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# Role of Bovine Bcl2A1 Gene in Staurosporine-Induced Apoptotic Cell Lines

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**Abstract**

Bcl2A1 (B-cell lymphoma 2 - related protein A1) is a member of a gene set that plays a critical role in regulating apoptosis of mammalian cells. The family is divided into 2 groups: (1) anti-apoptotic genes Bcl-2, Bcl-xL, Bcl2A1 and (2) pro-apoptotic genes Bid, Bax, Bak. Apoptosis in cells is regulated by a balanced expression of these groups. In this study, the ORF encoding the 175 amino acid bovine Bcl2A1 protein was amplified by reverse transcription polymerase chain reaction (RT-PCR), using specific primers deduced from the bovine Bcl2A1 mRNA sequence (www//ncbi.nlm.nih.org. Acc: AB195549) and which also contained appropriate restriction endonuclease cleavage sites for cloning. The product was then cloned into plasmid pcDNA3+ to construct the eucaryotic expression plasmid pcDNA3.Bcl2A1. Hela, L11, Vero and WSL cell lines were used to investigate apoptosis induced by staurosporine. Monitoring of cellular DNA fragmentation revealed that incubation with staurosporine at a concentration of 2 µM results in a sufficient level of apoptosis induction in Hela, L11, WSL cell cultures after 6 hours and in Vero cell cultures after 12 hours. Transfection of these cell lines with pcDNA3.Bcl2A1, using pseudorabies virus Us3 protein kinase (PrVUs3) which prevents staurosporine induced apoptosis by interacting with the pro-apoptotic proteins Bid and Bad as a control, proved that expression of the bovine Bcl2A1 gene blocks staurosporine-induced apoptosis in Hela and L11 cell lines. However, this activity was not observed in WSL and Vero cell cultures.

**Keywords:** Bovine Bcl2A1, Apoptosis, staurosporine, Transfection, DNA fragmentation, RT-PCR

## 1. Introduction

Apoptosis is the normal biological process of programmed cell death that plays an essential role in regulating growth, development and immune responses by ‘clearing’ redundant or abnormal cells in organisms. It can be induced by viral infection or exogenous stimuli such as

staurosporine, sorbitol and UV irradiation (Cartier A., 2003a/b). According to Kurschner (1996), apoptosis is associated with serious diseases among which cancer is a disease that is often characterized by too little or no apoptosis. In contrast, in neurodegenerative diseases such as Parkinson or Alzheimer, too much apoptosis in neuronal cells is thought to be part of the pathogenesis. Moreover, apoptosis is also important for normal placental development. During pregnancy, trophoblast cells from the placenta invade the uterine environment to help establish and maintain a successful pregnancy. The combination of apoptosis and cell proliferation is responsible for shaping tissues and organs in developing embryos. For example, apoptosis of cells located in-between the toes allows for their separation (Dash .P, http://www.sgul.ac.uk/depts/immunology/~dash/apoptosis). Studies of Koichi U., 2005 and Matwee C., 2000 and Yang MY., 2002 in cattle indicated association between apoptosis and bovine embryonic developmental stage as well as quality of the embryo. Hence, understanding of apoptosis and its regulation will open new research orientations in treatment of diseases and improvement of techniques relating to *in-vitro* fertilization.

So far, the molecular mechanism mediating apoptosis regulation has been considered to involve many factors in which apoptotic regulator family B-cell lymphoma 2 (Bcl2) plays a critical role (Choi SS., 1995; Boise LH., 1993).The Bcl2 family of apoptotic regulators is characterized by the presence of Bcl2 homology (BH) domains and can be subdivided into two groups: anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl2A1) and pro-apoptotic proteins (Bid, Bax, Bak) (Hagen SB, 2007; Koichi U, 2005; Reed, 2000, Joseph JK, 1997). They are intracellular membrane-associated proteins and most of them are found in the outer membrane of mitochondria, but some locate in nuclear envelope and endoplasmic reticulum membrane. According to published studies, the balance of expression between the two groups is the molecular basis for regulation of apoptosis (Joseph JK., 1997; Hagen SB., 2007). By cloning the bovine Bcl2A1 gene and analyzing the expression of the gene in trophoblastic binucleate cells of bovine placenta, Koichi U. concluded that bovine Bcl2A1 protein is a new candidate for anti-apoptotic maintenance of the binucleate cells that support placental functions throughout gestation in bovines.

To investigate the functions of anti-apoptotic genes, in this study, we aimed to overexpress bovine Bcl2A1 gene to analyze *in-vitro* its role in staurosporine-induced apoptotic mammalian cells.

## 2. Materials and Methods

### *2.1 Materials*

Ear tissue samples of Coc and HF cattle kept in the conservation farm, National Institute of Animal Sciences were taken and immediately stored in liquid nitrogen or RNA-later solution for further studies.

Cell lines such as Hela (Human cervix carcinoma) cells, L11 (ovine) cells, WSL (Wild boar lung) cells and Vero (monkey kidney) cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FCS, 2.4 mM L-glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml or in Minimum essential medium (MEM) supplemented with

10% FCS, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. Cell cultures were incubated at $37^0$C in a humidified atmosphere containing 5% $CO_2$.

*2.2 Methods*

2.2.1 Primer Design

The GenBank accession no. AB195549 sequence was used to design the primers flanking the ORF coding for bovine Bcl2A1 gene (Figure 1). The primers contain adaptors (capital letters) with restriction enzyme cleavage sites for cloning. The primers were commercially synthesized (IDT, USA)

Forward primer: 5'- accGCTAGCGAATTCggagaagatgactgacactgagtttg - 3'

Reverse primer: 5'- ggtGCGGCCGCAAGCTTttcagtcagtagtattgcttcag - 3'

2.2.2 RNA and DNA Isolation

About 30 mg of bovine ear tissues stored in liquid nitrogen or in RNA-later solution was used to extract total RNA, according to the protocol of the SV Total RNA Isolation System (Promega). Extraction of RNA from cell cultures was performed using the RNeasy Mini Kit (Quiagen).

To analyze DNA fragmentation, whole-cell DNA was isolated using the Qiamp DNA Blood Mini Kit (Quiagen).

2.2.3 Reverse Transcription-PCR (RT-PCR) and Specific PCR

To synthesize cDNA from RNA extracted from bovine ear tissues and cell culture, the Goscript Reverse Transcription System (Promega) and Super Scrip II reverse transcriptase (Invitrogen) were used, respectively.

The entire open-reading-frame (ORF) of bovine Bcl2A1 (Figure 1) was amplified by PCR in 1x PCR buffer supplemented with 1.5 mM $MgCl_2$, 200 µM dNTPs, 1 µM primers, 0.5 U GoTag Hot Start Polymerase (Promega) and 15 µl cDNA as template. Amplification conditions were: denaturation at $95^0$C for 2 min; 35 cycles in which each cycle was set at $95^0$C for 30 sec, $58^0$C for 45 sec and $72^0$C for 30 sec and a final extension at $72^0$C for 5 min.

2.2.4 Construction of Expression Vector pcDNA3.Bcl2A1

The PCR product containing the bovine Bcl2A1ORF and the plasmid vector pcDNA3+ were digested for 1 to 2 h with 5 U of each EcoRI and NotI (Promega) per µg DNA in the appropriate (1x) buffer according to the manufacturer's instructions. The plasmid DNA fragment was dephosphorylated by incubation at $37^0$C for 30 min with 6 to 8 U of calf intestinal phosphatase (CIP, Promega) per µg DNA in the presence of 1x CIP buffer according to the manufacturer's instructions. The reaction was terminated by addition of 300 µl of CIP stop buffer and incubated at $65^0$C for 15 min. After isolation of DNA fragments from agarose gels, a molar ratio of 1:3 of dephosphorylated vector and fragment DNA was used for sticky-end ligations Reactions were carried out with 0.1 U T4 DNA ligase (Promega) in 1x ligation buffer. Ligations were incubated at RT for 3 h and subsequently at $4^0$C overnight. The result was controlled by agarose

gel electrophoresis.

2.2.5 Transformation

Competent E.coli JM109 cells (Promega) were thawed on ice. 50 to 200 ng ligated DNA and controls were gently mixed with 50 µl JM109 cells (5 x $10^6$ cells) and incubated on ice for 20 min. The competent cells were then heated at $42^0$C for exact 2 min, subsequently incubated on ice for 5 min and 200 µl of LB medium containing 10 mM KCl and 10 mM $MgSO_4$ was added prior to incubation at $37^0$C for 1 h. The transformation mixtures were spreaded on LB agar plates containing 100 µg/ml ampicillin and incubated at $37^0$C overnight.

Figure 1. Nucleotide and deduced amino acid sequences of bovine Bcl2A1. The asterisks indicate the termination codon. The polyadenylation signal is underlined.

2.2.6 Extraction of Plasmid DNA

The selected colonies were grown at $37^0$C overnight in LB medium supplemented with 100 µg/ml ampicillin. Plasmid DNA was extracted, using the PureYield™ Plasmid Miniprep System (Promega).

2.2.7 Apoptosis assay

Apoptosis was induced by the addition of 2 µM staurosporine for the respective time, depending on the sensitive level of each cell lines, then followed by DNA laddering assay to detect apoptotic DNA fragmentation

2.2.8 DNA introduction into cells by transfection, using polyethylenimine (PEI)

For transient expression of the Bcl2A1 gene, 2.5 µg of plasmid DNA and 5 µg of PEI were

prepared separately in 200 µl of serum-and antibiotic-free medium and incubated at RT for 5 min. Subsequently, they were combined, gently mixed and further incubated at RT for 20 min. Confluent cell culture grown overnight in 6-well plates were washed once with 600 µl serum-and antibiotic-free medium and the same amount of the medium was added prior to dropwise addition of 400 µl sample. The cells were incubated at $37^0$C for 4 h, washed and further incubated in 2 ml/well culture medium containing serum and antibiotics.

2.2.9 Agarose gel electrophoresis

To analyze restriction enzyme cleavages, nucleic acid purification, PCR products as well as DNA fragment purification for cloning, agarose gel electrophoresis was used. The agarose gels were prepared at appropriate concentrations and then poured into a horizontal gel apparatus (Biorad). Samples and DNA marker fragments (1 kb ladder (Invitrogen) or smart ladder (Nippongene)) were loaded and the separation was performed at the respective voltage and time, depending on the size of the gel and DNA fragments in 1x running buffer TBE or TAE. Gels were stained in a solution containing 100 µg/ml of ethidium bromide for about 10 min. Documentation of the gel was carried out under UV light.

2.2.10 Sequencing

Plasmid DNA or PCR product was sequenced by automated fluorescent DNA sequencing technology (Perkin Elmer, Applied Biosystems). The sequencing reaction was performed using BigDye Terminator Cycle Sequencing Kit and BigDyeXTerminator Purification, according to the manufacturer's instructions. The cleaned products were sequenced by a Genetic Analyzer AB3130 and sequences were analyzed by BioEdit software.

## 3. Results and Discussion

### *3.1 RNA Extraction and Bovine Bcl2A1ORF Amplification*

To analyse the quality of the RNA extracted from the bovine ear tissues stored in liquid nitrogen or in RNA-later solution by, RNA was incubated at $70^0$C for 2 min prior to separation on 0.6% agarose for 30 min at 100 Volt. The resulting pattern (Figure 2) showed two distinct bands with of about 4.7 kb and 1.9 kb specific for 28S and 18S mammalian ribosomal RNA, respectively, indicating that the RNA remained intact. Since mRNA comprises only 1-3% of total RNA samples distinct mRNA species are not visible under these conditions.

After reverse transcription of COC and HF cattle RNA the cDNA was used as template in PCR reactions. Figure 3 shows that PCR products of the correct size (572 bp consisting of 528 bp of the bovine Bcl2A1ORF (Figure 1) and 44 bp of adaptor sequences) were amplified.

### *3.2 Cloning of Bovine Bcl2A1ORF*

These PCR products were digested with EcoRI and NotI and inserted into plasmid pcDNA3+ cleaved with the same enzymes resulting in expression plasmid pcDNA3.Bcl2A1 in which the transcription of the bovine Bcl2A1ORF is controlled by the human cytomegalovirus (HCMV) immediate early promoter and the bovine growth hormone (BGH) polyadenylation signal sequence. To monitor the cloning process, a PCR was applied directly from a part of colonies

growing on agar plates using the Bcl2A1ORF-specific primer pairs.

The result is shown in Figure 4A and indicated that the 572 bp PCR product of the bovine Bcl2A1ORF was amplified only from the plasmids with the Bcl2A1 insertion. This conclusion was confirmed by cleavage of a selected clone and the vector alone with restriction endonuclease HindIII. Since HindIII recognition sites flank the Bcl2A1ORF, two bands with sizes of 5.4 kb and 528 bp were obtained as expected (Figure 4B). In contrast, only one 5.4 kb band was present in the HindIII cleaved pcDNA3+.

Sequencing (Figure 4C) of both bovine Bcl2A1ORFs revealed homology of 100% to the published mRNA sequence (accession number AB195549). Since the sequences of Bcl2A1ORF of Coc cattle and HF cattle were identical, only the Bcl2A1ORF of Coc cattle was used for further studies.

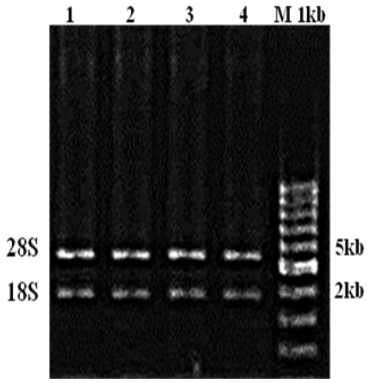


Figure 2. RNA extraction. Ear tissues of Coc and HF cattle stored in liquid nitrogen (1 and 3) and in RNA-later solution (2 and 4) were used for total RNA extraction. Result indicated the presence of two bands 28S and 18S specific for mammalian RNA molecule

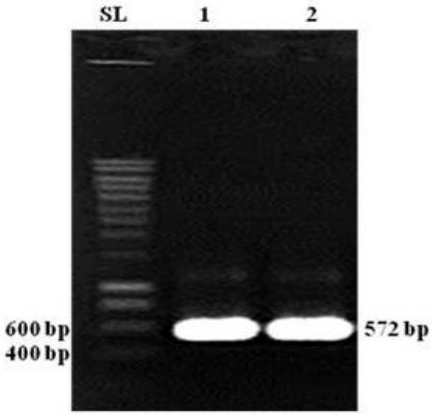


Figure 3. Amplificons of the Bcl2A1ORF from cDNA synthesized from total RNA isolated from Coc cattle (lane 1) and HF cattle (lane 2) ear tissues. DNA was size separated on 2% agarose gels, stained with ethidium bromide and photographed under UV-light. SL: smart ladder was used

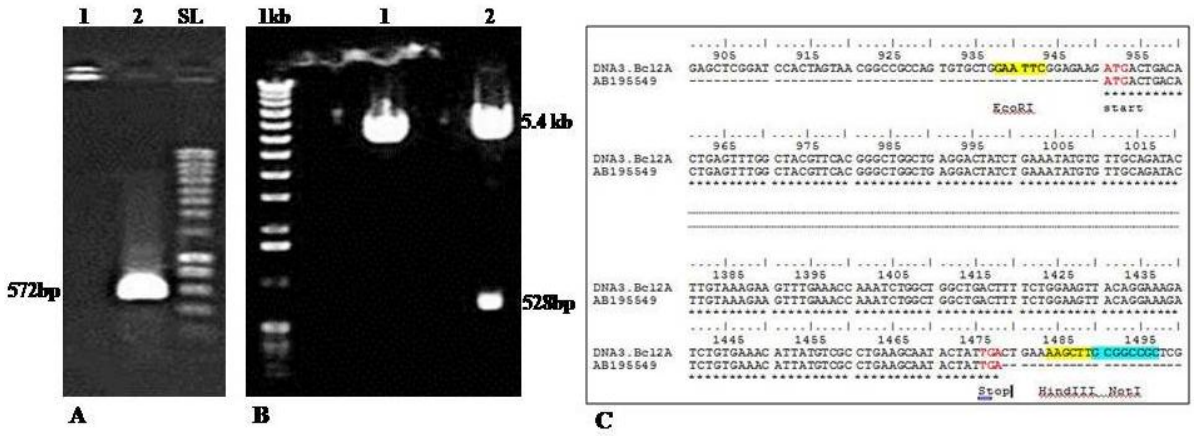


Figure 4. Cloning of bovine Bcl2A1 ORF. (A)-PCR was applied directly from a part of colonies formed by transformation of pcDNA3+ (lane 1) and pcDNA3.Bcl2A1 (lane 2); (B)-HindIII cleavage of pcDNA3+ (lane 1) and pcDNA3.Bcl2A1 (lane 2); and (C)-Comparision of bovine Bcl2A1 ORF amplified with bovine Bcl2A1 mRNA published (Acc.Nos. AB195549). SL= DNA Smart ladder.

*3.3 Apoptosis induction by Staurosporine*

Staurosporine ($C_{28}H_{26}N_4O_3$) is a natural product originally isolated in 1977 from *Streptomyces staurosporeus*. It is a fungal alkaloid that has for a long time been used *in-vitro* as an inducer of apoptosis in many different cell lines, but the mechanism involved remains poorly understood (Zhang XD, 2004). Hela, L11, Vero and WSL cell lines were grown in 6-well plates and induction of apoptosis with 2 µM staurosporine was monitored in time kinetics. At the indicated times (Figure 5), whole-cell DNA in the cells was isolated and separated on 2% agarose gels. Analysis of apoptosis induced by staurosporine was done by monitoring cellular DNA fragmentation. The method, as noted by Munger J. (2001), is simple and accurate. The mechanism by which cellular DNA is cleaved during apoptosis induction is briefly outlined as follows: upon receiving specific signals instructing the cells to undergo apoptosis, a number of distinctive changes occur in the cell. A family of cellular proteins known as caspases is typically activated in the early stages of apoptosis. These proteins cleave key cellular components including structural proteins in the cytoskeleton and nuclear proteins such as poly [ADP-ribose] polymerase 1 (PARP-1) that are required for correcting errors in cellular DNA (Boulares AH, 1999). The caspases can also activate other degradative enzymes such as caspase-activated DNases (CAD) that results in DNA degradation by cleavage in internucleosomal regions in apoptotic cells. Figure 5 shows the time kinetics of apoptosis induction, visualized by DNA fragmentation and revealed that DNA laddering is clearly demonstrated after 6 h incubation of Hela, L11 cells and WSL cells and after 12 h incubation of Vero cells with 2 µM staurosporine.

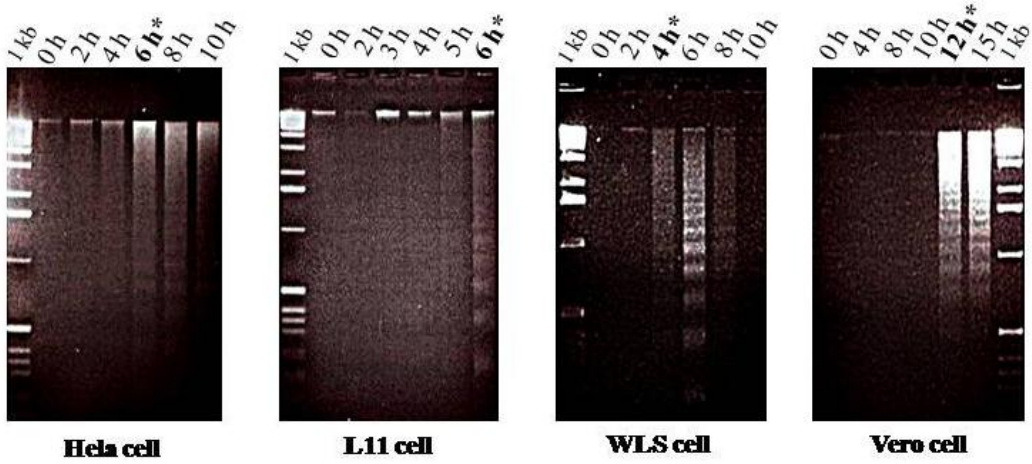


Figure 5. Time kinetics of apoptosis induced by 2 μM staurosporine and monitored by DNA fragmentation in Hela, L11, WSL and Vero cell cultures. Whole-cell DNA was extracted at the indicated time points and DNA was size separated on 2% agarose gels, stained with ethidium bromide and photographed under UV-light.

*3.4 Expression of Bovine Bcl2A1 Gene*

The bovine Bcl2A1 gene was introduced into the cell lines by transfection, using polyethylenimine (PEI). This method uses the cationic polymer polyethylenimine as a carrier, in which the negatively charged DNA binds to the polycation and the complex is taken up by the cell via endocytosis. To determine time and level of Bcl2A1 expression, transfected Hela, L11, WSL and Vero cell cultures were harvested at the indicated time points and wholle-cell RNA was extracted. The resulting RNA was reverse transcribed using oligo (dT) as primer and the Super Script II reverse transcriptase Kit (Invitrogen). The cDNAs were used as templates in PCR reactions with Bcl2A1ORF-specific primers. As shown in Figure 6, specific amplicons with the size of the 528 bp bovine Bcl2A1ORF were observed only when RNA from expression plasmid-transfected cells was used, demonstrating that the bovine Blc2A1 was expressed in all cell cultures at 4 h post-transfection (p.t). In detail, in Hela and L11 cells, expression of the target gene was strongest at 14 h.p.t whereas the best expression level in WSL and Vero cell cultures was reached later. This result demonstrated that the transfection procedure, resulted in efficient expression of the bovine Bcl2A1 gene all cell lines.

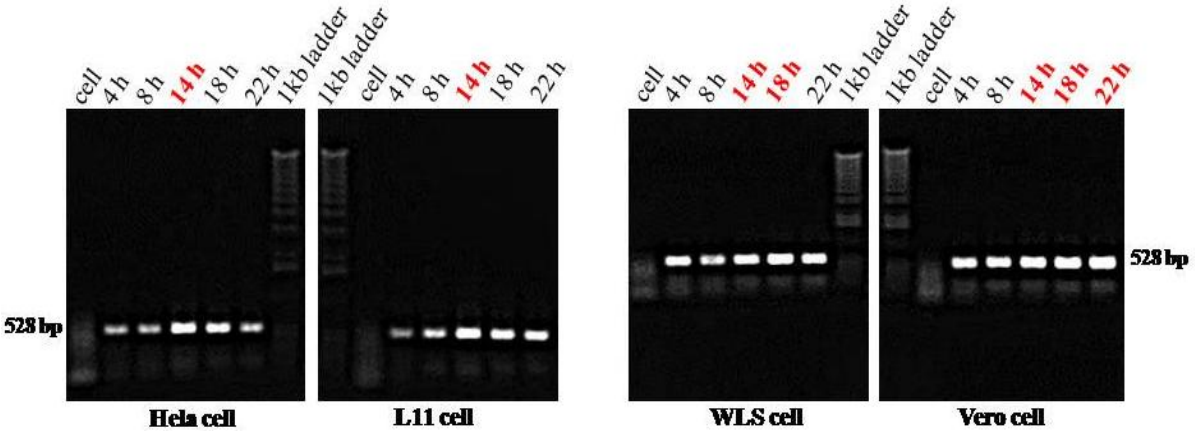

Figure 6. Analysis of expression of bovine Bcl2A1 mRNA. The bovine Bcl2A1 was introduced into the given cell lines by transfection. After the indicated times, transfected cells were harvested for total RNA extraction and then RT-PCR was applied to synthesize cDNA. The cDNA was used as a template in specific PCR to amplify the 528 bp bovine Bcl2A1ORF. Non-transfected cells were used as negative controls. DNA was size separated on 2% agarose gels, stained with ethidium bromide and photographed under UV-light.

### *3.5 Functions of Bovine Bcl2A1 in Staurosporine-Induced Apoptotic Cell Lines*

To investigate the role of bovine Bcl2A1 in apoptotic cells induced by staurosporine, Hela, L11, WSL and Vero cells were transfected with either pcDNA3.Bcl2A1 or pcDNA3.PrVUs3 (control). At 14 h post-transfection, the cells were treated with 2 μM staurosporine for 6 h (Hela, L11 and WSL cells) and 12 h for Vero cell or were left untreated (negative control). Whole-cell DNA was isolated to determine DNA fragmentation. As a control, expression of the pseudorabies virus Us3 protein kinase (PrVUs3) – a protein that prevents apoptosis (Kristin *et al*., 2005) – was used. Results presented in Figure 7 demonstrated that both PrVUs3 and bovine Bcl2A1 expression partly inhibited staurosporine-induced DNA fragmentation in Hela and L11 cells, providing evidence that bovine Bcl2A1 – like PrVUs3 – blocks apoptosis induced by staurosporine and therefore has anti-apoptotic activity. In contrast, in WSL and Vero cells cultures neither PrVUs3 nor bovine Bcl2A1 expression was capable to inhibit staurosporine-induced DNA fragmentation. The difference of anti-apoptotic function of bovine Bcl2A1 between these cell lines can be attributed to different sensitivities of the cell lines or to the sequence of protein-protein interactions with the cellular target component that Bcl2A1 will probably interact with and thus results in the different effects of bovine Bcl2A1 expression. Kristin *et al* (2005) showed that PrVUs3 is localized in mitochondria where the majority of apoptotic regulatory proteins reside and their interaction with the Us3 protein inhibits the activities of the pro-apoptotic proteins Bid, Bax. Prevention of pro-apoptotic functions of Bid and Bad by herpes simplex virus Us3 protein kinase (HSVUs3), the functional homolog of PrVUs3 has also been shown (Cartier *et al*, 2003a/b). Hence, the similarity of anti-apoptotic function between bovine Bcl2A1 and PrVUs3 in Hela and L11 cells leads us to hypothesize that the bovine Bcl2A1 moves to outer membrane of mitochondria where it probably interacts with pro-apoptotic protein(s), resulting in inhibition of apoptosis. This hypothesis is in agreement with studies which show that the balance of expression of anti-apoptotic and pro-apoptotic proteins is essential for regulation of apoptosis in cells (Joseph *et al*., 1997; Hagen *et al*., 2007; Koichi *et al*., 2005).

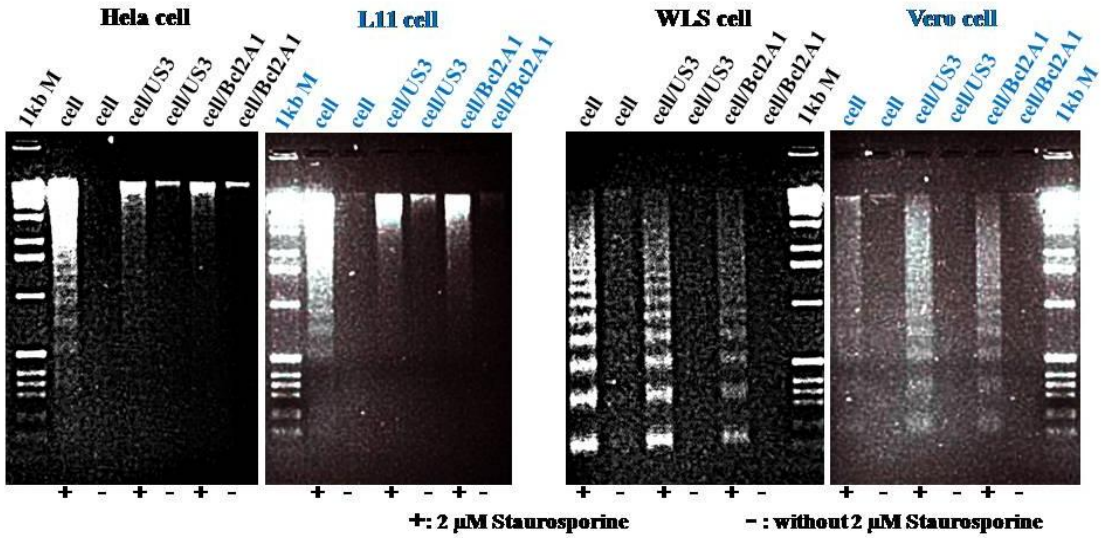


Figure 7. Effect of bovine Bcl2A1 expression in staurosporine-induced apoptotic cell lines. Hela, L11, WSL and Vero cells were transfected with pcDNA3.Bcl2A1 or pcDNA3.PrVUs3 (positive controls) at 14 h.p.t or left non-transfected (negative controls). Apoptosis was induced by incubating the cultures with 2 μM staurosporine (+) at the indicated times or left untreated (-). Whole-cell DNA was prepared and analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and photographed under UV-light.

## 4. Conclusion

In this study, we have amplified entire ORF of bovine Bcl2A1 gene (528 bp) that deduced 175 amino acids and cloned it into pcDNA3+ to form an expression vector pcDNA3.Bcl2A1. Transfection of mammalian cell lines with the expression vector and then induction of apoptosis by staurosporine demonstrated that the bovine Bcl2A1 was well expressed in the cell lines and it played the role as an anti-apoptotic protein in Hela and L11 cells. However, the function was not observed in transfected WSL and Vero cells.

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