | 3.5 | 0.8 | 1.5 |
| EB28 | 3D | 3D | 1L | 3D | 3D | 3D | 3D | 1L | 3D | 3D | 3.2 | 0.6 | 3 |
| EB97 | 3D | 3D | 1L | 3D | 3D | 3D | 3D | 3D | 3D | 1L | 2.8 | 0.5 | 4 |
| G2 | 1L | 3D | 1L | 1L | 1L | 1L | 3D | 1L | 1L | 1L | 2.6 | 0.2 | 5 |
| EB144 | 1L | 1L | 1L | 1L | 3D | 3D | 1L | 3D | 1L | 1L | 2.5 | 0.4 | 6 |
| EB31 | 1L | 1L | 1L | 3D | 1L | 1L | 1L | 3D | 3D | 1L | 2.3 | 0.3 | 7.5 |
| H15 | 1L | 3D | 3D | 1L | 3D | 3D | 3D | 3D | 3D | 1L | 2.3 | 0.0 | 7.5 |
| EB68 | 1L | 3D | 1L | 1L | 3D | 1L | 1L | 1L | 3D | 3D | 2.2 | 0.3 | 9.5 |
| KC19 | 1L | 1L | 1L | 1L | 3D | 1L | 3D | 1L | 3D | 1L | 2.2 | 0.0 | 9.5 |
| EB85 | 1L | 1L | 1L | 1L | 1L | 3D | 1L | 3D | 1L | 1L | 2.1 | 0.3 | 11 |
| H9 | 3D | 3D | 1L | 3D | 1L | 1L | 3D | 3D | 3D | 3D | 1.9 | 0.1 | 12 |
| EB113 | 1L | 1L | 1L | 3D | 1L | 1L | 1L | 1L | 3D | 1L | 1.8 | 0.1 | 13.5 |
| EB143 | 3D | 1L | 1L | 1L | 1L | 1L | 3D | 1L | 1L | 1L | 1.8 | 0.1 | 13.5 |
| EB98 | 1L | 3D | 1L | 1L | 1L | 1L | 1L | 3D | 1L | 3D | 1.7 | 0.3 | 15.5 |
| KC150 | 3D | 1L | 1L | 1L | 3D | 1L | 3D | 3D | 1L | 3D | 1.7 | 0.1 | 15.5 |
| H41 | 3D | 3D | 1L | 1L | 1L | 3D | 1L | 3D | 3D | 1L | 1.6 | 0.1 | 17 |
| EB120 | 3D | 3D | 3D | 1L | 1L | 3D | 3D | 3D | 1L | 1L | 1.5 | 0.1 | 18 |
| EB121 | 3D | 3D | 1L | 3D | 1L | 3D | 1L | 3D | 3D | 3D | 1.2 | 0.4 | 19 |
| KC9 | 3D | 3D | 3D | 3D | 3D | 3D | 1L | 3D | 3D | 3D | 1.0 | 0.2 | 20.5 |
| EB129 | 3D | 3D | 1L | 3D | 3D | 1L | 3D | 1L | 1L | 3D | 1.0 | 0.2 | 20.5 |
| EB138 | 3D | 1L | 1L | 3D | 3D | 1L | 3D | 3D | 1L | 1L | 0.9 | 0.2 | 22.5 |
| EB13 | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 3D | 1L | 0.9 | 0.1 | 22.5 | |
| EB145 | 3D | 3D | 3D | 3D | 3D | 1L | 1L | 3D | 3D | 3D | 0.7 | 0.3 | 24.5 |
| EB18 | 3D | 3D | 1L | 3D | 3D | 3D | 1L | 1L | 3D | 1L | 0.7 | 0.1 | 24.5 |

^a Parental origin of each gene segment: 1L, gene segment derived from T1L; 3D, gene segment derived from T3D.

^b MDCK cells were infected with viral strains at an MOI of 2 PFU per cell and incubated at 37°C for 24 h, and virus in cell lysates was titrated by plaque assay on L-cell monolayers. The results are expressed as the means of viral yields (viral titer at 24 h divided by viral titer at 0 h) for three independent experiments for each viral strain.

^c Viruses are ranked from highest to lowest based on yield of infectious virus after 24 h of viral growth.

The L1 and M1 genes are the primary determinants of differences in growth of reovirus strains T1L and T3D in MDCK cells. To determine whether viral genes associated with differences in the capacities of T1L and T3D to induce apoptosis of MDCK cells are also associated with differences in their growth in these cells, we tested the 25 T1L × T3D reassortant viruses shown in Table 1 for growth in MDCK cells. Reassortant viruses were adsorbed to MDCK cells at an MOI of 2 PFU per cell, and viral titers in the cell lysates were determined by plaque assay after 24 h of incubation (Table 2). In sharp contrast to viral genes associated with strain-specific differences in apoptosis induction, we found a highly significant association between the capacities of reassortant viruses to grow in MDCK cells and the T1L L1 gene (MW test, $P = 0.0024$; t test, $P = 0.0023$) and a moderate association between growth in MDCK cells and the T1L M1 gene (MW test, $P = 0.047$; t test, $P = 0.033$). No other gene segments were significantly associated with differences in viral growth in these cells ($P > 0.05$). By linear regression analysis, we obtained for the regression equation R^2 values of 67.0% ($P = 0.037$) for all 10 reovirus genes, 35.1% ($P = 0.009$) for the L1 and M1 genes, 32.7% ($P = 0.003$) for the L1 gene alone, and 18.8% ($P = 0.03$) for the M1 gene alone. These results indicate that the L1 gene is the principal determinant of differences in the capacities of

T1L and T3D to grow in MDCK cells; a smaller contribution is made by the M1 gene segment. Thus, strain-specific differences in apoptosis induction and strain-specific differences in viral growth segregate with different viral genes.

The L1 gene encodes core protein $\lambda 3$ (24), and M1 encodes core protein $\mu 2$ (24, 26). These proteins are present at approximately 12 copies per virion and are suggested to exist in a complex with core spike protein $\lambda 2$ at the vertices of the virion icosahedron (10). Although the functions of the $\lambda 3$ and $\mu 2$ proteins are not entirely understood, each is thought to play important roles in viral RNA synthesis. The $\lambda 3$ -encoding L1 gene determines strain-specific differences in the pH optimum of transcription (9), and the deduced amino acid sequence of $\lambda 3$ contains a sequence motif that is common to many RNA-dependent RNA polymerases (3, 16, 25). The $\lambda 3$ protein, when expressed in a vaccinia virus system, mediates poly(C)-dependent poly(G) polymerase activity but does not catalyze transcription of viral gene segments (37), indicating a requirement for additional proteins in synthesis of viral RNA. The $\mu 2$ -encoding M1 gene determines strain-specific differences in the temperature optimum of transcription (46), and a temperature-sensitive reovirus mutant that maps to M1 is not capable of double-stranded RNA synthesis at the restrictive temperature (7). These observations suggest that $\mu 2$ also participates in viral RNA-dependent RNA polymerase activity.

The L1 and M1 genes have been linked to strain-specific differences in reovirus growth in other cell types. Differences in the capacities of reovirus strains T1L and T3D to infect primary cultures of cardiac myocytes segregate with the M1 and L1 genes (23), and differences in growth in aortic endothelial cells segregate with the M1 gene (22). In addition to determining differences in viral yield in cultured cells, the L1 and M1 genes also play important roles in some models of reovirus pathogenesis. The L1 and M1 genes are linked to differences in the capacities of reovirus strains to produce myocarditis in immunocompetent mice (35, 36), and, along with the L2 and S1 genes, L1 and M1 are associated with strain-specific differences in reovirus virulence in severe combined immunodeficiency mice (13). It is not known how viral gene products involved in RNA synthesis influence viral growth and virulence; however, it has been suggested that they alter the kinetics of viral polymerase activity in particular host tissues or exert toxic effects on the cell (1, 13, 36).

Apoptosis induction and viral growth in MDCK cells are polygenic traits. We used a large panel of T1L × T3D reassortant viruses to identify viral genes that segregate with strain-specific differences in apoptosis induction and viral growth in MDCK cells. The reassortants do not segregate into discrete groups based on genotype; rather, they form a continuum with ranges exceeding those of either parent. Nonetheless, the use of a large reassortant panel and sensitive statistical techniques allowed us to identify the primary genetic determinants of the observed phenotypic differences, as has been done in several previous studies (13, 22, 23, 35, 36, 40, 41). Based on the R^2 values obtained by linear regression, we estimate that the viral S1 and M2 genes together account for 54% of the genetically determined variance in reovirus-induced apoptosis of MDCK cells and that the viral L1 and M1 genes together account for 52% of the genetically determined variance in reovirus growth in MDCK cells. Therefore, although the S1 and M2 genes are the primary determinants of apoptosis induction and the L1 and M1 genes are the primary determinants of viral growth, other reovirus genes contribute to strain-specific differences in both phenotypes. We also found that the phenotypes of some reassortant viruses were exceptions to the observed segregation patterns. Such phenotypic variation might be caused by

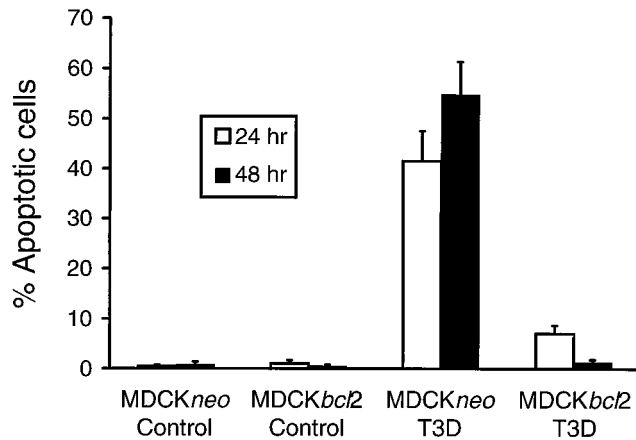


FIG. 4. AO staining of MDCKneo and MDCKbcl2 cells infected with T3D. Cells were either mock infected (control) or infected with T3D at an MOI of 100 PFU per cell. After incubation at 37°C for the indicated intervals, cells were harvested and processed for AO staining. The results are expressed as the means of the data obtained in three independent experiments. Error bars indicate standard deviations of the means.

experimental variability, the presence of novel mutations in some reassortant strains (36), or the effects of certain combinations of viral genes on expression of particular phenotypes (reviewed in reference 42). It is noteworthy that reassortant strains EB28 and EB97, which contain L1 and M1 genes derived from T3D yet produce high titers in MDCK cells, also grow well in cultured cardiac myocytes (23), a property that segregates with the T1L L1 and M1 genes.

Bcl-2 blocks reovirus-induced apoptosis of MDCK cells and has a minimal effect on viral growth. The Bcl-2 protein is a nuclear and mitochondrial membrane protein with an M_r of ~26,000 that was first recognized for its role in chromosomal translocations associated with human follicular B-cell lymphomas (38). Apoptosis induced by many types of stimuli is susceptible to blockade by overexpression of Bcl-2 (32), including apoptosis induced by several RNA-containing viruses (14, 20). To determine the effect of Bcl-2 overexpression on reovirus-induced apoptosis, MDCK cells engineered to overexpress the human *bcl-2* gene (MDCKbcl2 cells) and control MDCK cells (MDCKneo cells) (14) were infected with T3D, the prototype reovirus strain that induces maximum apoptosis, at an MOI of 100 PFU per cell. Cells were stained with AO at various intervals after adsorption, and apoptosis was quantitated by determining the percentage of cells containing condensed chromatin (Fig. 4). At both 24 and 48 h after adsorption, greater than 40% of infected MDCKneo cells had uniformly fluorescent, apoptotic nuclei. In contrast, less than 10% of infected MDCKbcl2 cells had condensed chromatin at the same time points. These data suggest that reovirus-induced apoptosis is blocked in cells overexpressing Bcl-2.

To confirm these results, we tested whether an endogenous endonuclease is activated in reovirus-infected MDCK cells overexpressing Bcl-2. MDCKbcl2 and MDCKneo cells were infected with T3D at an MOI of 40 PFU per cell. Cellular DNA was extracted from both types of cells at various intervals after adsorption and subjected to agarose gel electrophoresis (Fig. 5). Oligonucleosome-length ladders were visible in lysates prepared from infected MDCKneo cells 10 h after adsorption, peaking at approximately 12 h after adsorption. These data are similar to results obtained with untransfected MDCK cells (Fig. 1C). In contrast, the intensity of oligonucleosome-length ladders was substantially reduced in lysates prepared from

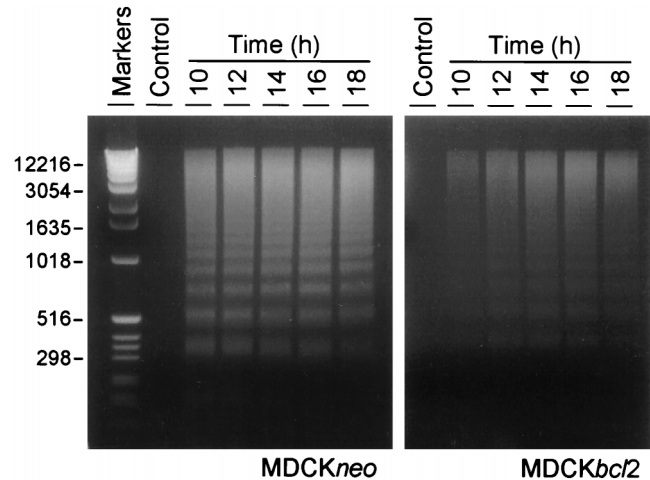


FIG. 5. Agarose gel electrophoresis of total cellular DNA extracted from MDCKneo and MDCKbcl2 cells infected with T3D. Cells were either mock infected (control) or infected with T3D at an MOI of 40 PFU per cell. After incubation at 37°C for the indicated intervals, purified cellular DNA was resolved by agarose gel electrophoresis and stained with ethidium bromide. Size markers (in base pairs) are indicated on the left.

infected MDCKbcl2 cells at all time points. These findings indicate that overexpression of Bcl-2 inhibits both morphologic and biochemical features of reovirus-induced apoptosis.

Apoptosis and viral growth. To determine the effect of Bcl-2 overexpression on reovirus growth in MDCK cells, MDCKbcl2 and MDCKneo cells were infected with T1L at an MOI of 2 PFU per cell, and viral titers in the cell lysates were determined by plaque assay at various intervals after adsorption (Fig. 6A). Strain T1L was chosen for these experiments because, unlike T3D, T1L grows well in MDCK cells. Yields of T1L in MDCKbcl2 cells were slightly lower than in MDCKneo cells, suggesting that blockade of apoptosis by the Bcl-2 protein has a minimal effect on viral replication. Since T1L does not induce apoptosis efficiently in MDCK cells, we sought to confirm these results by using a virus that grows well in MDCK cells yet induces apoptosis more efficiently than T1L. MDCKbcl2 and MDCKneo cells were infected with the T1L × T3D reassortant virus EB39 at an MOI of 2 PFU per cell, and the viral titers in the cell lysates were determined by plaque assay at various intervals after adsorption (Fig. 6B). Like T1L, EB39 produced equivalent yields in both cell lines, confirming that blockade of apoptosis by Bcl-2 has a minimal effect on reovirus growth in MDCK cells.

It has been proposed that apoptosis is a cellular defense mechanism that serves to limit viral replication (5, 21). If this were the case for reovirus-induced apoptosis, we would predict that strain-dependent differences in reovirus-induced apoptosis would be associated with strain-dependent differences in viral growth and that both of these phenotypes would segregate with the same viral genes. In fact, we found that differences in apoptosis induction and viral growth in MDCK cells do not segregate with the same viral genes. This finding suggests that the capacity of a reovirus strain to induce apoptosis is not a primary determinant of its capacity to produce infectious progeny. In support of this argument, we found that viral growth is not significantly altered in cells in which apoptosis is blocked by Bcl-2. Taken together, these results suggest that apoptosis does not serve as a mechanism to limit reovirus growth.

Findings made in this study are similar to those made in studies of influenza virus and Sindbis virus. Yields of influenza

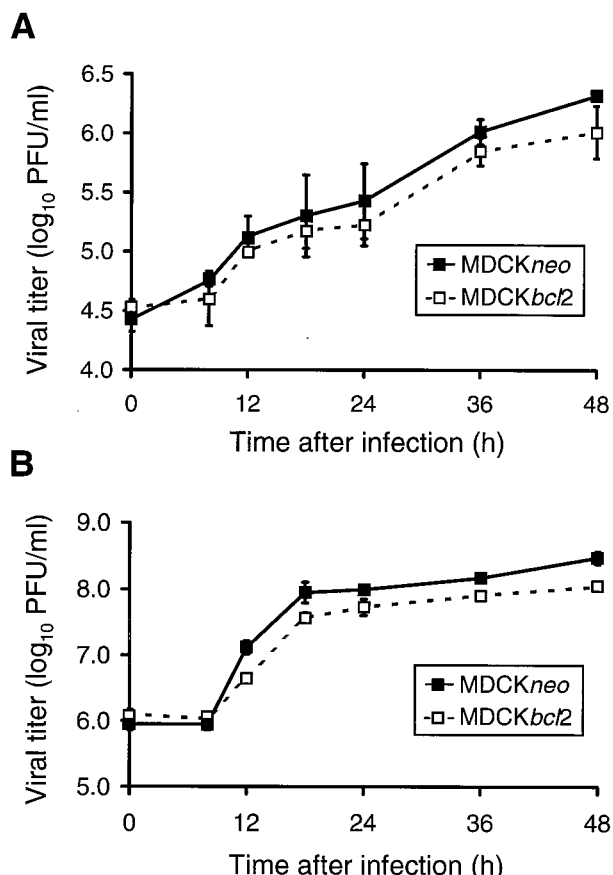


FIG. 6. Growth of T1L (A) and T1L × T3D reassortant virus EB39 (B) in MDCK_{neo} and MDCK_{bcl2} cells. Cells (2×10^5) were infected with either T1L or EB39 at an MOI of 2 PFU per cell. After a 1-h adsorption period, cells were incubated at 37°C for the indicated intervals and virus in cell lysates was titrated on L-cell monolayers by plaque assay. The results are presented as the means of the viral titers from three independent experiments. Error bars indicate standard deviations of the means.

virus in MDCK cells overexpressing Bcl-2 are significantly less than those in control cells (28). In comparison to wild-type Sindbis virus, a Sindbis virus chimera expressing Bcl-2 produces lower viral titers in brain tissues of infected mice and is less virulent (19). Thus, studies of reovirus, influenza virus, and Sindbis virus suggest a model in which apoptosis is a marker for a cellular process required for efficient viral replication rather than a mechanism to limit viral growth. It is possible that cellular responses leading to apoptosis result from induction of cellular factors associated with cell cycle control, which in turn serve to promote viral replication (40). Such factors might establish an environment that is more permissive for viral replication, perhaps by increasing nucleotide triphosphate pools, elaborating transcription factors, or increasing the efficiency of translation. Blockade of apoptosis in MDCK cells by Bcl-2 overexpression does not limit reovirus growth to the same extent as that of influenza virus. This suggests that replication of reovirus may be less dependent on cellular factors associated with apoptosis than replication of influenza virus. Alternatively, cellular factors required for reovirus growth might be induced at a point in the apoptosis pathway upstream of the site at which Bcl-2 exerts its inhibitory effects. Our ongoing studies of reovirus-induced apoptosis and the mechanisms used to trigger this response will contribute important

information about how viruses use cellular signaling pathways to enhance their replication.

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REFERENCES

- Baty, C. J., and B. Sherry. 1993. Cytopathogenic effect in cardiac myocytes but not in cardiac fibroblasts is correlated with reovirus-induced acute myocarditis. *J. Virol.* **67**:6295–6298.
- Brown, E. G., M. L. Nibert, and B. N. Fields. 1983. The L2 gene of reovirus serotype 3 controls the capacity to interfere, accumulate deletions and establish persistent infection, p. 275–287. *In* R. W. Compans and D. H. L. Bishop (ed.), *Double-stranded RNA viruses*. Elsevier Biomedical Press, New York, N.Y.
- Bruenn, J. A. 1991. Relationships among the positive strand and double-stranded RNA viruses as viewed through their RNA-dependent RNA polymerases. *Nucleic Acids Res.* **19**:217–226.
- Cerejido, M., E. S. Robbins, W. J. Dolan, C. A. Rotunas, and D. D. Sabatini. 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* **77**:853–888.
- Clouston, W. M., and J. F. Kerr. 1985. Apoptosis, lymphocytotoxicity and the containment of viral infections. *Med. Hypotheses* **18**:399–404.
- Cohen, J. J. 1991. Programmed cell death in the immune system. *Adv. Immunol.* **50**:55–85.
- Coombs, K. M. 1996. Identification and characterization of a double-stranded RNA⁻ reovirus temperature-sensitive mutant defective in minor core protein $\mu 2$. *J. Virol.* **70**:4237–4245.
- Coombs, K. M., B. N. Fields, and S. C. Harrison. 1990. Crystallization of the reovirus type 3 Dearing core. Crystal packing is determined by the $\lambda 2$ protein. *J. Mol. Biol.* **215**:1–5.
- Drayna, D., and B. N. Fields. 1982. Activation and characterization of the reovirus transcriptase: genetic analysis. *J. Virol.* **41**:110–118.
- Dryden, K. A., G. Wang, M. Yeager, M. L. Nibert, K. M. Coombs, D. B. Furlong, B. N. Fields, and T. S. Baker. 1993. Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. *J. Cell Biol.* **122**:1023–1041.
- Duke, R. C., and J. J. Cohen. 1992. Morphological and biochemical assays of apoptosis, p. 17.1–17.16. *In* J. E. Coligan et al. (ed.), *Current protocols in immunology*. John Wiley & Sons, New York, N.Y.
- Ernst, H., and A. J. Shatkin. 1985. Reovirus hemagglutinin mRNA codes for two polypeptides in overlapping reading frames. *Proc. Natl. Acad. Sci. USA* **82**:48–52.
- Haller, B. L., M. L. Barkon, G. P. Vogler, and H. W. Virgin IV. 1995. Genetic mapping of reovirus virulence and organ tropism in severe combined immunodeficient mice: organ-specific virulence genes. *J. Virol.* **69**:357–364.
- Hinshaw, V. S., C. W. Olsen, N. Dybdahl-Sissoko, and D. Evans. 1994. Apoptosis: a mechanism of cell killing by influenza A and B viruses. *J. Virol.* **68**:3667–3673.
- Jacobs, B. L., and C. E. Samuel. 1985. Biosynthesis of reovirus-specified polypeptides: the reovirus S1 mRNA encodes two primary translation products. *Virology* **143**:63–74.
- Koonin, E. V., E. E. Gorbalenya, and K. M. Chumakov. 1989. Tentative identification of RNA-dependent RNA polymerases of dsRNA viruses and their relationship to positive strand RNA viral polymerases. *FEBS Lett.* **252**:42–46.
- Krammer, P. H., I. Behrmann, P. Daniel, J. Dhein, and K. M. Debatin. 1994. Regulation of apoptosis in the immune system. *Curr. Opin. Immunol.* **6**:279–289.
- Lee, P. W., E. C. Hayes, and W. K. Joklik. 1981. Protein sigma 1 is the reovirus cell attachment protein. *Virology* **108**:156–163.
- Levine, B., J. E. Goldman, H. H. Jiang, D. E. Griffin, and J. M. Hardwick. 1996. Bcl-2 protects mice against fatal alphavirus encephalitis. *Proc. Natl. Acad. Sci. USA* **93**:4810–4815.
- Levine, B., Q. Huang, J. T. Isaacs, J. C. Reed, D. E. Griffin, and J. M.

- Hardwick**, 1993. Conversion of lytic to persistent alphavirus infection by the *bcl-2* cellular oncogene. *Nature* **361**:739–742.
21. **Martz, E., and D. M. Howell**. 1989. CTL: virus control cells first and cytolytic cells second? DNA fragmentation, apoptosis and the prelytic halt hypothesis. *Immunol. Today* **10**:79–86.
 22. **Matoba, Y., W. S. Colucci, B. N. Fields, and T. W. Smith**. 1993. The reovirus M1 gene determines the relative capacity of growth of reovirus in cultured bovine aortic endothelial cells. *J. Clin. Invest.* **92**:2883–2888.
 23. **Matoba, Y., B. Sherry, B. N. Fields, and T. W. Smith**. 1991. Identification of the viral genes responsible for growth of strains of reovirus in cultured mouse heart cells. *J. Clin. Invest.* **87**:1628–1633.
 24. **McCrae, M. A., and W. K. Joklik**. 1978. The nature of the polypeptide encoded by each of the ten double-stranded RNA segments of reovirus type 3. *Virology* **89**:578–593.
 25. **Morozov, S. Y.** 1989. A possible relationship of reovirus putative RNA polymerase to polymerases of positive-strand RNA viruses. *Nucleic Acids Res.* **17**:5394.
 26. **Mustoe, T. A., R. F. Ramig, A. H. Sharpe, and B. N. Fields**. 1978. Genetics of reovirus: identification of the dsRNA segments encoding the polypeptides of the μ and σ size classes. *Virology* **89**:594–604.
 27. **Nibert, M. L., L. A. Schiff, and B. N. Fields**. 1996. Reoviruses and their replication, p. 1557–1596. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fields virology*, 3rd ed. Lippincott-Raven, Philadelphia, Pa.
 28. **Olsen, C. W., J. C. Kehren, N. R. Dybdahl-Sissoko, and V. S. Hinshaw**. 1996. *bcl-2* alters influenza virus yield, spread, and hemagglutinin glycosylation. *J. Virol.* **70**:663–666.
 29. **Oppenheim, R. W.** 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14**:453–501.
 30. **Razvi, E. S., and R. M. Welsh**. 1995. Apoptosis in viral infections. *Adv. Virus Res.* **45**:1–60.
 31. **Sarkar, G., J. Pelletier, R. Bassel-Duby, A. Jayasuriya, B. N. Fields, and N. Sonenberg**. 1985. Identification of a new polypeptide coded by reovirus gene S1. *J. Virol.* **54**:720–725.
 32. **Sentman, C. L., J. R. Shutter, D. Hockenbery, O. Kanagawa, and S. J. Korsmeyer**. 1991. *Bcl-2* inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* **67**:879–888.
 33. **Shaw, J. E., and D. C. Cox**. 1973. Early inhibition of cellular DNA synthesis by high multiplicities of infectious and UV-inactivated reovirus. *J. Virol.* **12**:704–710.
 34. **Shen, Y., and T. E. Shen**. 1995. Viruses and apoptosis. *Curr. Opin. Genet. Dev.* **5**:105–111.
 35. **Sherry, B., and M. A. Blum**. 1994. Multiple viral core proteins are determinants of reovirus-induced acute myocarditis. *J. Virol.* **68**:8461–8465.
 36. **Sherry, B., and B. N. Fields**. 1989. The reovirus M1 gene, encoding a viral core protein, is associated with the myocarditic phenotype of a reovirus variant. *J. Virol.* **63**:4850–4856.
 37. **Starnes, M. C., and W. K. Joklik**. 1993. Reovirus protein $\lambda 3$ is a poly(C)-dependent poly(G) polymerase. *Virology* **193**:356–366.
 38. **Tsujimoto, Y., and C. M. Croce**. 1986. Analysis of the structure, transcripts, and protein products of *bcl-2*, the gene involved in human follicular lymphomas. *Proc. Natl. Acad. Sci. USA* **83**:5214–5218.
 39. **Tyler, K. L., and B. N. Fields**. 1996. Reoviruses, p. 1597–1623. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fields virology*. Lippincott-Raven, Philadelphia, Pa.
 40. **Tyler, K. L., M. K. T. Squier, A. L. Brown, B. Pike, D. Willis, S. M. Oberhaus, T. S. Dermody, and J. J. Cohen**. 1996. Linkage between reovirus-induced apoptosis and inhibition of cellular DNA synthesis: role of the S1 and M2 genes. *J. Virol.* **70**:7984–7991.
 41. **Tyler, K. L., M. K. T. Squier, S. E. Rodgers, B. E. Schneider, S. M. Oberhaus, T. A. Grdina, J. J. Cohen, and T. S. Dermody**. 1995. Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein $\sigma 1$. *J. Virol.* **69**:6972–6979.
 42. **Virgin, H. W., K. L. Tyler, and T. S. Dermody**. 1997. Reovirus, p. 669–699. *In* N. Nathanson (ed.), *Viral pathogenesis*. Lippincott-Raven, Philadelphia, Pa.
 43. **Weiner, H. L., K. A. Ault, and B. N. Fields**. 1980. Interaction of reovirus with cell surface receptors. I. Murine and human lymphocytes have a receptor for the hemagglutinin of reovirus type 3. *J. Immunol.* **124**:2143–2148.
 44. **Weiner, H. L., M. L. Powers, and B. N. Fields**. 1980. Absolute linkage of virulence and central nervous system tropism of reoviruses to viral hemagglutinin. *J. Infect. Dis.* **141**:609–616.
 45. **Wyllie, A. H., J. F. R. Kerr, and A. R. Currie**. 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68**:251–306.
 46. **Yin, P., M. Cheang, and K. M. Coombs**. 1996. The M1 gene is associated with differences in the temperature optimum of the transcriptase activity in reovirus core particles. *J. Virol.* **70**:1223–1227.