JOURNAL OF VIROLOGY, Oct. 1997, p. 8014–8018 Vol. 71, No. 10
0022-538X/97/$04.00+0

The Major Antigenic Protein of Infectious Bursal Disease Virus, VP2, Is an Apoptotic Inducer

ARMANDO FERNÁNDEZ-ARIAS,[1,2] SIOMARA MARTÍNEZ,[2] AND JOSE F. RODRÍGUEZ[1]*

Centro Nacional de Biotecnología, Cantoblanco, 28049 Madrid, Spain,[1] and Centro Nacional de Sanidad Agropecuaria, San José de las Lajas, La Habana, Cuba[2]

Received 28 April 1997/Accepted 28 May 1997

Infectious bursal disease virus (IBDV) is the causative agent of an economically important poultry disease. Vaccinia virus recombinants expressing the IBDV mature structural capsid proteins VP2 and VP3 were generated by using vectors for inducible gene expression. Characterization of these recombinant viruses demonstrated that expression of VP2 leads to induction of apoptosis in a variety of mammalian cell lines. Transfection of cell cultures with a expression vector containing the VP2 coding region under the control of the immediate-early promoter-enhancer region of human cytomegalovirus also triggers programmed cell death. The apoptotic effect of VP2 is efficiently counteracted by coexpression of the proto-oncogene *bcl-2*. The results presented demonstrate that VP2 is a bona fide apoptotic inducer. Evaluation of the significance of this finding for the virus life cycle must await further research.

Infectious bursal disease virus (IBDV), the prototype member of the *Avibirnavirus* genus of the *Birnaviridae* family (11, 12), is the causative agent of a highly infectious disease affecting young chickens. Two different serotypes (I and II) of IDBV have been identified. All known pathogenic IBDV strains belong to serotype I, whereas serotype II groups viruses capable of infecting chickens and turkeys but which lack clinical relevance. The cellular targets for IBDV replication are found in lymphoid organs, mainly the bursa of Fabricius. Chickens infected with IBDV develop an immune deficiency caused by depletion of B-lymphocyte precursors. This increases susceptibility to infections with opportunistic pathogens and reduces the growth rate of surviving animals (for reviews, see references 2 and 21). Recent reports have shown that infection with pathogenic serotype I IBDV strains leads to the induction of apoptosis both in vivo and in vitro (20, 35–37). It has been suggested that induction of apoptosis in infected lymphoid cells may contribute to the immune-suppressive effect of IBDV (36).

IBD affects all major poultry-producing areas worldwide and has an important economical impact (29). In recent years, the number of IBDV outbreaks with high mortality, caused by so-called "very virulent" IBDV strains, has been steadily increasing (4, 26).

IBDV virions are nonenveloped icosahedrons with a diameter of 60 to 65 nm (17). The three-dimensional structure of the IBDV capsids has been recently determined (3). The virus genome is formed by two segments of double-stranded RNA. Segment A (3.2 kb) contains two open reading frames (ORFs), A1 and A2. ORF A1 codes for a polyprotein of 108 kDa that, after proteolytic processing, yields three mature polypeptides: VP2 (37 to 40 kDa), VP3 (30 to 32 kDa), and VP4 (22 kDa). VP2 and VP3 form the virus capsid, and VP4 is responsible for the cleavage of the polyprotein (19, 25). ORF A2 codes for a 16-kDa protein of unknown function (27). Segment B (2.7 kb) codes for VP1 (90 kDa), a protein with RNA-dependent RNA polymerase activity that is encapsidated bound to the ends of both genomic RNA fragments (25).

The only practical method for the control of IBD is the use of vaccines consisting of either inactivated or attenuated virus. We sought to develop a system for the production of empty IBDV capsids that may eventually be applied as a subunit vaccine. Our strategy to achieve this goal was based on the expression of the mature IBDV capsid proteins VP2 and VP3 in mammalian cells by using the vaccinia virus (VV)-inducible expression system VOTE (38).

The IBDV Soroa strain, a pathogenic serotype I virus isolated in Cuba, was the starting genetic material for this study. The virus was adapted to grow in chicken embryo fibroblasts (CEF) and purified as previously described (14). Genomic RNA fragments were isolated from purified virus by phenol-chloroform extraction after treatment with proteinase K (31). A cDNA corresponding to the VP2 polypeptide was generated by reverse transcription-PCR (RT-PCR), using this genomic RNA as template and the primers 5′-ATATATGAATTCGA TCGCATCGATGACAAACCTGTCAGATCAAACCCAG CAG and 5′-CGCGCTCGAGTTACCTTATGGCCCGGATT ATGTCT according to a previously described protocol (15). After PCR, the DNA fragment, containing the first 452 codons of the polyprotein followed by an artificial stop codon, was subjected to digestion with *Cla*I, treatment with Klenow enzyme, and restriction with *Xho*I. The DNA fragment was then ligated to pVOTE.2 (38) (previously cleaved with *Nde*I, treated with Klenow enzyme, and subjected to digestion with *Xho*I) to generate the plasmid pVOTE.2/VP2. Similarly, the region corresponding to VP3 was generated by RT-PCR using the primers 5′-GCGCCATGGGTTTCCCTCACAATCCACGCGAC and 5′-GCGCGGATCCTCACTCAAGGTCCTCATCAGAG ACA. The resulting cDNA fragment contained the last 289 codons of the polyprotein preceded by an artificial ATG to allow for initiation of translation. The PCR-generated VP3 DNA fragment was restricted with *Bam*HI, partially digested with *Nco*I, and cloned into pVOTE.1 (38) which had been previously digested with the same restriction enzymes, giving rise to the plasmid pVOTE.1/VP3. The plasmid vectors pVOTE.2/VP2 and pVOTE.1/VP3 were purified and utilized to generate the recombinant viruses VT7LacOI/VP2 and VT7LacOI/VP3, respectively. Generation and amplification of recombinant viruses was carried out as previously described (13). The plasmid vectors pVOTE.1 and pVOTE.2 as well as the VV recombinant VT7LacOI were kindly provided by Bernard Moss (NIH, Bethesda, Md.). The only difference between

* Corresponding author. Phone: 34-1-5854549. Fax: 34-1-5854506. E-mail: jfrodrig@cnb.uam.es.

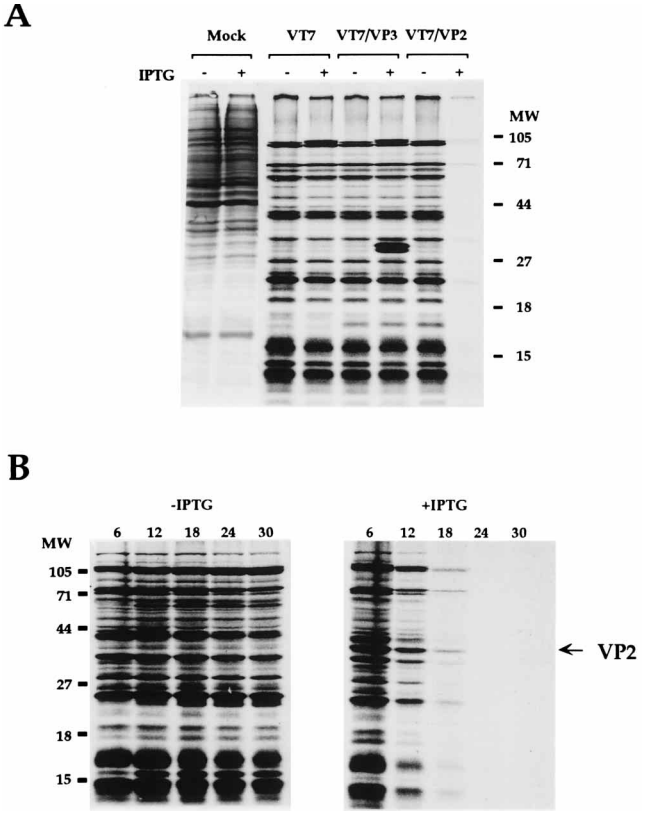

FIG. 1. Characterization of proteins expressed in cells infected with recombinant VV. (A) Recombinants expressing the IBDV structural polypeptides VP2 and VP3. BSC40 cells mock infected or infected with the viruses VT7LacOI (VT7), VT7LacOI/VP2 (VT7/VP2), or VT7LacOI/VP3 (VT7/VP3), either untreated (lanes −) or treated (lanes +) with the inducer IPTG, were metabolically labeled with [^{35}S]methionine at 20 h p.i. and analyzed by SDS-PAGE (12% polyacrylamide) and autoradiography. The positions of molecular mass markers are indicated (MW). (B) Time-course analysis of the inhibition of protein synthesis in cells expressing VP2. BSC40 cells infected with the recombinant virus VT7LacOI/VP2, either treated or untreated with the inducer IPTG, were metabolically labeled with [^{35}S]methionine at the indicated times (h p.i.) and analyzed by SDS-PAGE (12% polyacrylamide) and autoradiography. The positions of molecular mass markers are indicated (MW). The position of the band corresponding to VP2 is indicated by an arrow.

pVOTE.1 and pVOTE.2 lies in the sequences of their multiple cloning sites (38).

The expression of VP2 and VP3 was analyzed in African green monkey kidney epithelial BSC40 cells, derived from the original BSC-1 cell line (American Type Culture Collection) infected with the recombinant viruses VT7LacOI/VP2 and VT7LacOI/VP3. For this purpose, cells infected at a multiplicity of infection of 10 PFU/cell were maintained either in the presence or the absence of the inducer isopropyl β-D-thiogalactosidase (IPTG) (2 mM final concentration). At 20 h postinfection (p.i.), cells were metabolically labeled for 30 min with 50 μCi of [^{35}S]methionine per ml, washed twice with phosphate-buffered saline, and resuspended in Laemmli buffer. Protein samples (15 μg/sample) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography (Fig. 1A). As expected, induction of cells infected with the parental virus VT7LacOI led to expression of an 85-kDa polypeptide corresponding to the inducible version of the T7 DNA polymerase gene inserted in the genome of VT7LacOI (38). Induction of cells infected with VT7LacOI/VP3 resulted in the selective expression of three polypeptides, the T7 RNA polymerase and a doublet of 30 and 32 kDa that was identified as VP3 by Western blot analysis using an anti-IBDV-specific serum (data not shown). Unexpectedly, the addition of IPTG to VT7LacOI/VP2-infected cultures caused a severe reduction in the incorporation of [^{35}S]methionine, suggesting that expression of VP2 in infected cells results in a strong inhibition of total protein synthesis.

To further characterize this phenomenon, a time-course analysis was carried out. Cells were infected at a multiplicity of infection of 10 with VT7LacOI/VP2 and incubated either with or without IPTG. At different times p.i., cells were metabolically labeled with [^{35}S]methionine and processed as described above. Inhibition of protein synthesis was clearly detectable as early as 12 h p.i. (Fig. 1B) in the IPTG-induced VT7LacOI/VP2-infected cultures, and protein synthesis was almost completely silenced between 18 to 24 h p.i. A prominent radioactive band of 37 kDa was present in samples from IPTG-induced cultures. This band was identified as VP2 by Western blot analysis (data not shown). Taken together, these data indicate that expression of VP2 shuts off total protein synthesis. The specificity of this effect is confirmed by results obtained with both VT7LacOI and VT7LacOI/VP3, which demonstrate that neither the expression of T7 RNA polymerase nor the accumulation of cap-independent transcripts, produced by transcription of recombinant genes by T7 RNA polymerase (38), caused detectable changes in the rate of protein synthesis.

The cytopathic effect observed in IPTG-induced VT7LacOI/VP2-infected cultures was reminiscent of that described in apoptotic cells, i.e., shrinkage and premature detachment of the infected cells from the culture surface. To verify whether expression of VP2 leads to apoptosis, the effect of VP2 induction on the fate of both rRNA and cellular DNA was investigated. rRNA was isolated from either infected or mock-infected cultures by using the Ultaspec-II RNA isolation system (Biotecx Laboratories, Inc.) and analyzed by 1% formaldehyde-ethidium bromide-agarose gel electrophoresis (31) (Fig. 2A). After electrophoresis of samples from mock-infected, VT7LacOI/VP3-infected, and uninduced VT7LacOI/VP2-infected cultures, two prominent bands corresponding to 28S and 18S rRNA species were detectable, indicating that in these samples, the rRNA remained intact. A different migration pattern was observed in samples from IPTG-induced VT7LacOI/VP2-infected cells. In this case, the 28S and 18S bands were fainter and other bands, likely to correspond to 28S and 18S degradation products, were clearly detectable, indicating that induction of VP2 expression is also associated with degradation of rRNA.

Low-molecular-weight DNA from cell cultures was extracted as described previously (18) and analyzed by 1% agarose-ethidium bromide gel electrophoresis (31). The results of this analysis are shown in Fig. 2B. While no sign of DNA degradation was observed in samples obtained from uninduced VT7LacOI/VP2-infected cells, a distinct laddering effect, indicative of nucleosomal degradation, was detectable in DNA samples obtained from IPTG-induced cultures at 48 and 72 h p.i. The effect of VP2 expression on chromosomal DNA was also assessed at a cellular level. VT7LacOI/VP2-infected cells were fixed, stained with the DNA-binding fluorescent dye Hoechst 33258 (Hoechst Behring), and observed under a UV-light microscope as previously described (23). A large majority of the cells in VT7LacOI/VP2-infected IPTG-induced cultures began to shrink and detach from the culture surface at 24 h p.i. These cells showed extensive chromatin condensation indicative of apoptosis (data not shown).

Several VV genes are involved in the control of programmed cell death. VV genes B13R and B22R encode polypeptides belonging to the superfamily of serine proteinase inhibitors (22, 33) with similarity to the cowpox *crmA* gene (30). It has

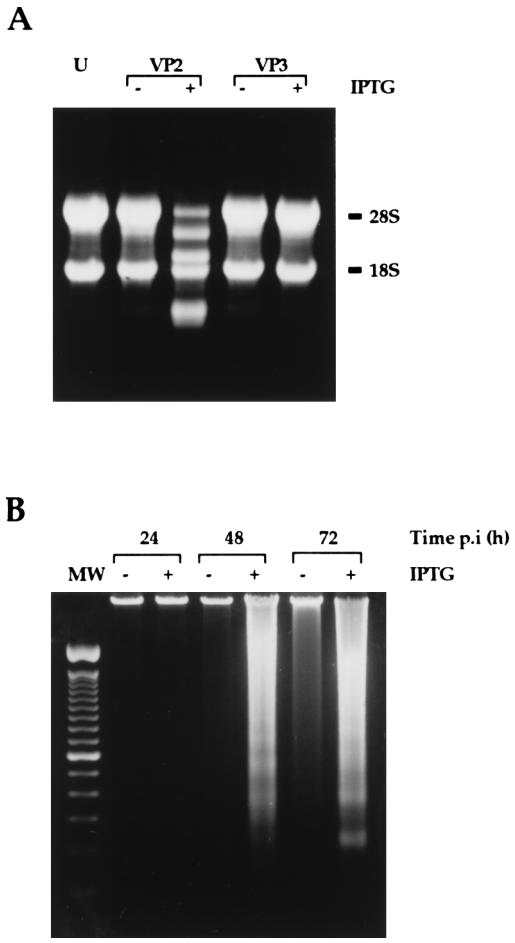

FIG. 2. Induction of apoptosis in BSC40 cells expressing VP2. (A) Effect of VP2 expression on rRNA. rRNA purified from mock- (U), VT7LacOI/VP2 (VP2)-, or VT7LacOI/VP3 (VP3)-infected cells either untreated (lanes −) or treated (lanes +) with the inducer IPTG was extracted at 24 h p.i. and analyzed by 1% formaldehyde-ethidium bromide-agarose gel electrophoresis. The positions of the 28S and 18S rRNAs are indicated. (B) Effect of VP2 expression on cellular DNA. Low-molecular-weight DNA from cells infected with the recombinant virus VT7LacOI/VP2 either untreated (lanes −) or IPTG-treated (lanes +) was extracted at 24, 48, and 72 h p.i., respectively, and analyzed by 1% agarose-ethidium bromide gel electrophoresis alongside a 100-bp DNA ladder marker (lane MW) (Gibco BRL).

been shown that expression of B13R prevents Fas-induced and tumor necrosis factor-induced apoptosis, probably by blocking the activity of members of the caspase family (10). Genes K3L and E3L encode inhibitors of p68 kinase (1, 5, 7, 9). Inactivation of the E3L gene enhances the apoptotic potential of VV, probably by activation of the endogenous p68 kinase (23). It is interesting to note that expression of VP2 triggers apoptosis despite the presence of the VV anti-apoptotic repertoire. This might indicate that VP2 induces apoptosis through a pathway different from those controlled by VV genes. Alternatively, it is possible that the high levels of VP2 expression obtained after IPTG induction may overcome the capability of mechanisms displayed by VV to prevent apoptosis.

The possibility existed that VP2 might induce apoptosis either in cooperation with a VV polypeptide(s) or by interfering with the action of an anti-apoptotic VV gene product(s). To analyze this possibility, a VP2 transient expression analysis in BSC40 cells was carried out. For this analysis, two plasmid expression vectors, pcDNA-VP2 and pcDNA-LacZ, were constructed. These plasmids contain the coding region of the VP2 of IBDV or the *lacZ* gene of *Escherichia coli*, respectively, under the transcriptional control of the immediate-early promoter-enhancer region of human cytomegalovirus within the mammalian expression vector pcDNA3 (Invitrogen Corporation). The VP2 coding region was generated by RT-PCR by using the primers described above for the generation of the plasmid pVOTE.2/VP2. The resulting DNA product was digested with *Eco*RI and *Xho*I and ligated to pcDNA3 treated with the same the restriction enzymes. The *lacZ* gene was obtained by restriction of the plasmid pCH110 (Pharmacia Biotech) with the enzymes *Hin*dIII and *Bam*HI. The resulting 3.7-kb DNA fragment containing the *lacZ* coding region was recovered from an agarose gel and then ligated to pcDNA3 digested with *Hin*dIII and *Bam*HI. The pcDNA-VP2 and pcDNA-LacZ plasmids were purified and used to transfect preconfluent monolayers (2.5×10^5 cells/35-mm-diameter plate) of BSC40 cells grown on glass coverslips with lipofectamine reagent (Gibco BRL). To avoid experimental artifacts arising from potential toxic effects of the lipofectamine, cells were transfected with different amounts (5 to 25 μg/plate) of plasmid DNA. A constant lipofectamine/DNA ratio was maintained in these assays.

After transfection, the cultures were washed three times and covered with 3 ml of Dulbecco modified Eagle medium containing 2% fetal calf serum. A slight toxicity was detected only in cells from cultures transfected with the highest amount (25 μg/plate) of the control plasmid, pcDNA-LacZ. Accordingly, we decided to analyze the results obtained in cultures transfected with 10 μg of plasmid/plate. At 48 h posttransfection, the coverslips were recovered and the cells were fixed with methanol for 20 min at −20°C, stained with Hoechst 33258, and observed under phase contrast and UV microscopy. As shown in Fig. 3, cells from monolayers transfected with the control plasmid pcDNA-LacZ had a normal epitheliallike morphology. In contrast, a proportion (approximately 15%) of cells from cultures transfected with the VP2 expression vector pcDNA-VP2 showed extensive morphological changes which were associated with gross alterations in the pattern of nuclear staining. The bright rounded cells showed extensive chromatine condesation and nuclear fragmentation indicative of apoptosis. Under these conditions the efficiency of transfection, determined by 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) staining (6) of cells transfected with pcDNA-LacZ, was approximately 15 to 20%. These results indicate that in the absence of VV-encoded polypeptides VP2 also induces a programmed cell death response in BSC40 cells.

The data reported here demonstrate that VP2 acts as an efficient apoptotic inducer in African green monkey BSC40 cells. The effect of VP2 expression on the rate of protein synthesis was analyzed in a number of mammalian cell lines infected with the recombinant virus VT7LacOI/VP2: Vero (African green monkey), COS-1 (African green monkey), HuTK$^-$143B (human), HeLa (human), 3T3 (mouse), and L249 (mouse) (American Type Culture Collection). Although some differences in the levels of inhibitory effect were detected, induction of VP2 expression resulted in a strong inhibition of total protein synthesis in all cell lines tested (data not shown).

An extensive database search analysis with the VP2 amino acid sequence did not reveal significant homologies to currently known apoptotic inducers.

It has been shown that the expression of the proto-oncogene *bcl-2* prevents apoptosis induced by numerous effectors, including infection with different viruses (reviewed in reference 32). We carried out several experiments to analyze whether VP2-induced apoptosis could also be blocked by expression of *bcl-2*. BSC40 cells were infected with either the recombinant

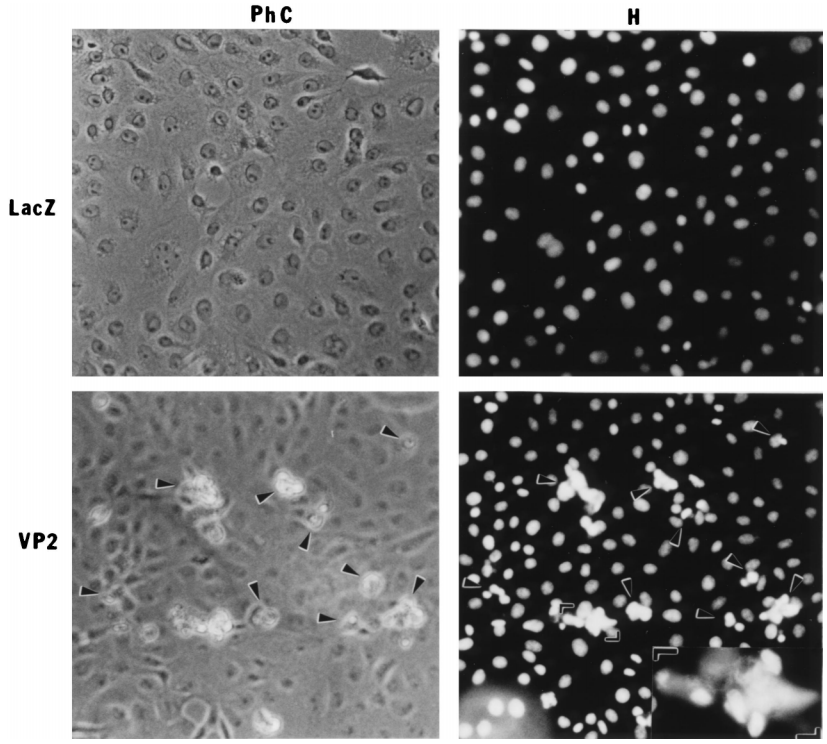

FIG. 3. Induction of apoptosis in BSC40 cells transfected with the expression vector pcDNA-VP2. Cells were transfected with DNA corresponding to either pcDNA-VP2 or pcDNA-LacZ. At 48 h posttransfection, cells were fixed, stained with Hoechst 33258, and visualized and photographed either by phase contrast (PhC) or fluorescence microscopy (H). Representative examples of cells found in these samples are shown. Arrows, cells showing morphological and nuclear staining alterations. Inset, magnified view of the indicated section of the field.

VT7LacOI/VP2 (10 PFU/cell) alone or with a mixture of VT7LacOI/VP2 and VV-bcl-2 (10 PFU of each virus/cell). VV-bcl-2 is a recombinant VV that inducibly expresses the human *bcl-2* gene (24) and was kindly supplied by Mariano Esteban (Centro Nacional de Biotecnologia, Madrid, Spain). After infection, cells were maintained either in the presence or the absence of IPTG. At 24 h p.i., cultures were metabolically labeled with [^{35}S]methionine and processed for SDS-PAGE and autoradiography as described above. The results of this study (Fig. 4) show that expression of the Bcl-2 protein abrogates the inhibitory effect of VP2 on total protein synthesis. Bcl-2 expression also caused a delay in the onset of degradation of both rRNA and nuclear DNA (data not shown). These results indicate that the step of the apoptotic pathway induced by VP2 expression is located upstream of Bcl-2.

Two reports have described the induction of programmed cell death of chicken peripheral blood lymphocytes (36), CEF, and Vero cells (35) after infection with IBDV. Lymphoid cells showing the hallmarks of apoptosis have also been found in young chickens and embryos experimentally infected with pathogenic serotype I IBDV strains (20, 37). Thus, it was expected that expression of VP2 in CEF infected with VT7LacOI/VP2 would trigger apoptosis. However, under our experimental conditions, expression of VP2 does not cause an apoptotic response in this cell type (data not shown). The insensitivity of CEF to the induction of apoptosis by VP2 expression suggest that the results obtained in mammalian cells do not reflect the authentic role of VP2 during the natural IBDV infection. An alternative interpretation might be that, as it has been shown with other apoptotic inducers, i.e., the VP3 protein of chicken anemia virus (28), some cell types, including CEF, are resistant to VP2-induced apoptosis. The inability of VP2 to induce apoptosis in CEF could be related to the tropism of the IBDV Soroa strain used in this study. As has been observed with most field isolates, the original Soroa isolate obtained from the bursa of a naturally infected chicken did not replicate well in CEF. In order to improve the yields, the virus was adapted to CEF by serial passage, a process known to reduce virus pathogenicity and that, in some cases, results in

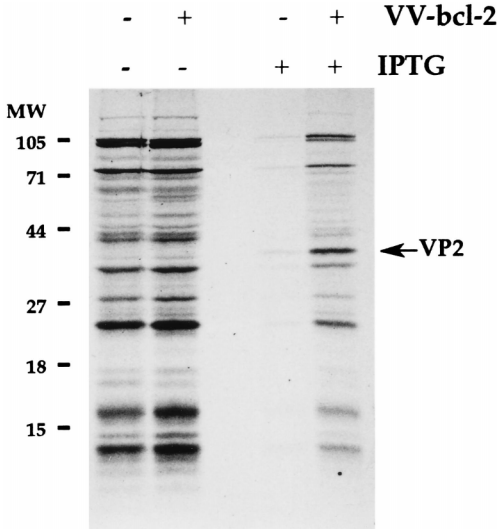

FIG. 4. Effect of *bcl-2* expression on inhibition of protein synthesis induced by VP2. BSC40 cultures were infected with VT7LacOI/VP2 [VV-bcl-2 (−)] or with VT7LacOI/VP2 and VV-bcl-2 [VV-bcl-2 (+)]. After infection, cells were maintained either in the absence (lanes −) or the presence of IPTG (lanes +). At 24 h p.i., cultures were metabolically labeled with [^{35}S]methionine and analyzed by SDS-PAGE and autoradiography. The positions of molecular mass markers are indicated (MW). The position of the band corresponding to VP2 is indicated by an arrow.

the introduction of amino acid changes within VP2 (39). It is possible that serial passage might have also altered the apoptotic potential of the virus in this specific cell type. In agreement with this, the Soroa strain does not trigger programmed cell death in CEF (data not shown).

The generation of recombinant fowlpox virus and herpesvirus of turkeys expressing VP2 has been described (8, 16). In these reports, there is no indication about the induction of programmed cell death in cells infected with these recombinant viruses. This might also be related with the fact that these recombinant viruses were obtained and characterized in CEF.

It has been suggested that the immunosuppressive effect of some IBDV isolates might be related to their ability to trigger apoptosis of infected B-lymphocyte precursors. Infection with those viruses would destroy the bursa of Fabricius in the absence of an inflammatory response, leading to immunosuppression in infected chickens (36). There is increasing evidence that apoptosis plays a key role in the life cycle of numerous viruses (32, 34). In most cases, the viral gene products responsible for the induction of apoptosis are unknown (34). The finding that VP2 is an apoptotic inducer might shed some light on the search for factors involved in the pathogenicity of the virus. However, further experimentation is required to evaluate the true significance of our finding on the life cycle of the virus.

We thank Diane Wilcock, and Jose Sánchez-Serrano for critical readings of the manuscript and Lolo Rodríguez, Juan Ramón Rodríguez, Marisé Borja, and Carmen Rivas for helpful discussions. We also thank Inés Poveda and Angel Sanz for excellent photography work. We are indebted to Julia Noda Gómez for the Soroa strain of IBDV and the anti-IBDV antiserum, Bernard Moss for the VOTE expression system, and Mariano Esteban for the VV-bcl-2 recombinant virus.

This work was supported by grant BIO-95-2075-E from the Comisión Interministerial de Ciencia y Tecnología.

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