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2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin Modifies Expression and Nuclear/Cytosolic Localization of Bovine Herpesvirus 1 Immediate-Early Protein (bICP0) During Infection

Filomena Fiorito,[1] Gabriella Marfè,[2] Giovanna E. Granato,[3] Roberto Ciarcia,[3] Emma De Blasio,[1] Marco Tafani,[4] Salvatore Florio,[3] Luisa De Martino,[1*] Gianmarco Muzi,[5] Ugo Pagnini,[1] and Antonio Giordano[5,6]

[1] *Department of Pathology and Animal Health, University of Naples "Federico II," Naples, Italy*
[2] *Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata," Rome, Italy*
[3] *Department of Structures, Function and Biological Technologies, University of Naples "Federico II," Naples, Italy*
[4] *Department of Cellular and Molecular Pathology, IRCCS San Raffaele Pisana, Rome, Italy*
[5] *Department of Human Pathology & Oncology, University of Siena, Siena, Italy*
[6] *Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, Pennsylvania*

ABSTRACT

Our previous studies have demonstrated that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) increases Bovine Herpesvirus 1 (BHV-1) replication through a dose-dependent increase in cytopathy and increased viral titer. Furthermore, TCDD was able to trigger BHV-1-induced apoptosis by up-regulating the activation of initiator caspases 8 and 9, as well as of effector caspase 3. Since TCDD activates caspase 3 after 4 h of infection, we have hypothesized an involvement of BHV-1 infected cell protein 0 (bICP0) in this process. Such protein, the major transcriptional regulatory protein of BHV-1, has been shown to indirectly induce caspase 3 activation and apoptosis. In order to elucidate the role of bICP0 in this apoptotic pathway, here we have analyzed the effects of TCDD on bICP0 expression. Following infection of bovine cells with BHV-1, we detected apoptotic features already at 12 h after infection, only in TCDD exposed groups. Furthermore, in the presence of different doses of TCDD, we observed a time-dependent modulation and increase of bICP0 gene expression levels, as revealed by RT-PCR analysis. Western blot analysis and immunocytochemistry revealed that TCDD induced an increase of bICP0 protein levels in a dose-dependent manner, compared to unexposed groups. Moreover, Western blot analysis of nuclear and cytosolic fractions of infected cells revealed that TCDD anticipated the presence of bICP0 protein in the cytoplasm. In conclusion, both the increase of replication of BHV-1 and anticipation of BHV-1-induced apoptosis could be the result of a relationship between TCDD and bICP0. J. Cell. Biochem. 111: 333–342, 2010.

KEY WORDS: TCDD; BHV-1; APOPTOSIS; bICP0 GENE EXPRESSION; bICP0 PROTEIN NUCLEAR AND CYTOSOLIC LOCALIZATION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the congeners of polychlorodibenzodioxin, a class of highly toxic and persistent environmental contaminants. Several studies suggest that the exposure to TCDD leads to an enhanced mortality in mice infected with influenza virus [Burleson et al., 1996], increased gene expression of human immunodeficiency virus type-1 (HIV-1) in chronically infected cells [Gollapudi et al., 1996], or an activation of cytomegalovirus replication in human fibroblasts [Murayama et al., 2002]. Furthermore, TCDD activates HIV-1 replication in OM 10.1 cells, promyelocytic cell line latently infected with HIV-1 [Ohata et al., 2003], and increases Bovine Herpesvirus type-1 (BHV-1) replication in bovine cells [Fiorito et al., 2008a]. Recently, high levels of TCDD have been detected in dairy products from some areas of Campania Region (Italy) [Diletti et al., 2003; Santelli et al., 2006], where BHV-1 is widespread and its eradication still represents an important goal [2004/558/EC; Ackermann and Engels, 2006].

BHV-1 is an important viral pathogen that, in cattle, can cause severe respiratory infection, conjunctivitis, abortions, and shipping

*Correspondence to: Dr. Luisa De Martino, Department of Pathology and Animal Health, University of Naples "Federico II," 80137 Naples, Italy. E-mail: luisa.demartino@unina.it
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fever, which is a complicated infection of the upper respiratory tract. BHV-1 initiates the disorder through immunosuppression that could render the animals more susceptible to secondary bacterial infections, leading to pneumonia and occasionally to death [Tikoo et al., 1995; Jones, 2003]. Virus establishes latency in ganglionic neurons of the infected host. Reactivation from latency can be stimulated by immunosuppression conditions, such as dexamethasone administration or increases in natural corticosteroids, resulting in virus spread to susceptible hosts [Jones, 2003].

The genes of BHV-1, like other members of the Alphaherpesvirus family, are expressed in three temporally distinct phases identified as immediate-early (IE), early (E), and late (L). BHV-1 infected cell protein 0 (bICP0), the bovine homologue of HSV-1 ICP0, regulates all three these phases by acting as a strong activator or as a repressor of specific viral promoters [Wirth et al., 1991, 1992; Jones et al., 2006].

Infection of bovine cells [Devireddy and Jones, 1999; Geiser et al., 2008; Fiorito et al., 2008b] or calves [Winkler et al., 1999] with BHV-1 leads to rapid cell death, partially due to apoptosis. Previously, we have demonstrated that, in Madin-Darby Bovine Kidney (MDBK) cells, TCDD exposure is able to enhance both BHV-1 replication, through a dose-dependent increase in cytopathy and increased viral titer [Fiorito et al., 2008a], and anticipation of BHV-1 induced apoptosis, by up-regulating the activation of initiator caspases 8 and 9, as well as of executioner caspase 3 [Fiorito et al., 2008b].

It is known that bICP0, in the absence of any viral gene expression, indirectly induces caspase 3 activation and apoptosis in mouse neuroblastoma cells (neuro-2A) and bovine turbinate cells [Henderson et al., 2004]. Moreover, a late caspase 3 activation occurs during BHV-1 productive infection [Devireddy and Jones, 1999; Lovato et al., 2003; Fiorito et al., 2008b], suggesting that bICP0, in part, activates caspase 3 [Jones et al., 2006]. Since bICP0 also triggers viral gene expression and productive infection, that in turn promotes cell death [Geiser et al., 2008], we have supposed that the observation of an anticipated activation of caspase 3 after 4 h of BHV-1 infection in the presence of TCDD [Fiorito et al., 2008b], could be due to a relationship between TCDD and bICP0. To test this hypothesis, in the present study, we have evaluated apoptotic cell death in MDBK cells infected with BHV-1 and exposed to TCDD. Then, we have analyzed the effects of TCDD on bICP0 expression during infection using semi-quantitative RT-PCR, Western blot analysis and immunocytochemistry. Furthermore, Western blot analysis of nuclear and cytosolic fractions of infected cells was performed in order to evaluate bICP0 localization during productive infection.

MATERIALS AND METHODS

MATERIALS

MDBK cells (CCL22, American Type Culture Collection) were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 2% foetal calf serum (FCS), 1% L-glutamine, 1% penicilline/streptomycine, 0.2% sodium pyruvate and 0.1% tylosine. Cells were maintained in an incubator at 37°C (in 5% CO_2/95% air). This cell line was maintained free of mycoplasma and of bovine viral diarrhoea virus.

The BHV-1 Cooper strain was used throughout the study. Virus stocks were routinely grown on MDBK cells and were also used for determination of virus titers [De Martino et al., 2003].

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) in toluene was purchased from Supelco (St. Louis, MO). All other chemicals were of the highest commercially available purity.

CELLS, VIRUS INFECTION, AND TCDD EXPOSURE

MDBK cells, at confluency, were washed with DMEM and then infected or not with Bovine Herpesvirus type 1 (BHV-1), at multiplicity of infection (MOI) of 5, in presence or in absence of different concentrations of TCDD (0.01, 1, and 100 pg/ml). After 1 h of adsorption at 37°C, the cells were incubated for indicated times post-infection and then processed.

DNA FRAGMENTATION ASSAY

Monolayers of MDBK cells were grown in 25 cm^2 flask, after 24 h, at confluency, were infected with BHV-1 and exposed to TCDD (0.01, 1, or 100 pg/ml). After 4, 8, 12, 24, 36, and 48 h post-infection, DNA from treated or not-treated infected cells was extracted using a commercial Qiagen DNeasy tissue kit (Qiagen), according to the manufacturer's instructions for cultured cells. Five micrograms of each DNA sample was electrophoresed on a 1.5% agarose gel containing 0.1 mg of ethidium bromide per ml. The DNA was visualized under UV light, and the sizes of the respective amplified products were estimated by comparing the mobilities with a 100-bp ladder (Invitrogen, Milan, Italy). The images of PCR products specific bands on agarose gels were imported into a computer by scanner captured as digital TIFF images.

EXAMINATION OF CELL MORPHOLOGY

Cell morphology was examined by light microscopy following Giemsa staining. Briefly, monolayers of MDBK cells (10^5 cells per well), in 16-well culture chambers, were infected with BHV-1, in the presence or absence of different concentrations of TCDD (0.01, 1, or 100 pg/ml) and incubated at 37°C. After 4, 8, 12, 24, 36, and 48 h post-infection, cells were washed twice with PBS. Cells were fixed with 95% ethanol, drained, and dried. Afterward, cells were stained with a 5% Giemsa solution (Merck, Darmstadt, Germany). After 30 min cells were rinsed with tap water and H_2O. The slides were mounted in Entellan (Merck) and coverslipped. Light microscopic studies and photomicrographs observations were carried out.

SEMI-QUANTITATIVE RT-PCR ANALYSIS

MDBK cells were grown in 24 well plates, after 24 h, confluent cells, were washed with DMEM and then infected with Bovine Herpesvirus type 1 (BHV-1), at multiplicity of infection (MOI) of 5, in the presence or absence of different concentrations of TCDD (0.01, 1, or 100 pg/ml). After 1 h of adbsorption at 37°C, cells were incubated for 1, 2, 3, 4, 8, 12, 24, 36, and 48 h and then processed. Total RNA from infected and treated or infected and not-treated MDBK cells was extracted using a commercial High Pure RNA Isolation kit (total RNA isolation system from Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions for cultured cells. Two micrograms of total RNA were reverse transcribed using Oligo-dT (Invitrogen) with reverse transcriptase from Invitrogen as instructed

by the supplier. The first-strand cDNA was amplified using the primers previously described for bICP0 and β-actin respectively by Delhon et al. [2002] and Ciacci-Zanella et al. [1999]. The cDNA was amplified in a Eppendorf thermal cycler under the experimental conditions previously described [Schang and Jones, 1997]. PCR products were analyzed by electrophoresis on 1.5% ethidium bromide-containing agarose gels and visualized under UV light. The sizes of the respective amplified products were estimated by using a 100-bp ladder (Invitrogen). The images of PCR products specific bands on agarose gels were imported into a computer by scanner captured as digital TIFF images and quantified using UV-GEL TEC program.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

MDBK cells in 75 cm^2 flask, at confluency, were infected with BHV-1 at MOI 5, in the presence or absence of different concentrations of TCDD (0.01, 1, or 100 pg/ml). After 1, 2, 3, 4, 8, 12, 24, 36, and 48 h post-infection, adherent cells were washed twice with PBS and removed from the flask by treatment with trypsin-EDTA solution. Then cells were mixed with cells previously collected by centrifugation in supernatant from the same flask and resuspended at an adequate concentration in PBS. The pellets, obtained by centrifugation, were stored at −20°C.

Cells were homogenized directly into lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate). The lysates were clarified by centrifugation at 14,000 rpm × 10 min. Protein concentrations were estimated by an assay (Bio-Rad, Hercules, CA) and boiled in Laemmli buffer [0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue] for 5 min before electrophoresis. Proteins were subjected to SDS-PAGE (12.5% polyacrylamide). After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon, Millipore Corp., Bedford, MA); complete transfer was assessed using pre-stained protein standards (Bio-Rad). After blocking with Tris-buffered saline-BSA [25 mM Tris (pH 7.4), 200 mM NaCl, and 5% BSA], the membrane was incubated with the primary antibodies. The following antibodies, dissolved in 5% bovine serum albumin-TBST, were used: polyclonal rabbit anti-BICP0 (a.a. 663–676) serum, kindly provided by M. Schwyzer (University of Zürich, Switzerland) [Fraefel et al., 1994], (dilution 1:800), and anti-ß-actin MAb (dilution 1:7,500), purchased from Cell Signaling (Italy). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:10,000) (at room temperature), and the reaction was detected with an enhanced chemiluminescence system (Amersham Life Science, Buckinghamshire, UK). The images of Western immunoblot specific bands on X-ray films were imported into a computer by a scanner and captured as digital TIFF images. Furthermore, the results were plotted in a graph after densitometric analysis of the blots obtained.

ISOLATION OF NUCLEAR AND CYTOSOLIC FRACTIONS

Cells were harvested and washed twice with ice cold PBS, then centrifuged at 1,200 rpm for 7 min at 4°C. Pellets were re-suspended in 0.33 M sucrose, 10 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 0.1% Triton X-100 in 5:1 v/v (ice cold and in presence of protease inhibitors). After 15 min on ice, aggregates were broken up gently with thin glass rod and centrifuged at 3,000 rpm for 5 min at 4°C. The supernatants representing the cytosolic fractions and the pellets containing a pure preparation of nuclei without the nuclear membrane were obtained and stored at −70°C. Nuclear pellets were gently re-suspended in ice cold 0.45 M NaCl, 10 mM Hepes pH 7.4 (in the presence of protease inhibitors). The suspension was incubated on ice for 15 min. After centrifugation at 14,000 rpm for 5 min at 4°C, supernatant representing the nuclear extract was collected and then Western blot analysis of nuclear and cytosolic fractions were performed to detect bICP0.

IMMUNOCYTOCHEMISTRY

Cells were processed for immunocytochemistry essentially as described by Kousoulas et al. [1984]. However, cells were fixed with methanol to permeabilize the cell membrane. Briefly, monolayers of MDBK cells (10^5 cells per well), in 16-well plates, were infected with BHV-1 at MOI 5, in the presence or absence of different concentrations of TCDD (0.01, 1, or 100 pg/ml) and incubated at 37°C. After 1, 2, 3, 4, 8, 12, 24, 36, and 48 h post-infection, cells were washed twice with PBS. Cells were fixed with acetone and washed with PBS. Afterward, 1% H_2O_2 in methanol was added at 50 μl per well. After 10 min, cells were washed with PBS. Then, 25 μl of 10% normal goat serum per well was added and chambers were incubated at 37°C. After 30 min, 25 μl of diluted (1:400) polyclonal rabbit anti-BICP0 serum per well was added and chambers were incubated for 60 min at 37°C. Cells were washed three times with PBS and incubated for 40 min at 37°C with 25 μl of biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA) per well. After washing with PBS, ABC solution (avidin-biotinylated horseradish peroxidase macromolecular complex—Vectastain kit), prepared as instructed by the supplier (Reactolab S.A.), was added to the cells at 50 μl per well. After 30 min at 37°C, the ABC solution was removed and cells were washed twice with PBS. Afterwards, a substrate consisting of nickel-DAB solution (3,3′-diaminobenzidine tetrahydrochloride; 10 mg/15 ml, of PBS; Sigma) and 0.025% H_2O_2 was added at 100 μl per well. Staining reaction was controlled under microscope and stopped after 5–10 min in tap water. The slides were counterstained with hematoxylin. After that cells were dehydrated in graded alcohols, cleared in xylene and mounted in Entellan (Merck) and coverslipped. Some groups of cells incubated with normal goat serum instead of primary antibody were used as negative controls, which failed to stain gray-black with nickel-DAB. Light microscopic studies and photomicrographs observations were carried out.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. The *paired* Student's *t*-test was used for comparison between control and experimental groups. *P*-value <0.05 was considered statistically significant.

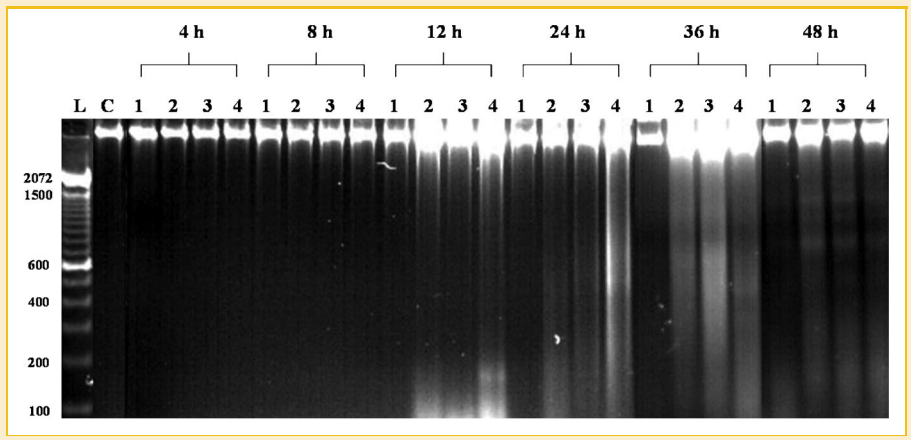

Fig. 1. TCDD exposure anticipates apoptosis in BHV-1 infected cells compared to controls. BHV-1 infected cells have displayed DNA degradation already at 12 h p.i. in TCDD exposed cells, while only at 48 h p.i. in not exposed cells. Agarose gel electrophoresis of DNA extracted from MDBK cells at 4, 8, 12, 24, 36, and 48 h p.i. unexposed (Lane 1) and exposed to TCDD (0.01, 1, and 100 pg/ml, Lanes 2, 3, and 4 respectively). Lane C, DNA prepared from control cells at 48 h; Lane M, 100-bp ladder. Numbers on the left indicate base pairs. Data from one of three experiments is shown.

RESULTS

TCDD ANTICIPATES BHV-1 INDUCED APOPTOSIS IN MDBK CELLS

The effects of different concentrations of TCDD (0.01, 1, and 100 pg/ml) on BHV-1 induced apoptosis were evaluated in MDBK infected cells at various times post-infection. These experiments showed that TCDD exposure anticipates apoptosis in BHV-1 infected cells compared to controls, as demonstrated by DNA laddering already at 12 h p.i. (Fig. 1). BHV-1 infected but unexposed group revealed DNA laddering at 48 h p.i. (Fig. 1), as already demonstrated in previous studies [Devireddy and Jones, 1999]. Moreover, a greater number of cell shrinkage and pycnotic nuclei was detected, using light microscopy of Giemsa-stained cells, already at 12 h p.i. in TCDD exposed cells compared to control cells (Fig. 2).

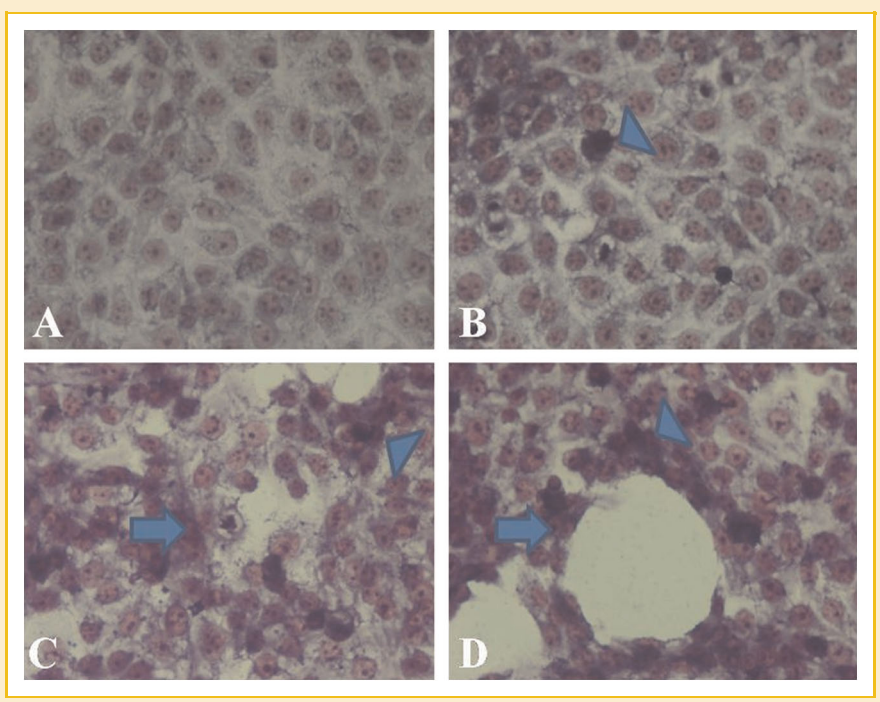

Fig. 2. Photomicrographs showing morphology of MDBK cells stained with Giemsa. MDBK cells were infected with BHV-1, at MOI of 5, in presence or absence of different concentrations of TCDD and incubated at various time post-infection. Cells were fixed, stained with Giemsa solution and then observed under light microscope, as described in the Materials and Methods Section. A: MDBK untreated cells (control). B: BHV-1 infected cells after 12 h of infection displayed a small number of pycnotic nuclei (arrowheads). All groups of BHV-1 infected cells exposed to TCDD (0.01 pg/ml) (C), or to (100 pg/ml) (D) showed a large number of both cell shrinkage (arrows) and pycnotic nuclei (arrowheads) (magnification, 400×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TCDD EXPOSURE INCREASES GENE EXPRESSION OF bICP0

In order to evaluate the role of TCDD on bICP0 gene expression during BHV-1 infection, MDBK cells were infected with BHV-1, in the presence or absence of different concentrations of TCDD (0.01, 1, or 100 pg/ml).

Figure 3 shows that bICP0 gene expression levels decreases from 2 to 4 h p.i., followed by an increase from 8 to 12 h p.i., and then a further decrease from 24 h to the end of infection (Fig. 3A,B). By contrast, in the presence of TCDD, we observed that bICP0 mRNA was never transcribed at detectable levels, before 2 h of infection in all treated groups (Fig. 3). Furthermore, in the presence of all doses of TCDD tested, we observed a significant ($P < 0.001$, $P < 0.01$, and $P < 0.05$) increase of bICP0 gene expression levels in BHV-1 infected groups compared to unexposed controls (Fig. 3). In particular, levels of bICP0 gene increased from 4 h to 12 h p.i., undergoing a steady but small reduction at 24, 36, and 48 h p.i. (Fig. 3A,B). Moreover, we detected a dose-dependent increase in the levels of bICP0 gene expression in all exposed groups, as compared to unexposed controls (Fig. 3A,B).

Our results indicated that TCDD exposure during BHV-1 infection significantly increased bICP0 gene expression levels, in a dose-dependent manner, compared to untreated controls.

TCDD EXPOSURE INCREASES PROTEIN EXPRESSION OF bICP0

We examined the effects of different concentrations of TCDD (0.01, 1, or 100 pg/ml) on the expression of bICP0 protein by Western blot analysis. As expected, in unexposed groups, bICP0 protein expression, was detected 2 h after infection, decreased (50%) at 3 h p.i. and progressively increased from 4 h p.i., reaching the highest expression at 24 h p.i., then decreased from 36 h until the end of infection (Fig. 4A,B). These data agree to previous studies which demonstrated a similar trend of bICP0 expression, even though using a different dilution (1:1,000) of the same anti-bICP0 serum [Inman et al., 2001b]. TCDD, in a dose dependent manner, induced a

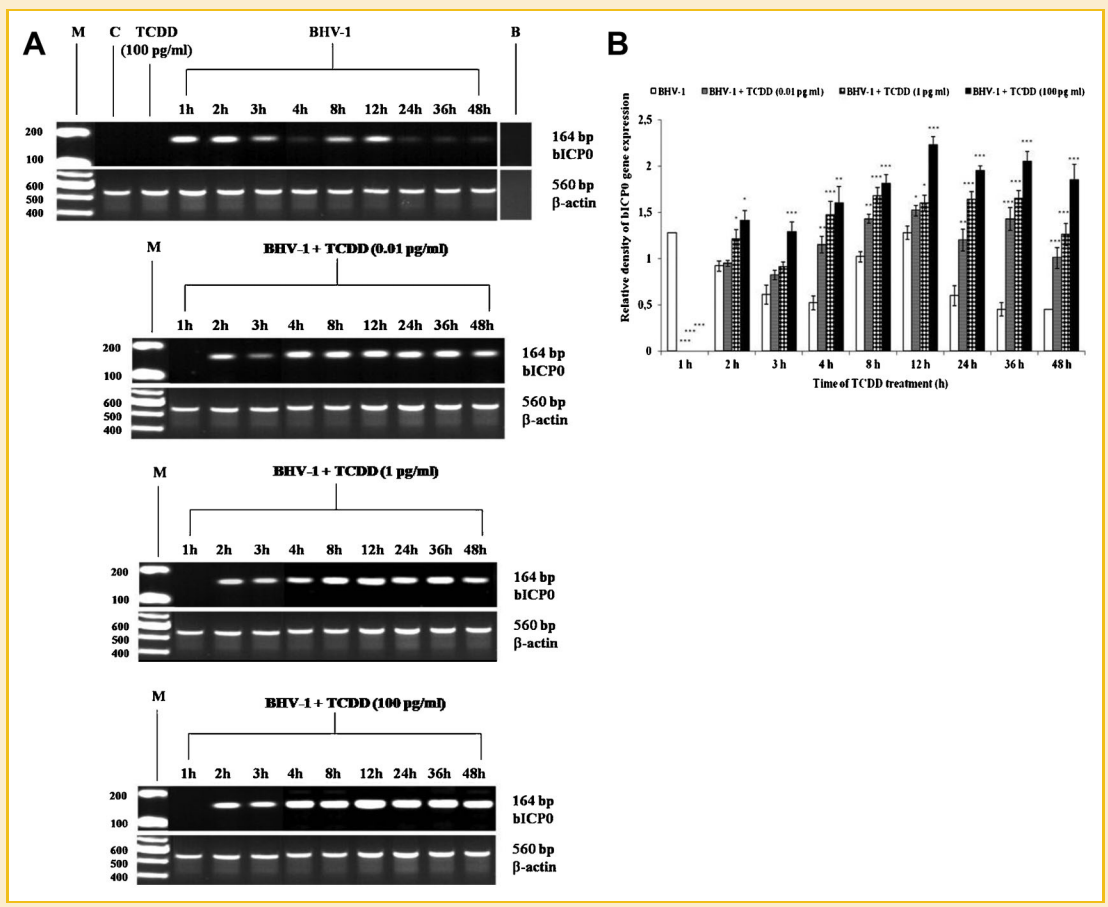

Fig. 3. Effects of TCDD on bICP0 gene expression in MDBK cells infected with BHV-1. A: RT-PCR was performed with RNA prepared from untreated cells (control) (lanes C), infected cells (lane BHV-1), or infected and exposed to different concentrations of TCDD (lane BHV-1 + TCDD 0.01 pg/ml), (lane BHV-1 + TCDD 1 pg/ml) or (lane BHV-1 + TCDD 100 pg/ml) cells after 1, 2, 3, 4, 8, 12, 24, 36, or 48 h p.i. Lane B is the no-template reaction, lane M is a 100-bp molecular weight marker. Numbers on the left are sizes of the markers in base pairs. RT-PCR was performed as described in the Materials and Methods Section. Amplified products were electrophoresed on 1.5% agarose gels. RT-PCR was performed with primers bICP0 and with primers β-actin. Primers bICP0 amplify a 164-bp product [Delhon et al., 2002], and primers β-actin amplify a 540-bp product [Ciacci-Zanella et al., 1999]. B: Densitometry analysis of the gels shown in (A). Results are the mean ± SD of three separate experiments. Significant differences between unexposed infected groups and TCDD-exposed and infected groups are indicated by probability p. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; Student's *t*-test.

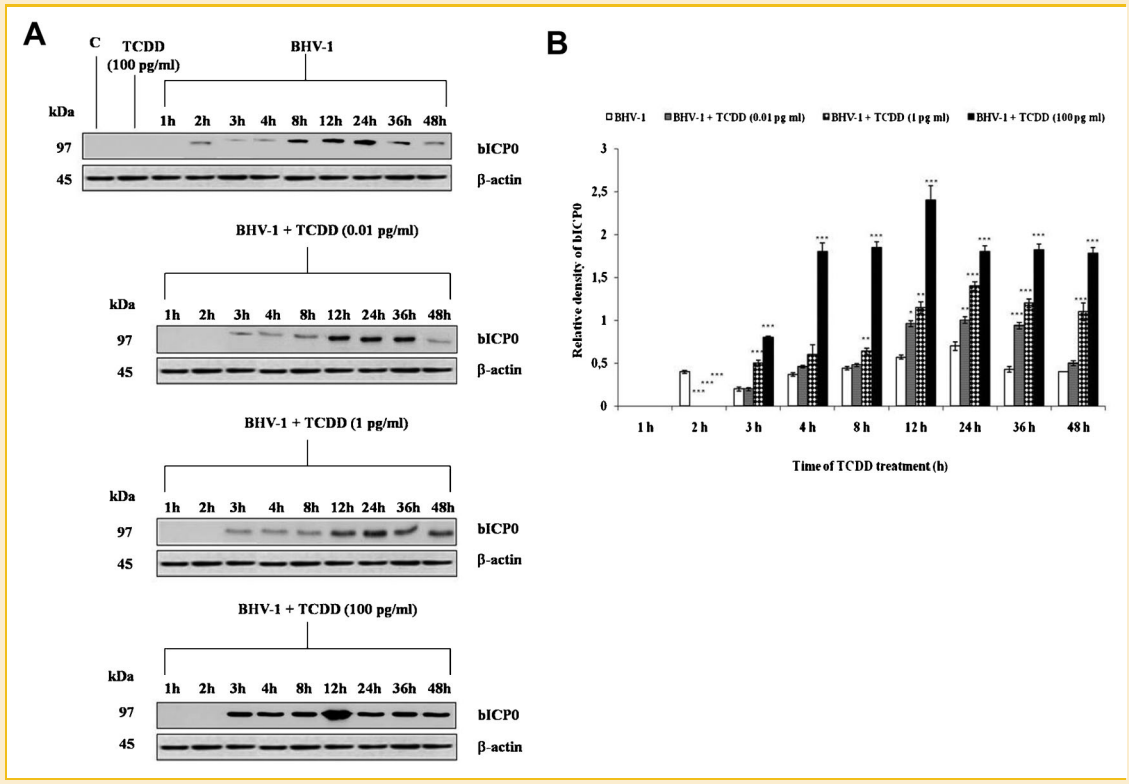

Fig. 4. Effects of TCDD on bICP0 protein expression. A: Whole-cell lysate were prepared from untreated cells (control) (lanes C), infected cells (lane BHV-1), or infected and exposed to different concentrations of TCDD (lane BHV-1 + TCDD 0.01 pg/ml), (lane BHV-1 + TCDD 1 pg/ml) or (lane BHV-1 + TCDD 100 pg/ml) cells and, after 1, 2, 3, 4, 8, 12, 24, 36, or 48 h p.i. Western blot analysis was performed with an antibody which specifically recognized bICP0 or β-actin, as described in the Materials and Methods Section. β-Actin was used as an internal loading control. The molecular weight (in kDa) of protein size standards is shown on the left hand side. Blots are representative of at least three separate experiments. B: Densitometry analysis of the blots shown in (A). Results are the mean ± SD of three separate experiments. Significant differences between unexposed infected groups and TCDD-exposed and infected groups are indicated by probability *P*. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; Student's *t*-test.

significant increase in bICP0 protein levels in infected groups, as compared to unexposed controls (Fig. 4). Western blot analysis of BHV-1 infected cells showed that bICP0 was detected in TCDD exposed groups starting from 3 h p.i. (Fig. 4A,B). Then, TCDD drastically and significantly ($P < 0.001$, $P < 0.01$, and $P < 0.05$) induced an increase in bICP0 levels, in a time dependent manner, in all infected groups from 4 to 48 h p.i. (Fig. 4A,B). In particular, 0.01 or 1 pg/ml of TCDD, even though increasing bICP0 protein levels, did not change bICP0 protein kinetic: reaching the maximum after 24 h of infection (Fig. 4A,B) then decreasing from 36 h to the end of infection. In contrast, 100 pg/ml of TCDD increased bICP0 protein expression and changed bICP0 protein kinetic. In fact, bICP0 protein expression peaked at 12 h of infection (Fig. 4A,B), then decreased reaching a plateau at 24–48 h p.i.

To confirm the results reported above and to evaluate cellular distribution of bICP0 protein at various times post-infection, we performed immunocytochemistry assay in MDBK cells infected with BHV-1, in the presence or absence of different concentrations of TCDD (0.01, 1, and 100 pg/ml). Here, we displayed only the results obtained after 12 h p.i., a time in which, in the same experimental conditions, apoptotic features occurred in BHV-1 infected cells exposed to TCDD, as previously shown [Fiorito et al., 2008b]. The typical color developed with nickel-DAB (gray-black) indicated that the serum specifically reacted with antigens localized in the nuclei (Fig. 5C—arrowheads), as well as, in the cytoplasm (Fig. 5C—arrows) of cells infected with BHV-1 (Fig. 5) showing that bICP0 entered the nucleus as well as the cytoplasm of infected cells. Such results demonstrated that TCDD exposure increased both nuclear (Fig. 5D,E,F—arrowheads) and cytosolic (Fig. 5D,E,F—arrows) accumulation of bICP0 protein after 12 h of infection. In particular, in presence of TCDD, a dose-dependent increase in nuclear and cytosolic accumulation of bICP0 protein was detected. In contrast, MDBK control cells or cells infected but incubated with normal goat serum instead of primary antibody (negative control), counterstained with hematoxylin, displayed only typical light pink color at the same time (Fig. 5A,B).

These experiments showed that TCDD, in a dose-dependent manner, caused a significant increase in the levels of bICP0 proteins during BHV-1 infection.

TCDD EXPOSURE MODIFIES NUCLEAR AND CYTOSOLIC DISTRIBUTION OF BICP0 PROTEIN

To determine the effects of TCDD in bICP0 intracellular distribution during BHV-1 infection, Western blot analysis was performed on both cytosolic and nuclear extracts. Figure 6 shows that bICP0 protein was expressed in the nuclear fraction of infected cells starting at 2 h p.i., and decreasing from 3 to 48 h p.i. (Fig. 6A,B). A

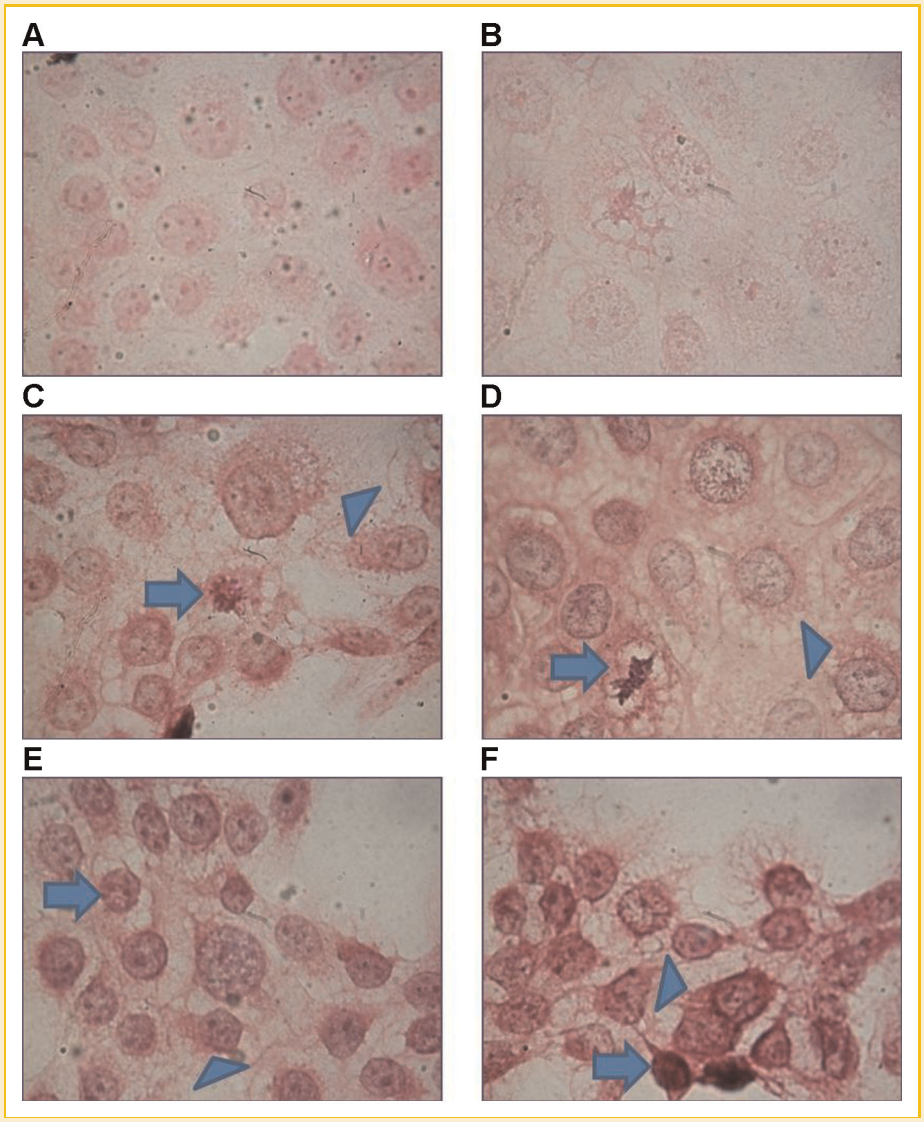

Fig. 5. Photomicrographs showing immunocytochemistry assays for bICP0. MDBK cells were infected with BHV-1, at MOI of 5, in the presence or absence of different concentrations of TCDD (0.01, 1, or 100 pg/ml). After 12 h post-infection, fixed cells were incubated with polyclonal rabbit anti-bICP0 serum (1:800), stained with biotinylated goat anti-rabbit immunoglobulin G and the peroxidase-labeled avidin. The color was developed with nickel-DAB (gray-black), the slides were counterstained with hematoxylin and then observed under light microscope, as described in the Materials and Methods Section (magnification, 1,000×). A: MDBK untreated cells (control). B: BHV-1 infected cells incubated with normal goat serum instead of primary antibody (negative control) at 12 h of infection (negative control). After 12 h of infection, all groups of BHV-1 infected cells unexposed (C) or exposed to TCDD (0.01 pg/ml) (D), (1 pg/ml) (E) or to (100 pg/ml) (F) showed both nuclear (arrowheads) and cytosolic (arrows) accumulation of bICP0 protein. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significant increase of bICP0 protein levels in the nuclear fraction was evident at 3 h p.i. in BHV-1 infected cells treated with TCDD compared to unexposed controls (Fig. 6A,B). In particular, 100 pg/ml TCDD drastically and significantly ($P < 0.001$) enhanced the bICP0 protein expression in infected cells from 3 to 4 h p.i. (Fig. 6A,B). Analyzing treated cells at 0.01 and 100 pg/ml of TCDD compared to 1 pg/ml of TCDD (Fig. 6A,B), we distinguished after 8 h of infection an U-shaped dose responsiveness with a significant ($P < 0.001$ and $P < 0.01$) increase of bICP0 protein levels in treated cells. This modulation with an U-shaped dose responsiveness induced by TCDD was consistent with results of previous studies [Murayama et al., 2002; Ahn et al., 2005; Fiorito et al., 2008a,b]. Our results suggested that TCDD caused a significant ($P < 0.001$, $P < 0.01$, and $P < 0.05$) dose-dependent increase in the bICP0 protein levels from starting from 12 h p.i. to the end of infection (Fig. 6A,B). Furthermore, expression of bICP0 protein in the cytosolic fraction of infected cells was already evident at 8 h p.i., with an increasing trend between 12 and 24 h and a decrease from 36 h p.i. on Figure 6A–C. In the same experimental conditions and in the presence of the highest dose of TCDD (100 pg/ml), we found a detectable expression of bICP0 protein in cytosolic fraction after 3 h of infection (Fig. 6A–C). After 4 h of infection, in all TCDD exposed groups, we observed a significant ($P < 0.001$, $P < 0.01$, and $P < 0.05$) increase in bICP0 protein levels compared to infected but unexposed control groups

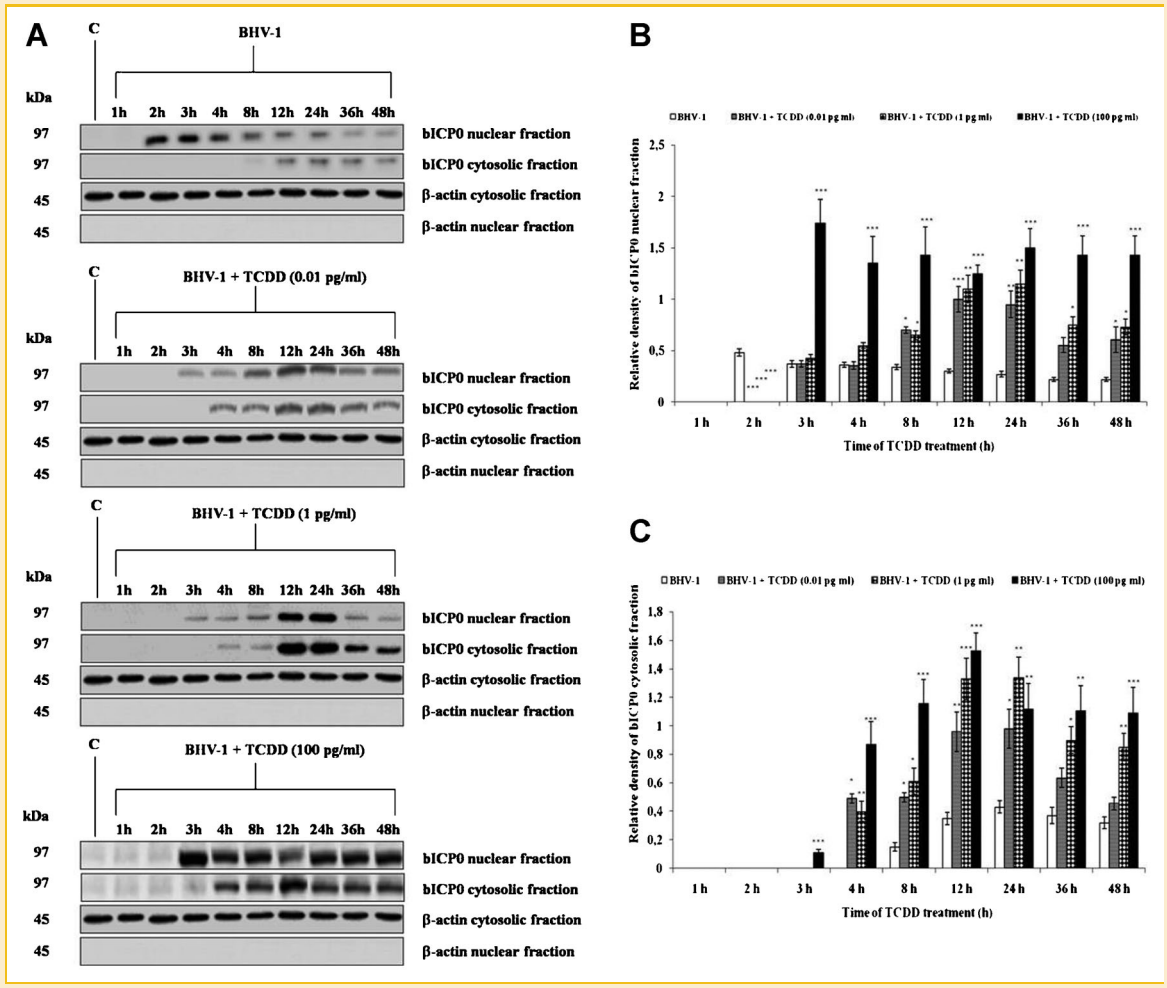

Fig. 6. Effects of TCDD on bICP0 nuclear/cytosolic localization. A: Nuclear and cytosolic fractions was prepared from untreated cells (control) (lanes C), infected cells (lane BHV-1), or cells infected and exposed to different concentrations of TCDD (lane BHV-1 + TCDD 0.01 pg/ml), (lane BHV-1 + TCDD 1 pg/ml) or (lane BHV-1 + TCDD 100 pg/ml), after 1, 2, 3, 4, 8, 12, 24, 36, or 48 h p.i. Western blot analysis was performed with an antibody which specifically recognized bICP0 or β-actin, as described in the Materials and Methods Section. β-Actin was used as an internal loading control. The molecular weight (in kDa) of protein size standards is shown on the left hand side. Blots are representative of at least three separate experiments. B: Densitometry analysis of the nuclear fractions blots shown in (A). C: Densitometry analysis of the cytosolic fractions blots shown in (A). Results are the mean ± SD of three separate experiments. Significant differences between unexposed infected groups and TCDD-exposed and infected groups are indicated by probability *P*. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; Student's *t*-test.

(Fig. 6A–C). When we compared the treated cells at 0.01 and 100 pg/ml of TCDD to those treated at 1 pg/ml (Fig. 6A–C), we distinguished, after 4 h of infection and at 24 h p.i., a typical modulation respectively with an U-shaped and an inverted U-shaped dose responsiveness with a significant ($P < 0.001$, $P < 0.01$, and $P < 0.05$) increase of bICP0 protein levels in treated cells, as reported above. In addition, after 12 h p.i. and from 36 h to the end of infection, TCDD, in a dose-dependent manner, caused a significant ($P < 0.001$, $P < 0.01$, and $P < 0.05$) increase in the bICP0 protein levels, as revealed by western blot analysis (Fig. 6A–C). These data demonstrated that TCDD induced both a different cellular distribution of bICP0 protein and an increase of its level in bovine cells infected with BHV-1, Cooper strain, at MOI of 5. In particular, bICP0 protein levels were detected in the nuclear fraction from TCDD exposed cells later respect to the same fraction from unexposed cells. In contrast, bICP0 protein was identified in the cytosolic fraction from TCDD exposed cells earlier respect to the same fraction from unexposed cells. Our findings showed that, in unexposed groups, translocation of bICP0 from the nucleus to the cytoplasm was detected already at 8 h of infection in according with previous studies [Lopez et al., 2001; Van Sant et al., 2001]. Furthermore, these data confirmed that TCDD enhances bICP0 protein expression during infection with BHV-1 in cultured cells. In addition, the presence of TCDD increases nuclear and cytosolic accumulation of bICP0 protein at all doses tested.

DISCUSSION

This report describes the effects of TCDD on bICP0, gene and protein expression, following infection of BHV-1 in bovine cells. The immediate early protein bICP0 is a promiscuous activator of both

viral and cellular genes [Wirth et al., 1992] and play a critical role in virus life cycle [Wirth et al., 1992; Fraefel et al., 1994; Inman et al., 2001a]. Furthermore, bICP0 promotes productive infection in differentiated cells, but the mechanism by which bICP0 stimulates productive infection or activates viral gene expression is poorly understood [Jones et al., 2006]. In a previous study we demonstrated that TCDD increases BHV-1 replication through a dose-dependent increase in cytopathy and increased viral titer in bovine cells [Fiorito et al., 2008a]. Since TCDD plays a role in BHV-1 infection [Fiorito et al., 2008a,b], herein, we have shown that TCDD increased the levels of both bICP0 gene expression and protein expression during infection. In particular, the semi-quantitative RT-PCR analysis indicated that different concentrations (0.01, 1, and 100 pg/ml) of TCDD modified the times of bICP0 gene expression and increased bICP0 gene expression, in a dose-dependent manner respect to controls, at all time intervals studied (Fig. 3). Therefore, the effects of TCDD exposure on bICP0 gene expression, characterized by a delayed onset of transcription and increased detectable levels of bICP0, herein explored, could be due to the fact that TCDD, by altering the kinetic of bICP0 gene, increases viral DNA replication. In support of this hypothesis, a previous study reported, in presence of TCDD, an increase of cytomegalovirus replication in infected human fibroblasts with a concomitant increase in viral DNA replication [Murayama et al., 2002].

Our data, the first reported so far on bICP0 nucleo-cytoplasmic trafficking, evidenced the ability of TCDD to induce a different cellular distribution of bICP0 protein, during BHV-1 infection, with detectable levels of bICP0 protein in the nuclear fraction appearing later than unexposed groups, whereas in cytosolic fraction bICP0 was detected earlier than in unexposed groups. Furthermore, TCDD caused a significant increase in the levels of bICP0 proteins during BHV-1 infection, on both nuclear and cytosolic fractions (Fig. 6). In fact, in TCDD exposed groups, we found in cytosolic fraction a detectable expression of bICP0 protein from 3 or 4 h of infection whereas, in the same experimental conditions, in unexposed groups, bICP0 protein was detected from 8 h of infection. As reported above, BHV-1 shares certain biological properties with HSV-1 and HSV-2. In particular, bICP0 is bovine homologue of HSV-1 ICP0. Immediately after its synthesis, ICP0 is transported to the nucleus of the infected cell [Van Sant et al., 2001]. Lopez et al. [2001] showed that translocation of ICP0 from the nucleus to the cytoplasm of HEL fibroblasts takes place between 5 and 9 h after infection with wild-type virus, that is, at the time of or after the onset of viral DNA synthesis and, more likely, the expression of γ2 proteins whose synthesis requires the synthesis of viral DNA. Late in infection, ICP0 is actively sequestered in the cytoplasm by a process mediated by proteasomes [Lopez et al., 2001]. The peregrinations of ICP0 appear to depend on both viral and cellular factors [as reviewed in Hagglund and Roizman, 2004]. ICP0 binds cyclin D3 [Kawaguchi et al., 1997], in particular, the overexpression of cyclin D3 accelerates the translocation of ICP0 into the cytoplasm [Van Sant et al., 2001]. However, the mechanism by which cyclin D3 enables the export of ICP0 to the cytoplasm is unknown.

TCDD has been previously shown to alter the cellular concentration of several regulatory cell-cycle proteins, including cyclins, in several cell lines [Barnes-Ellerbe et al., 2004; Akintobi et al., 2007]. In our previous study we showed that TCDD induces cell proliferation and increased cell viability in MDBK cells [Fiorito et al., 2008a]. Consequently, the anticipated times of bICP0 translocation, respect to unexposed groups, that we evidenced, could be due to the action of TCDD on cell cycle. It is known that bICP0 is involved in virus-induced apoptosis [Henderson et al., 2004; Geiser et al., 2008]. It is well-established that BHV-1 induces apoptosis in infected cells [Devireddy and Jones, 1999; Geiser et al., 2008; Fiorito et al., 2008b]. Herein, we confirmed the detection of apoptotic features already at 12 h after infection, only in TCDD exposed groups (Figs. 1 and 2), as previously shown using other methods [Fiorito et al., 2008b]. However, little is known about the viral factor/event which triggers the apoptotic process. Previous studies reported that the plasmid ΔbICP0 was predominantly localized to the cytoplasm of transfected neuro-2A cells, whereas bICP0 was primarily localized to the nucleus [Inman et al., 2001a], suggesting that interactions between cytoplasmic factors and bICP0 stimulated toxicity and apoptosis [Henderson et al., 2004]. Furthermore, bICP0, in the absence of any viral gene expression, indirectly induces caspase 3 activation and apoptosis in mouse neuroblastoma cells (neuro-2A) and bovine turbinate cells [Henderson et al., 2004]. Then, during BHV-1 productive infection, caspase 3 is activated relatively late [Devireddy and Jones, 1999; Lovato et al., 2003; Fiorito et al., 2008b], suggesting that bICP0, in part, activates caspase 3 [Jones et al., 2006]. In addition, it has been demonstrated that ICP0 gene expression acts as an initial inducer of apoptosis during HSV-1 infection [Sanfilippo and Blaho, 2006]. Consequently, we suppose that the anticipated caspase 3 activation after 4 h p.i., during treatment with TCDD [Fiorito et al., 2008b], may be due to the fact that, as shown here, after 4 h of exposure, TCDD causes a cytosolic accumulation of bICP0 that, in turn, would activate caspase 3.

In conclusion, our study suggests that TCDD causes a dose-dependent increase in cytopathy, an increased viral titer [Fiorito et al., 2008a] and anticipation of BHV-1-induced apoptosis [Fiorito et al., 2008b; and here], by modifying bICP0 expression and nuclear/cytosolic localization during infection. Future studies will be crucial to elucidate the mechanism by which TCDD interacts with bICP0 protein in bovine cells.

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