JOURNAL OF VIROLOGY, Apr. 1998, p. 3018–3028 Vol. 72, No. 4
0022-538X/98/$04.00+0

Human Parvovirus B19 Nonstructural (NS1) Protein Induces Apoptosis in Erythroid Lineage Cells

STANLEY MOFFATT, NOBUO YAEGASHI, KOHTARO TADA, NOBUYUKI TANAKA, AND KAZUO SUGAMURA*

Department of Microbiology and Immunology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-77, Japan

Received 29 September 1997/Accepted 19 December 1997

Infection of erythroid-lineage cells by human parvovirus B19 is characterized by a gradual cytocidal effect. Accumulating evidence now implicates the nonstructural (NS1) protein of the virus in cytotoxicity, but the mechanism underlying the NS1-induced cell death is not known. Using a stringent regulatory system, we demonstrate that NS1 cytotoxicity is closely related to apoptosis, as evidenced by cell morphology, genomic DNA fragmentation, and cell cycle analysis with the human erythroleukemia cell line K562 and the erythropoietin-dependent megakaryocytic cell line UT-7/Epo. Apoptosis was significantly inhibited by an interleukin-1β (IL-1β)-converting enzyme (ICE)/CED-3 family protease inhibitor, Ac-DEVD-CHO (CPP32; caspase 3), whereas a similar inhibitor of ICE (caspase 1), Ac-YVAD-CHO, had no effect. Furthermore, stable expression of the human Bcl-2 proto-oncogene resulted in near-total protection from cell death in response to NS1 induction. Mutations engineered into the nucleoside triphosphate-binding domain of NS1 significantly rescued cells from NS1-induced apoptosis without having any effect on NS1-induced activation of the IL-6 gene expression which is mediated by NF-κB. Furthermore, using pentoxifylline, an inhibitor of NF-κB activation, we demonstrate that the NF-κB-mediated IL-6 activation by NS1 is uncoupled from the apoptotic pathway. This functional dissection indicates a complexity underlying the biochemical function of human parvovirus NS1 in transcriptional activation and induction of apoptosis. Our findings indicate that NS1 of parvovirus B19 induces cell death by apoptosis in at least erythroid-lineage cells by a pathway that involves caspase 3, whose activation may be a key event during NS1-induced cell death.

Human parvovirus B19 is a small single-stranded DNA virus that causes a wide variety of human diseases including fifth disease in children, arthritis in adults, chronic anemia in immunocompromised hosts, and probably also nonimmune hydrops fetalis (48). Broad studies of parvovirus B19 have been hampered by the strict specificity of the virus for erythroid-lineage cells, which is due in part to the limited distribution of its receptor, P antigen (5). So far, parvovirus B19 shows a cytotoxic effect on human primary erythroid-lineage cells in bone marrow (34), fetal liver (46), and *Cynomolgus* monkey bone marrow cells (14). The NS1 protein of other related viruses, rat H-1 virus (24) and minute virus of mice (21), has also been reported to possess cytotoxic activities. A variety of viruses are known to be cytotoxic for their host cells, and evidence is accumulating that virus-induced cytotoxicities result from programmed cell death, also known as apoptosis. Many viruses, including herpes simplex virus (22), human immunodeficiency virus type 1 (HIV-1) (25), influenza virus (29), and alphavirus (39), cause their host cells to undergo apoptosis. Most of these viruses encode proteins that, by themselves, could also initiate the apoptotic process; these include E1A protein of adenovirus (8), apoptin (VP3) of chicken anemia virus (19), Tat protein of HIV-1 (23), and Tax protein of human T-cell leukemia virus type 1 (HTLV-1) (47). Since it has been reported that NS1 of parvovirus B19 has a cytotoxic effect on a human erythroid cell line (34) and since parvovirus-infected cells have ultrastructural features resembling those associated with apoptosis (28), it is possible that NS1 is also an apoptosis-inducing factor.

Apoptosis is a physiological mechanism of cell depletion during differentiation and development (35); it defends hosts against emerging malignant cells (45) and may be viewed as a host response to virus infection. A wide range of studies have strongly implicated the interleukin-1β (IL-1β)-converting enzyme (ICE)/CED-3 family proteases as key participants in apoptotic cell death (11, 12, 31). On the other hand, Bcl-2, originally discovered as a result of its translocation to the immunoglobulin (Ig) heavy-chain enhancer in the t(14;18) translocation and present in more than 80% of human follicular lymphomas (6), is known to suppress apoptosis triggered by different stimuli in a wide variety of cells, including p53 (7), Epstein-Barr virus (17), adenovirus E1A (20), c-*myc* (43), ceramide (50), and growth factor withdrawal in hematopoietic cells (18, 41). In the present study, we first demonstrate that NS1 induces apoptosis in human erythroid cell lines and that the apoptosis is mitigated by a caspase 3 inhibitor and Bcl-2. NS1 is also known to be involved in viral replication and gene expression (9). We previously demonstrated that NS1 has a *trans*-acting transcriptional activity for the IL-6 cellular gene, which is mediated by NF-κB (26). The present study also suggests that there is a mechanistic dissociation between NS1-induced apoptosis and activation of IL-6 gene expression.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-77, Japan. Phone: 81-22-717-8096. Fax: 81-22-273-2787. E-mail: sugamura@mail.cc.tohoku.ac.jp.

MATERIALS AND METHODS

Cell culture. K562 is an erythroleukemia cell line, and UT-7/Epo is a megakaryocytic cell line adapted for growth in erythropoietin (a gift from Kirin

Brewery Pharmaceutical Research Laboratory, Tokyo, Japan)-containing medium (38). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and cultured in a humidified incubator at 37°C with a 7% CO_2–air atmosphere. A final concentration of 2 U of erythropoietin per ml was included in the medium for UT-7/Epo cells. The cells were starved to a final concentration of 0.2% FCS for different time intervals for examination of apoptosis.

Site-directed mutagenesis and plasmid construction. The Quikchange site-directed mutagenesis kit (Stratagene) was used to engineer both K334E and T332E mutations into the wild-type expression vector pOPRSV-NS1 with the following oligonucleotide primers: 5′-GGGCCGCCAAGTACTGGAGAAACA AACTTGGC-3′ (forward primer) and 5′-CCAAGTTTGTTTTTCCTTCAC TTGGGGGCCCATAAAACC-3′ (reverse primer) for the lysine-to-glutamate (K334E) change and 5′-GGTTTTATGGCCCCCAAGTGAAGGAAAAACAA ACTTGG-3′ (forward primer) and 5′-CCAAGTTTGTTTTTCCTTCACTTGG GGGCCCATAAAACC-3′ (reverse primer) for the threonine-to-glutamate (T332E) change. PCR was then performed as specified by the manufacturer. Successful mutagenesis was verified by sequencing.

DNA transfection. The procedure for the derivation of the *lac* repressor system for UT-7/Epo cells was the same as that previously reported for K562 (26), except that the cells were pulsed (352 μF and 500 V) and selected with hygromycin B (500 μg/ml; Sigma) for the *lac* repressor and neomycin (G418) (700 μg/ml; Sigma) for NS1. K562 and UT-7/Epo cells stably expressing NS1 under tight control of the repressor were isolated and designated KLNS and ULNS, respectively. All the cells expressed NS1 upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG). A human Bcl-2-expressing plasmid, pSVBT (provided by Y. Tsujimoto, Osaka University Medical School), was linearized with *Sac*I, and 20 μg of DNA was cotransfected with a blasticidine resistance gene into KLNS and ULNS cells under the same electroporating conditions as described for K562 (26) and selected for resistance to blasticidine (3 μg/ml). Cells stably expressing Bcl-2 were analyzed with a Bcl-2 monoclonal antibody (MAb), Bcl-2(100) IgG1 (Santa Cruz).

Luciferase assays. The cells were transfected by the DEAE-dextran method as reported previously (26). IPTG at a final concentration of 10 mM was added for the last 24 h. The cells were lysed with 150 μl of lysis buffer for 10 min at room temperature and centrifuged, and the soluble extracts were recovered for a luciferase assay with a PicaGene assay kit (PKG-L100; Toyo Inc.). The light intensity was measured with a luminometer (LB9501; Berthold, Wildbad, Germany). The protein concentration was determined with a protein assay kit (Bio-Rad) and used for normalization of the luciferase assays.

Immunoblotting and immunoprecipitation. Total-cell lysates (5×10^6 cells) were obtained with RIPA buffer (pH 7.5) (10 mM Tris-HCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 1 mM EDTA, 10 μg of aprotinin per ml). The lysates were then immunoprecipitated with a polyclonal antiserum (Strategene) to the *lac* repressor or MAb ParNS1 specific for NS1 coupled to protein A-Sepharose beads and anti-mouse IgG (Zymed), and immunoprecipitates were separated by polyacrylamide gel electrophoresis as described previously (26). Human Bcl-2 was detected with Bcl-2 (100) mouse MAb (Santa Cruz).

Cell viability assay. Cells (5×10^5) were cultured for the indicated times in the appropriate medium supplemented with 0.2% FCS with or without IPTG induction. Viable-cell numbers were determined by trypan blue exclusion with a hemocytometer. The percent cell viability was calculated by finding the ratio between viable cell number and total cell number. To analyze the effect of the caspase inhibitors, the cells were preincubated with various concentrations of the tetrapeptide caspase 1 ICE inhibitor, acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO), and the caspase 3 (CPP32/Yama/Apopain) inhibitor, acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) (Peptide Institute, Osaka, Japan) for a maximum of 72 h, and the viability was again determined by trypan blue exclusion.

DNA fragmentation analysis. DNA fragmentation was assessed as previously described (15) with minor modifications. Briefly, 3×10^6 cells were washed with phosphate-buffered saline (PBS) and pre-fixed in 70% ethanol. The cells were pelleted, ethanol was removed, and the cells were resuspended in 50 μl of phosphate-citrate buffer (14 parts 0.2 M Na_2HPO_4, 1 part 0.1 M citric acid) at room temperature for 30 min. Upon centrifugation, the supernatants were removed, extracted with phenol-chloroform (1:1), and precipitated with 2.5 volumes of pure ethanol. The pellet was rinsed in 70% ethanol, dried, and dissolved in 20 μl of Tris-HCl–1 mM EDTA (pH 7.5)–RNase A (100 μg/ml). After incubation for 1 h at 37°C, the supernatants were loaded onto a 1.5% agarose gel for electrophoresis. The DNA bands were then visualized under UV light.

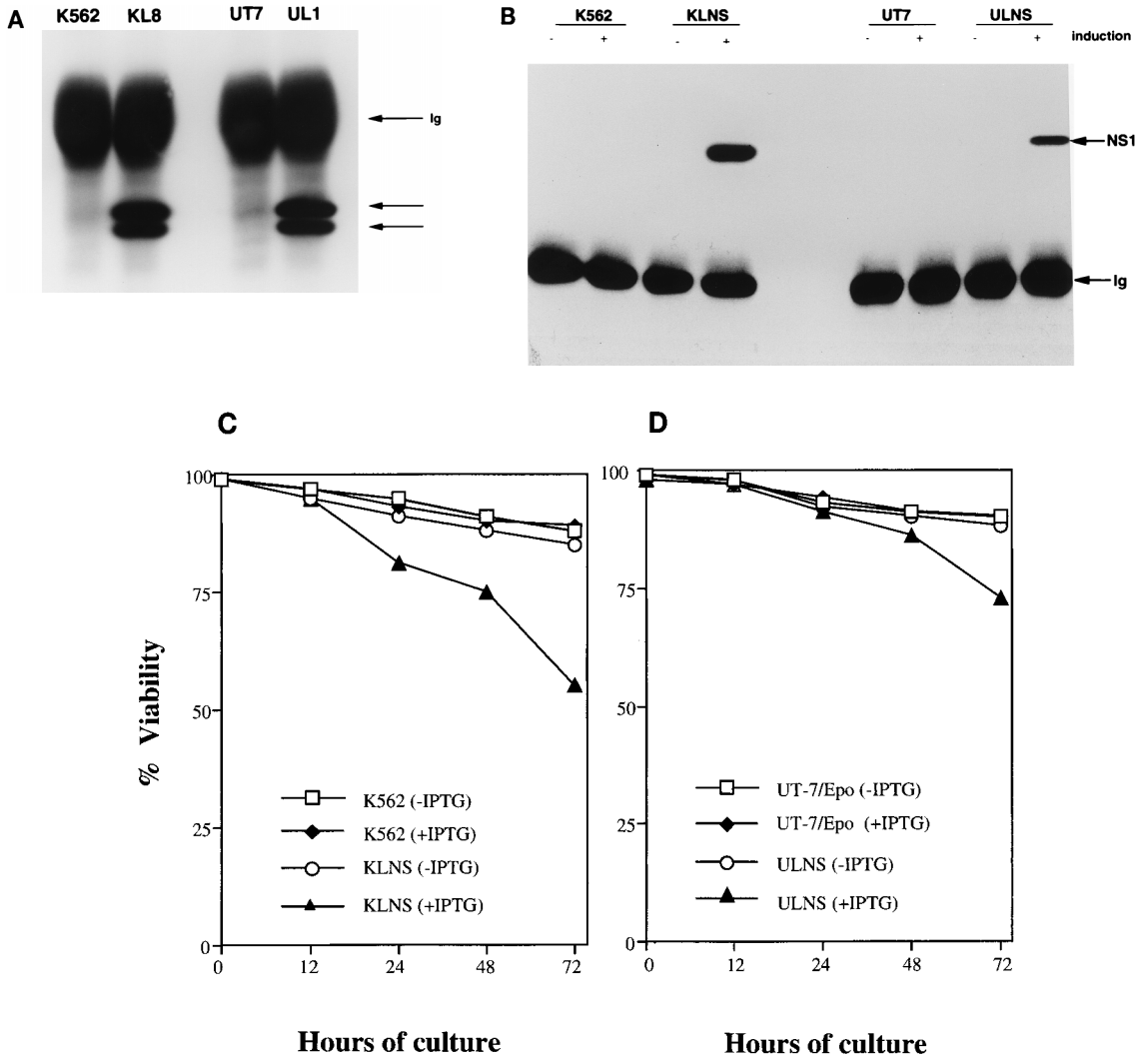
Annexin V-FITC and flow cytometry. For flow cytometry, we relied on the phospholipid-binding affinity of annexin V for the cell membrane phospholipid phosphtidylserine, which was translocated to the plasma membrane of cells undergoing apoptosis (42). Annexin V-FITC was then used to quantitatively determine the percentage of cells undergoing apoptosis. Cells (10^6) were washed twice with phosphate-buffered saline (PBS), resuspended in 100 μl of binding buffer (10 mM HEPES-NaOH [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$), stained with 5 μl of fluorescein isothiocyanate (FITC)-conjugated annexin V (Pharmingen) and 10 μl of propidium iodide (PI) (50 μg/ml) to measure the DNA content per nucleus, vortexed gently, and incubated for 15 min at room temperature in the dark. Labelled cells were subjected to analysis with a FACSort (Becton-Dickinson, Mountain View, Calif.) flow cytometer for measurement of fluorescence. Compartments were established so that fractions of intact cells (double negative for PI and annexin V), early-phase apoptotic cells (PI positive), late-phase apoptotic cells (double positive), and necrotic cells (annexin V positive) were gated in compartments 1, 2, 3, and 4 respectively.

TUNEL immunostaining. Terminal deoxynucleotidyltransferase end labeling (TUNEL) was performed as reported previously (40). In brief, biotin 16-dUTP is added to the double- and single-stranded DNA by using terminal deoxynucleotidyltransferase with an assay kit (GIBCO BRL) to detect the free 3′ ends of newly cleaved DNA in situ. Goat anti-biotin followed by biotinylated anti-goat IgG and FITC-avidin was then used to immunostain the nuclei with 3′-OH ends of cleaved DNA. Approximately 3×10^5 cells were seeded on polyethyleneimine-coated eight-chamber glass slides at room temperature overnight and fixed in 4% formalin in PBS for 1 h, 0.5% Triton X-100 for 10 min, 1 mg of RNase A per ml for 1 h, and 500 ng of proteinase K for 1 h, with each step followed by washing in PBS. The slides were mounted with 50% glycerol in PBS (pH 8.5). Fluorescence photography was performed with a BX60 Olympus fluorescence confocal microscope. Image-analyzed terminal deoxynucleotidyltransferase-stained cells were then examined for FITC- and PI-stained nuclei by observation of cell morphology followed by photography.

RESULTS

NS1 expression is adequate for initiation of apoptosis in serum-starved erythroleukemia cells. Taking into consideration the specificity of B19 parvovirus infection in erythroid-lineage cells, K562 and UT-7/Epo cells were used to examine the cytotoxic activity of NS1. We had noted previously the impossibility of generating cell lines stably expressing NS1 because of its cytotoxic activity. The *Escherichia coli lac* repressor-operator system we reported previously (26) was then used to control NS1 expression. Figure 1A shows the expression of the *lac* repressor protein in *lac*-derived cells but not in the parental cells. The expression of NS1 in newly derived clones of K562 (KLNS) or UT-7/Epo (ULNS) under the control of IPTG was observed only under inducing conditions (Fig. 1B). These cells were quiescent and displayed no significant level of cell death at or before the induction of NS1 expression. The cells were induced to express NS1, and the level of cell death was monitored over time together with that in the parental cells under serum starved conditions (Fig. 1C and D). The cells showed moderate death (23 and 45% for ULNS and KLNS cells, respectively) in repeated experiments; nevertheless NS1 was still expressed even after 72 h upon induction (data not shown). In our initial experiments, we observed that the initiation of cell death was unusually slow, from 5 to 7 days, in the presence of optimum concentration of serum (data not shown), suggesting that NS1 may potentially trigger cell death in the absence of growth factors. Therefore, we attempted to explore the possibility that death of these erythroid cells after the induction of NS1 occurred as a result of apoptosis. Induction of KLNS and ULNS cells with IPTG generated a death pattern which was apoptotic in appearance, with cell rounding, chromatin condensation, cytoplasmic blebbing, and the characteristic DNA fragmentation into a 180- to 200-bp ladder as shown by gel electrophoresis (Fig. 1E). Parental cells under the same conditions did not cause any demonstrable DNA laddering (data not shown). To further demonstrate apoptosis in these cells, we used TUNEL staining of free 3′-OH ends of cellular DNA to identify cells in situ with fragmented nuclear DNA (i.e., apoptotic nuclei). Using this method, we detected cells positively stained for fragmented DNA (Fig. 1F). These results suggest that NS1 mediates the apoptosis of these erythroid cell lines.

Exogenous expression of Bcl-2 results in nearly total protection from apoptosis. The Bcl-2 proto-oncogene is known to be highly effective in blocking many apoptotic pathways but not others. Our initial experiments revealed that neither K562 nor UT-7/Epo parental cells expressed detectable levels of Bcl-2



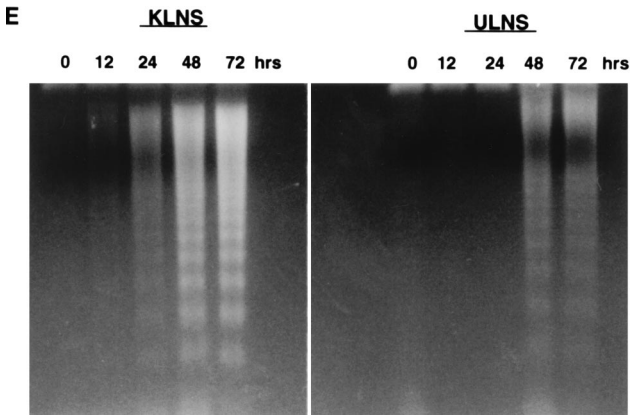


FIG. 1. (A and B) Stable introduction of the *lac* repressor (A) is adequate for efficient regulation of NS1 protein expression (B) in both K562 and UT-7/Epo cells. Total-cell lysates from parental and *lac* transfectant 5×10^6 cells were analyzed by immunoblot analysis with polyclonal antiserum to the *lac* repressor or MAb ParNS1 in the presence or absence of IPTG. Double arrows in panel A indicate the doublet bands of the *lac* repressor. (C to F) Expression of NS1 under low-serum conditions is sufficient to induce apoptosis, as measured by percent cell viabilities (C and D) DNA fragmentation (E), and TUNEL immunostaining (F). Cell viabilities were measured for both KLNS and ULNS cells by trypan blue exclusion with approximately 5×10^5 cells. Data are the means of triplicate determinations with very similar results. TUNEL immunostaining (F) shows the parent (panels 1), K334E mutants (panels 2), T332E mutants (panels 3), and the wild-type NS1 (panels 4) cells with (lower panels) or without (upper panels) IPTG induction. DNA fragmentation and TUNEL-positive cells (magnification, ×380) were detected as described in Materials and Methods.

and that NS1 did not affect the expression of Bcl-2 in these cells (Fig. 2A). Furthermore, Bcl-2 did not affect the steady-state levels of NS1 when the level of NS1 expression in NS1 and NS1/Bcl-2 transfectants was compared. To determine whether NS1-induced apoptosis represents a Bcl-2-inhibitable pathway, we generated stable Bcl-2 transfectants in both KLNS and ULNS cells and examined them for NS1-induced apoptosis. The transfectants were treated with IPTG for 72 h, and cell viability was determined. The effect of Bcl-2 was distinctly observable within 48 h of culture, leading to 49 and 43% rescue of apoptosis for KLNS and ULNS cells, respectively. At 72 h of culture, there was a significant suppression of cell death (74 and 72% for KLNS and ULNS cells, respectively) (Fig. 2B and C). Although some cell death still occurred after 72 h, a pronounced difference between Bcl-2-positive and -negative transfectants was observed, resulting in nearly total protection from

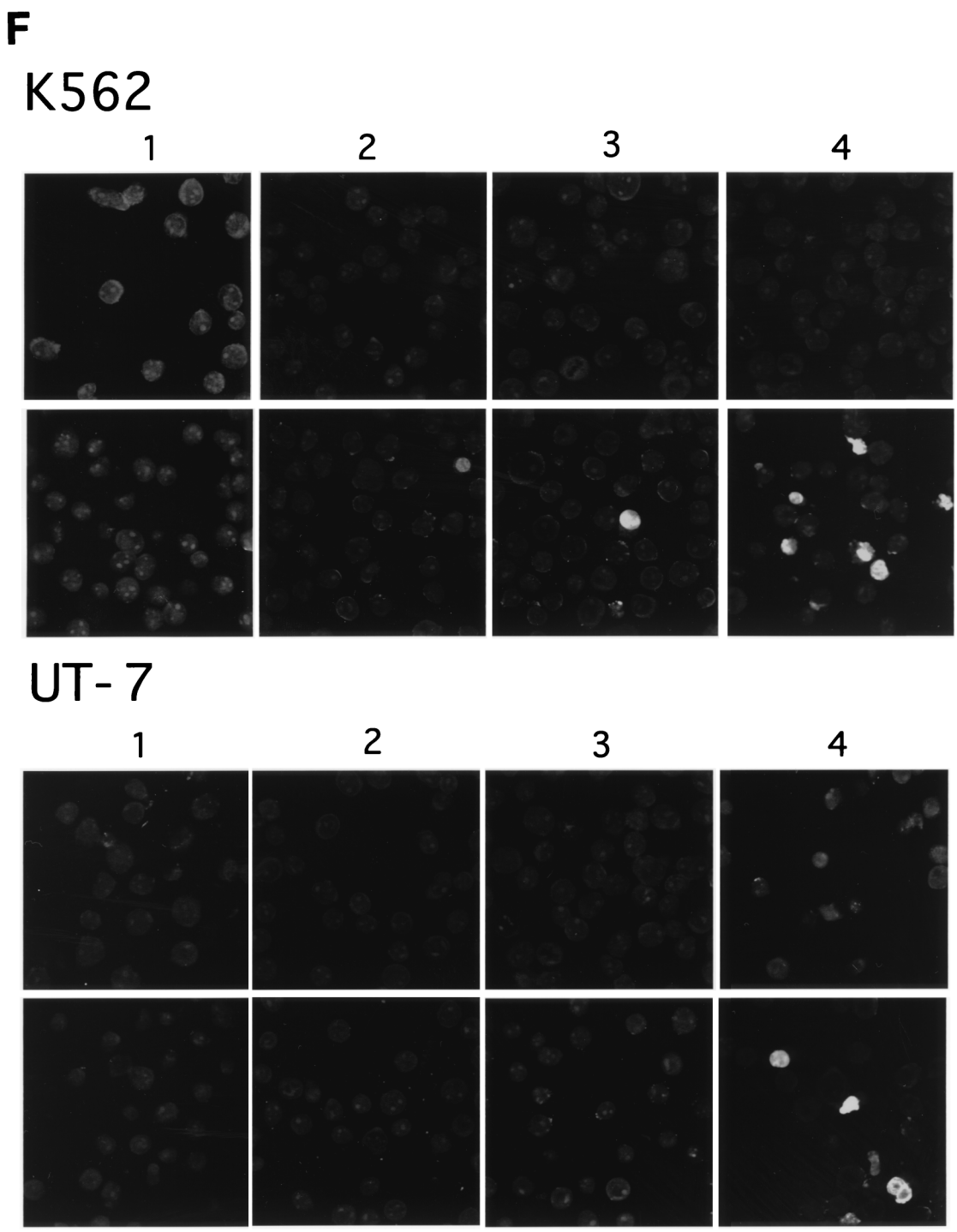


FIG. 1—*Continued.*

apoptosis by exogenous introduction of Bcl-2. These results indicate that NS1 induces cell death in a Bcl-2-inhibitable pathway.

Selective inhibition of NS1-induced apoptosis by inhibition of CPP32/caspase 3 but not ICE/caspase 1. To investigate whether the ICE-like proteases (caspases) participate in apoptosis mediated by NS1 in the erythroid cell lines, Ac-YVAD-CHO and Ac-DEVD-CHO, which are inhibitors of caspase 1 and caspase 3, respectively, were examined for their effects on the NS1-induced apoptosis of KLNS and ULNS cells. They were treated with IPTG to induce NS1 expression, and cell viabilities were assessed after 72 h of culture under serum-starved conditions in the presence or absence of various concentrations of the caspase inhibitors. The caspase 3 inhibitor, Ac-DEVD-CHO, blocked the ability of NS1 to induce apopto-

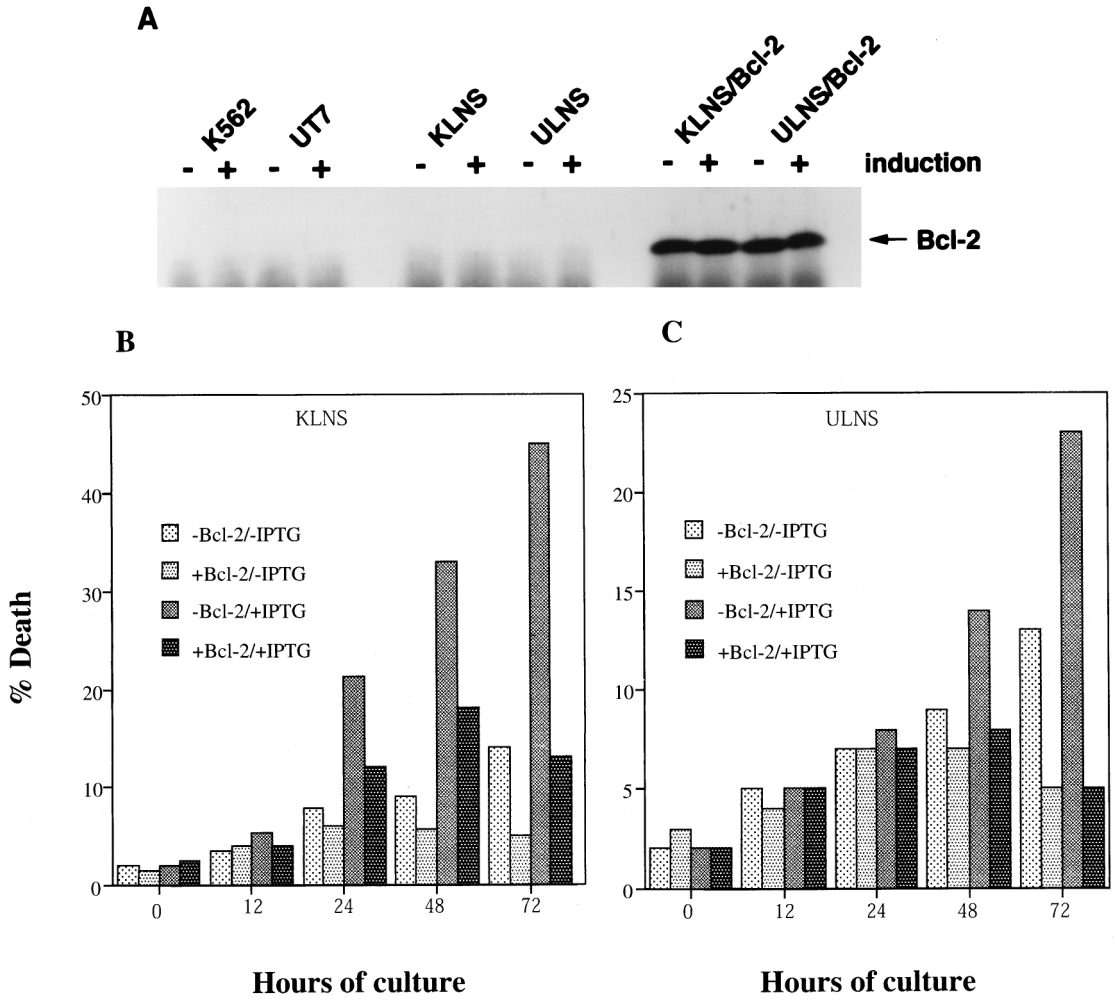


FIG. 2. Stable expression of human Bcl-2 significantly reduces NS1-induced cell death. (A) Representative Bcl-2-stable transfectants of both KLNS and ULNS cells were detected with a Bcl-2 MAb, Bcl-2 100, by immunoblotting with 5×10^6 cells in the presence or absence of IPTG. The position of the Bcl-2 protein corresponding to approximately 26 kDa is indicated. (B and C) The kinetics of Bcl-2 inhibition of NS1-induced cell death was measured by trypan blue exclusion for both KLNS (B) and ULNS (C) cells with 5×10^5 cells for each cell line. The experiments were performed twice, with no significant difference between results.

sis in KLNS and ULNS cells in a dose-dependent fashion, but the caspase 1 inhibitor, Ac-YVAD-CHO, did not (Fig. 3). These data suggest that NS1 initiates apoptosis by activating caspase 3 and that caspase 1 may not be essential.

Disruption of the NTP-binding domain is expected to impair apoptosis. NS1 contains a nucleoside triphosphate (NTP)-binding motif in the middle of the protein, and the cytotoxicity mediated by NS1 is abolished by various mutations within the NTP-binding domain (16, 27). We extended this line of study by asking whether disruption of this domain could ameliorate NS1-mediated apoptosis by designing two single-point mutations; K334E (lysine to glutamate at position 334) and T332E (threonine to glutamate at position 332) with the original NS1 expression vector as a template. Upon verification of the DNA sequences, the mutations were transfected into KL8 and UL1 cells expressing the *lac* repressor gene, and the mutants were subjected to selection for neomycin resistance. After screening, mutant NS1-expressing cells were further selected for cloning. Representative clones expressing mutant NS1 upon induction with IPTG were designated KLNS.K334E and KLNS.T332E (for K562) and ULNS.K334E and ULNS.T332E (for UT-7/Epo), respectively (Fig. 4A and B). We then assessed the kinetics of cell death with respect to the wild-type NS1 transfectants. The mutants with a disruption in the NTP-binding domain dramatically suppressed the cytotoxic activity of NS1, although complete abrogation of cell death was not observed, whereas the wild-type NS1-carrying cell lines showed a continued increase in cytotoxicity (Fig. 4C and D). Further quantitation of the progression of apoptotic cells in both parental cells and NS1 transfectants by annexin V-FITC staining showed a significant increase in the population of apoptotic (double-positive) cells (37.7%) in the wild-type NS1-transfected cells, in contrast to 2.3, 2.3, 2.1, and 2.7% double-positive cells for K562, KLNS.K334E, KLNS.T332E, and KLNS.Bcl-2 cells, respectively. Similarly, the population of apoptotic ULNS cells were 20.2%, compared with 2.2, 2.1, 2.0, and 2.2% for UT-7/Epo, ULNS.K334E, ULNS.T332E, and ULNS.Bcl-2 respectively (Fig. 4E). These results suggest that the NTP-binding domain of NS1 is essential for the induction of apoptosis and cell cycle arrest. UT-7/Epo and K562 cells and their respective NS1 transfectants do not express detectable levels of Fas by Western blotting (data not shown), and neither does a Fas ligand-blocking antibody (kindly provided by S. Nagata, Osaka University Medical School) block apoptosis by

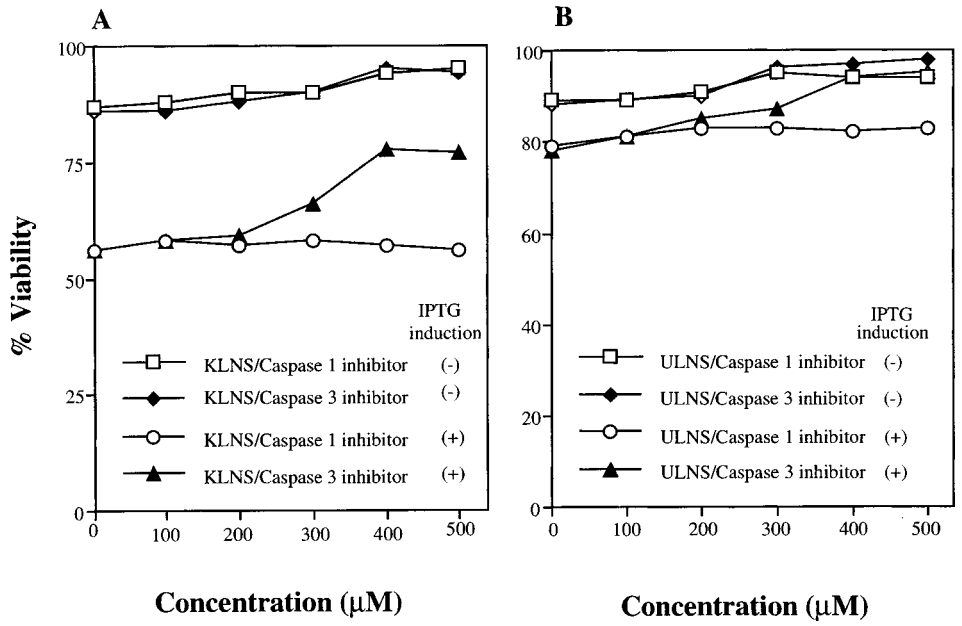


FIG. 3. CPP32 (caspase 3) but not ICE (caspase 1) inhibitor selectively represses NS1-induced apoptosis. About 5×10^5 of KLNS cells (A) or ULNS cells (B) were incubated with different concentrations of either ICE/caspase 1 (Ac-YVAD-CHO) or CPP32/caspase 3 (Ac-DEVD-CHO) inhibitor, and cell viabilities were quantitated after 72 h of culture as described in Materials and Methods. Data are the means of triplicate determinations with identical results.

NS1 (data not shown), indicating that NS1-induced cell death is not mediated by the Fas-Fas ligand system.

Distinct effects of pentoxifylline between NS1-induced IL-6 gene activation and apoptosis. We previously reported that human parvovirus B19 NS1 induced the activation of transcription of the IL-6 gene through the transcription factor NF-κB (26). We therefore examined the relationship between NS1-induced apoptosis and *trans*-acting activation of transcription. We first asked whether the NS1 mutants could by themselves *trans*-activate the wild-type IL-6 promoter. Both the wild type and the mutants of NS1 significantly induced the activation of the IL-6 promoter in transfectants of K562 and UT-7/Epo cells, although there were intercellular differences in fold induction (Fig. 5A and B). The fold induction was 5.2, 4.6, and 3.9 for KLNS, KLNS.K334E, and KLNS.T332E, respectively. Similarly, the fold induction was 3.6, 2.5, and 2.6 for ULNS, ULNS.K334E, and ULNS.T332E, respectively. Endogenous secretion of IL-6 into the supernatants of the mutant and wild-type NS1 transfectants after IPTG induction was also detected at comparable levels by enzyme-linked immunosorbent assays (Table 1), confirming the above observation. As expected, the induction of the IL-6 promoter carrying a mutation in the NF-κB site was dramatically inhibited by 4.7-, 3.9-, and 3.8-fold for KLNS, KLNS.K334E, and KLNS.T332E, respectively, and 2.5-, 3.5-, and 2.6-fold for ULNS, ULNS.K334E, and ULNS.T332E respectively, indicating the essential role of NF-κB in mediating IL-6 activation by NS1. Pentoxifylline (Ptx), a methyl xanthine derivative that is an antioxidant, has an inhibitory effect on NF-κB (36). We next examined the effect of Ptx on IL-6 gene activation by wild-type NS1 in KLNS and ULNS cells with both wild-type and mutant IL-6 promoters. Our results showed that the addition of Ptx was accompanied by a dose-dependent inhibition of IL-6 promoter activation (Fig. 5C and D), indicating that the activation of NF-κB contributes to IL-6 gene activation by NS1. To gain insight into the effect of Ptx on apoptosis by NS1, we used different concentrations of Ptx and monitored the cell viability after 72 h of culture with or without IPTG induction. The results show that Ptx does not block apoptosis (Fig. 5E and F), suggesting that NF-κB is dissociated from the apoptotic pathway mediated by NS1.

DISCUSSION

Previous studies have demonstrated that the cytopathic effect of parvoviruses positively correlated with the intracellular accumulation of the NS1 proteins of parvoviruses (21, 24, 33). These observations, together with the inability to generate stable NS1 transfectants, have raised the possibility that the NS1 proteins of parvoviruses were equipped with an intrinsic cytotoxic activity. Nevertheless, the major unanswered question is the mechanism of NS1 cytotoxicity. In this communication, we provide direct evidence that the erythroid-lineage cell lines K562 and UT-7/Epo in which NS1 was stably incorporated died by apoptosis in an IPTG-inducible fashion. IPTG by itself had no discernible effect on cell death, and there was no requirement for the other capsid proteins, VP1 and VP2, for cell death, suggesting that NS1 can induce apoptosis even in the absence of the capsid proteins.

Our initial observations obtained with our present system under normal cell culture conditions indicated that cell lysis begins after 5 to 7 days of continuous IPTG induction, suggesting a possible decay of an essential cell death factor whose replenishment is slowed in the presence of growth factors, such as serum. Another possibility is that a factor present in serum generates an intracellular signal which activates a pathway to suppress NS1-induced cytotoxicity (a study which is under way). Hence, our data is significant and serves as an essential paradigm for the actual mechanism of cell death by NS1 under normal physiological conditions. NS1-induced apoptosis was verified by DNA fragmentation, cell morphology, and annexin V-FITC staining. The results of this study thus elucidate the precise mechanisms involved in parvovirus B19 NS1-induced cell death.

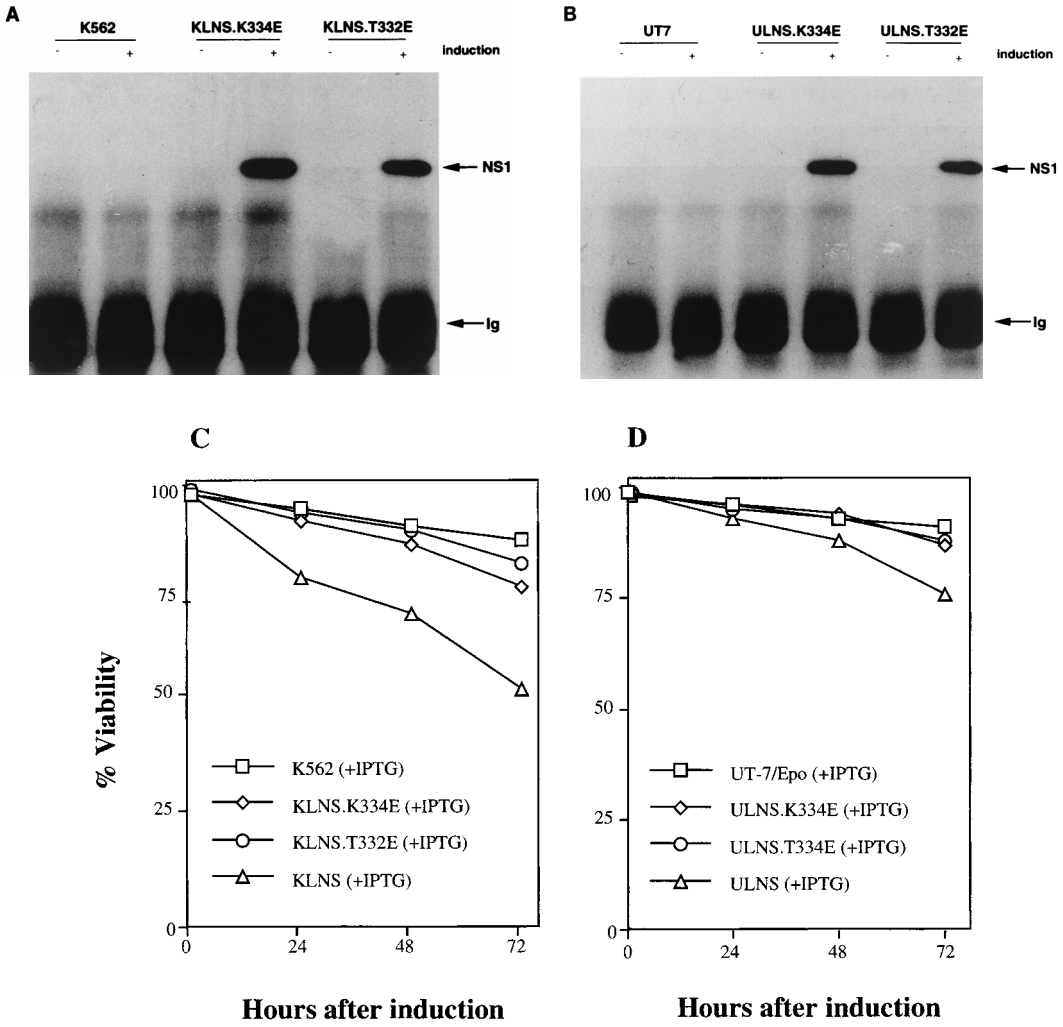


FIG. 4. Disruption of the NTP-binding domain abolishes apoptosis by NS1. Stable transfectants of K334E and T332E mutants of the NTP-binding domain of NS1 were obtained for both KLNS cells (A) and ULNS cells (B) by the *lac* repressor-operator system. The detection method was the same as described for Fig. 1B. The mutants are expressed only in the presence of IPTG. The kinetics of the rate of cell death (C and D) was determined in a similar manner to that in Fig. 1C and D, and the effect of K334E and T332E mutants on the progression of cell death (E), measured alongside those of the wild-type NS1 as well as the Bcl-2 transfectants, was determined as described in Materials and Methods. Cells cultured in the presence of IPTG under serum-starved conditions for 72 h were stained with annexin V-FITC and PI and analyzed for the degree of apoptosis (fluorescence) triggered by NS1. A total of 10^6 cells were analyzed in the assay to create each scatter diagram. Cells in compartments 1, 2, 3, and 4 represent double-negative, PI-positive, double-positive, and annexin V-positive cells, respectively. Double-positive cells indicate cells in the late phase of apoptosis, with fragmented DNA and destruction of the cellular membrane.

Apoptosis is also induced by other viral peptides, including E1A of adenovirus (8), Tat of HIV-1 (23) and Tax of HTLV-1 (47), as well as by a number of cellular proteins like c-Jun (4), p53 (7), and c-Myc (43) and by serum withdrawal (18), which are all blocked by the expression of Bcl-2. In the present study, we demonstrated that NS1-induced apoptosis of the erythroid cell lines is also inhibitable by Bcl-2. There was no significant difference between NS1 and NS1/Bcl-2 transfectants in the level of NS1 expression (data not shown), even though the Bcl-2 transfectants rescued the cells from apoptosis, indicating that NS1 expression is not affected by the cell cycle in these cells. The moderate levels of cell death in both K562 and UT-7/Epo cells are therefore not due to a down-regulation by Bcl-2 but reflect the activation of possible intracellular factors mediating NS1-induced cell death. Interestingly, even 72 h of culture, NS1 could still be detected (data not shown). Taken together, our results indicate that Bcl-2 coexpression circumvents NS1-induced arrest, suggesting that Bcl-2 restores normal proliferation of the cells even in the presence of NS1. However, other apoptotic systems have been reported to be independent of Bcl-2 (32, 37). The NS1-induced apoptosis may have a mechanism in common with apoptosis induced by HIV-1 Tat (23) and HTLV-1 Tax (47), because apoptosis mediated by all these viral peptides is detectable only under the serum-deprived conditions and is inhibitable by Bcl-2. The common cell death mechanism that possibly mediates apoptosis by these viral peptides may be suppressed by serum stimulation, suggesting the possible existence of an antiapoptotic factor(s) which is inducible upon stimulation of hematopoietic cells with serum. Since parental K562 and UT-7/Epo cells, as well as their NS1 transfectants, do not express detectable levels of Bcl-2 and Bcl-x_L, also an antiapoptotic factor (3), even after stimulation with serum (data not shown), serum stimulation of cells may induce a common suppressive factor(s) for the viral peptide-induced apoptosis which is distinct from Bcl-2 and Bcl-x_L. On the other hand, NS1 transfectants of Raji cells did

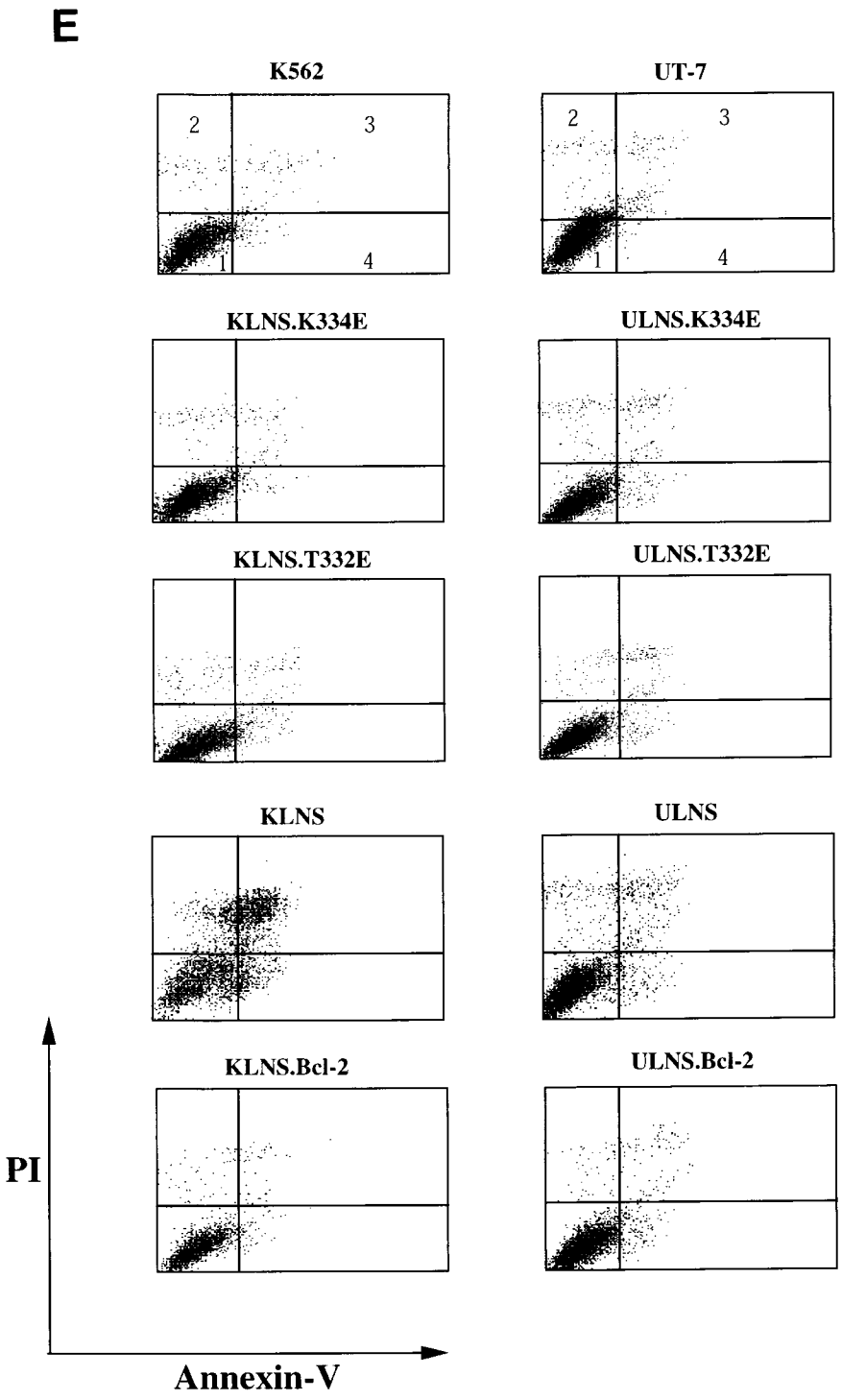


FIG. 4—*Continued.*

not show any detectable level of apoptosis upon NS1 induction (data not shown). The reason why NS1 does not trigger apoptosis in Raji transfectant cells may be the higher level of Bcl-2 expression (data not shown) and the existence of other anti-apoptotic factors.

In mammals, different homologs of the ICE/Ced-3 protease (caspases) family are required for induction of apoptosis in different cell lines (31, 49). It has recently been reported that ICE-related proteases, CPP32/caspase 3 and Mch2/caspase 6, are the major active caspases in apoptotic cells and are activated in response to distinct apoptosis-inducing stimuli in a wide variety of cells (13). We therefore tested whether NS1 induces apoptosis through activation of proteases, particularly ICE/caspase 1 and CPP32/caspase 3. Incubation of KLNS and ULNS cells with effective doses of caspase 3 inhibitor significantly rescued the cells from NS1-induced apoptosis, whereas

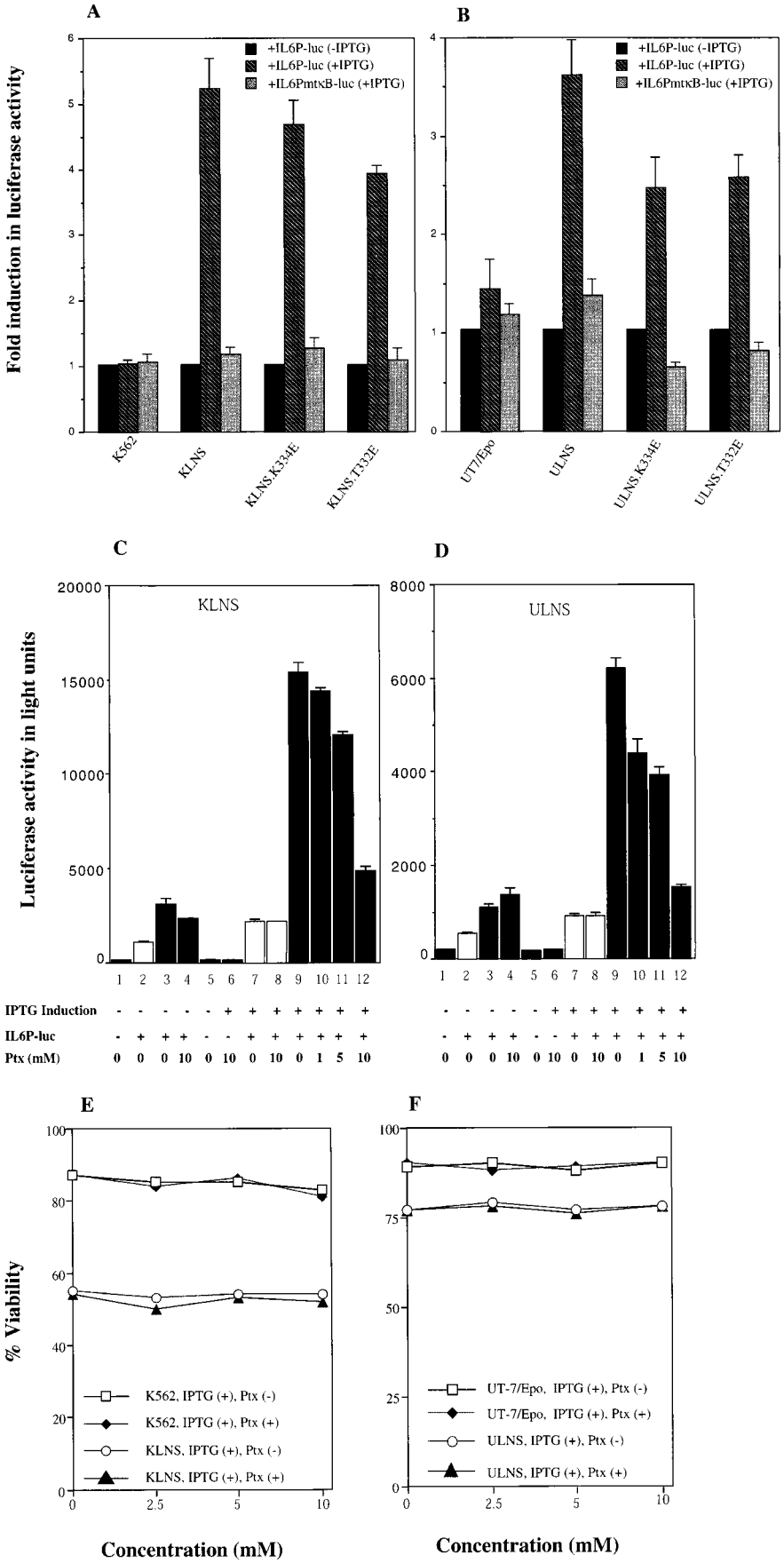

A
B
Fold induction in luciferase activity
+IL6P-luc (-IPTG)
+IL6P-luc (+IPTG)
+IL6PmtκB-luc (+IPTG)
K562
KLNS
KLNS.K334E
KLNS.T332E
UT7/Epo
ULNS
ULNS.K334E
ULNS.T332E
C
D
KLNS
ULNS
Luciferase activity in light units
IPTG Induction
IL6P-luc
Ptx (mM)
E
F
% Viability
K562, IPTG (+), Ptx (-)
K562, IPTG (+), Ptx (+)
KLNS, IPTG (+), Ptx (-)
KLNS, IPTG (+), Ptx (+)
UT-7/Epo, IPTG (+), Ptx (-)
UT-7/Epo, IPTG (+), Ptx (+)
ULNS, IPTG (+), Ptx (-)
ULNS, IPTG (+), Ptx (+)
Concentration (mM)
Concentration (mM)

FIG. 5. Effect of Ptx on NS1-mediated gene activation and apoptosis. (A and B) A 5-μg portion of the IL-6 gene promoter with or without the mutant NF-κB-binding site was transiently transfected by the DEAE-dextran method into 5×10^6 cells of the mutant and wild-type NS1-stable transfectants of both K562 (A) and UT-7/Epo (B) cells. Then 10 mM IPTG was added to the cultures for the last 24 h, and the cultures were harvested and assayed for luciferase activity. Fold inductions were calculated as the ratios between normalized results with IPTG and those without IPTG. (C and D) Ptx dose-dependently inhibits NS1-mediated IL-6 gene activation induced by NS1. Cells were transfected with the IL-6 promoter with (open bars) or without (solid bars) the mutant NF-κB site, and luciferase activities were determined after 48 h of culture. Incubation with IPTG and Ptx is described in Materials and Methods. The results are shown as the means and standard deviations for three identical experiments. (E and F) Ptx does not block NS1-induced apoptosis. Cells were induced with IPTG in the presence (solid symbols) or absence (open symbols) of different concentrations of Ptx, and cell viabilities were determined after 72 h of culture. Cell death was quantitated by trypan blue exclusion, and there was no significant difference in the results of duplicate experiments.

ICE/caspase 1 inhibitor had virtually no effect on apoptosis. While admitting that our assay system does not provide information about the exact mechanism of action of these caspases in eliciting cell death, it nevertheless identifies the pool of caspase 3-like proteases activated in NS1-mediated cell death and hence lays a solid foundation for future examination of the action of caspase 3 in NS1-induced apoptosis. Our data agree with recent evidence that caspase 1 itself is unlikely to be a major participant in cell death, since targeted disruption of the *Ice* gene in mice did not dramatically alter the phenotype of these animals (10, 49). Caspase 3 activation in NS1-induced apoptosis is intruiging and prompts the speculation that a defect in caspase 3 processing may be directly related to cell proliferation or transformation and may thus provide an attractive therapeutic strategy for severe diseases caused by parvovirus B19 infection, such as aplastic crisis. The caspase 1-caspase 3 cascade is thought to be the main pathway for apoptosis signaling. Therefore, if NS1-induced apoptosis utilizes this pathway, caspase 1 may lie far upstream of the cascade activation site of NS1 in the pathway. Alternatively, it is possible that NS1 induces the activation of caspase 3 in a pathway independent of the caspase 1-caspase 3 cascade. K562 and UT-7/Epo and their respective NS1 transfectants do not express Fas as shown by both Western blotting and flow cytometric analysis (data not shown), neither does a Fas ligand-blocking antibody block NS1-mediated apoptosis, implying that the Fas-Fas ligand pathway is not involved in NS1-induced cell death.

In an attempt to define the functional domain for cell death, we designed point mutations into the NTP-binding domain of the NS1 open reading frame. This investigation was prompted by the reported role of this domain in NS1-induced cytotoxicity (27). In the present study, mutation of threonine 332 or lysine 334 to glutamate greatly but not completely reduced the cytotoxicity of NS1. This is in contrast to an earlier report, where these mutations resulted in near 100% suppression of cytotoxicity by NS1. One reason for this discrepancy may be the stringency of uptake of trypan blue in quantitating cell death as opposed to colony formation as reported (27). These results are nevertheless consistent with an earlier report and implicate the NTP-binding domain in the apoptotic function of NS1.

TABLE 1. IL-6 induction into the supernatants of mutant or wild-type NS1 transfectants in the absence or presence of IPTG

Cell line	Concn of induced IL-6 (pg/ml)[a]	
	Without IPTG induction	With IPTG induction
None (medium)	0.2	0.2
UT-7/Epo	1.6	1.7
ULNS.K334E	1.6	71.5
ULNS.T332E	1.6	70.4
ULNS	1.5	76.3
None (medium)	0.2	0.2
K562	1.7	1.7
KLNS.K334E	1.6	81.7
KLNS.T332E	1.6	80.3
KLNS	1.7	88.7

[a] Cells (3×10^6) were cultured without or with 10 mM IPTG for 24 h. Culture supernatants were recovered for the IL-6 assay by sandwich enzyme-linked immunosorbent assay. Values are the mean concentrations derived from duplicate experiments.

Another point of interest was to see the relationship between the *trans*-acting transcriptional activation and apoptosis of B19 parvovirus NS1. We previously reported a novel function of B19 parvovirus NS1 in *trans*-activating a cellular gene, the IL-6 gene (26). Mutations of the NTP-binding domain of NS1 proteins of rat H-1 virus (24) and the minute virus of mice (21) affect viral DNA replication and transcription. These observations, together with the significant involvement of the NTP-binding domain of B19 parvovirus NS1 in cytotoxicity, suggest that the NTP-binding domains of parvoviruses may influence key functions in *trans*-activation as well as cytotoxicity. There was, however, no significant difference between the NTP-binding domain of mutant and wild-type of B19 parvovirus NS1 in *trans*-activating the IL-6 promoter in transient-transfection assays, indicating that this assumption does not seem to be the case in the *trans*-activation function of B19 parvovirus NS1. Consistent with this, we believe that while the NS1 proteins of mouse and human parvoviruses may be structurally similar, there are likely to be functional differences between them as reported for their *trans*-activation activities (24, 26, 30). Furthermore, the NF-κB binding site was indispensable for both the mutant and wild type to *trans*-activate the IL-6 promoter, as evidenced by Ptx inhibition assays; however, inhibition of NF-κB activation had no apparent effect on cytotoxicity. These results suggest that the NF-κB pathway for IL-6 *trans*-activation is dissociated from the apoptotic pathway. Our results are interesting in the light of the emerging role of NF-κB as an antiapoptotic transcription factor (1, 2, 44) and suggest that a rather complex cascade is involved in the execution of B19 NS1 biochemical functions. Work is under way in our laboratory to find the precise mechanism used by other transcription factors involved in rescuing cells from NS1-induced apoptosis.

ACKNOWLEDGMENTS

We thank Setsuya Aiba, Dermatology Department, Tohoku University School of Medicine, and Hiroko Kato and Satoshi Ijuin of Leica Co., Tokyo, Japan for excellent assistance with confocal microscopy. We are also indebted to S. Nagata, Osaka University Medical School, for provision of the Fas and Fas ligand expression plasmids as well as for the Fas ligand-blocking antibody.

This work was supported in part by CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation (JST); a grant-in-aid for scientific research on priority areas from the Ministry of Education, Science, Sports and Culture of Japan; and a grant from the special coordination funds of the Science and Technology Agency of Japan.

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