

# E4orf4, a Novel Adenovirus Death Factor That Induces p53-independent Apoptosis by a Pathway That Is Not Inhibited by zVAD-fmk

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**Abstract.** In the absence of E1B, the 289-amino acid product of human adenovirus type 5 13S E1A induces p53-independent apoptosis by a mechanism that requires viral E4 gene products (Marcellus, R.C., J.C. Teodoro, T. Wu, D.E. Brough, G. Ketner, G.C. Shore, and P.E. Branton. 1996. *J. Virol.* 70:6207–6215) and involves a mechanism that includes activation of caspases (Boulakia, C.A., G. Chen, F.W. Ng, J.G. Teodoro, P.E. Branton, D.W. Nicholson, G.G. Poirier, and G.C. Shore. 1996. *Oncogene.* 12:529–535). Here, we show that one of the E4 products, E4orf4, is highly toxic upon expression in rodent cells regardless of the p53 status, and that this cytotoxicity is significantly overcome by coexpression with either Bcl-2 or Bcl-X<sub>L</sub>. Conditional expression of E4orf4 induces a cell death process that is characterized by apoptotic hallmark

features, such as externalization of phosphatidylserine, loss of mitochondrial membrane potential, cytoplasmic vacuolation, condensation of chromatin, and internucleosomal DNA degradation. However, the wide-spectrum inhibitor of caspases, tetrapeptide zVAD-fmk, does not affect any of these apoptogenic manifestations, and does not alter the kinetics of E4orf4-induced cell death. Moreover, E4orf4 expression does not result in activation of the downstream effector caspase common to most apoptosis-inducing events, caspase-3 (CPP32). We conclude, therefore, that in the absence of E1A, E4orf4 is sufficient by itself to trigger a p53-independent apoptosis pathway that may operate independently of the known zVAD-inhibitable caspases, and that may involve an as yet uncharacterized mechanism.

**T**ISSUE homeostasis is maintained by a tight balance between cellular proliferation and cell death processes such as apoptosis. Apoptosis is a cell suicide mechanism culminating in an autolytic cellular degradation that can be triggered by a wide range of stimuli, such as viral infection (White and Gooding, 1994; Teodoro and Branton, 1997), growth factor withdrawal (Evan et al., 1992; Rao et al., 1992; Wagner et al., 1994; Lin and Benchamol, 1995; Sakamuro et al., 1995), TNF- $\alpha$  and Fas ligand (for review see Nagata, 1997), and DNA damage after either irradiation (Clarke et al., 1993; Lowe et al., 1993b; Strasser et al., 1994; Han et al., 1995), or treatment with chemotherapeutic drugs (Lowe et al., 1993a). Apoptotic cell death may occur in either a p53-dependent or -independent manner (for review see Liebermann et al., 1995). The role of p53 as a general tumor suppressor gene is well established, and its ability to function as a sequence-specific transcription factor appears to be directly linked to its role in G1-arrest of the cell cycle in response to detrimen-

tal conditions (Bates and Vousden, 1996). The mechanism by which p53 regulates induction of apoptosis, however, remains unclear, but may rely on the ability of p53 to control expression of proapoptotic inducers such as Bax (Reed, 1997). The molecular determinants of p53-independent apoptotic responses remain poorly understood.

Although the initial triggering phase of apoptosis may take several routes, in most cases the execution phase of this process converges on a common pathway characterized by activation of the caspase family of interleukin-1 $\beta$ -convertase enzyme (ICE)<sup>1</sup>-like cysteine proteases (Oltvai and Korsmeyer, 1994; Steller, 1995; Chinnaiyan and Dixit, 1996; Fraser and Evan, 1996). Additionally, however, recent evidence suggests that other pathways, which do not rely on activation of the known caspases, can also lead to cell death and manifestation of the morphological features of apoptosis (Xiang et al., 1996; Boise and Thompson, 1997). In both cases, irreversible commitment to cell death

1. *Abbreviations used in this paper:* CPP32, caspase-3; DAPI, 4',6-diamidino-Z-phenylindole; HA, hemagglutinin; ICE, interleukin-1 $\beta$ -convertase enzyme; PARP, poly(ADP-ribosyl) polymerase; PP, protein phosphatase; rTA, reverse tetracycline; TMRE, tetramethylrhodamine ethyl ester perchlorate.

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may involve a loss of the mitochondrial membrane potential ( $\Delta\psi_m$ ) through opening of the permeability transition pore complex (Kroemer et al., 1995; Petit et al., 1995; Zamzami et al., 1995a,b; Castedo et al., 1996; Zamzami et al., 1996; Kroemer, 1997). Regardless of the biochemical transformations leading to apoptosis, however, the final phase — the degradation phase — is usually characterized by typical morphological changes, including plasma membrane blebbing, cytoplasmic boiling and vacuolation, chromatin condensation, internucleosomal cleavage of DNA, cell shrinkage, and fragmentation of the cell into membrane-bound apoptotic bodies that are ultimately engulfed by other cells (Wyllie, 1980). Not surprisingly, both the initiation and execution of apoptosis are tightly regulated. One element of this control is provided by a family of proteins related to Bcl-2, which act as general suppressors of apoptosis in many contexts, including those leading to p53-dependent or -independent death (for review see Farrow and Brown, 1996; Yang and Korsmeyer, 1996).

Adenoviruses have provided important model systems to study the regulation of DNA synthesis, cell proliferation, transformation, and apoptosis (Dyson and Harlow, 1992; Moran, 1993; White and Gooding, 1994; Teodoro and Branton, 1997). The products of early region 1A (E1A 13S and E1A 12S) are the triggers of these cellular effects, determined by the context in which E1A genes operate. In the presence of E1B products, for example, the cytotoxic consequences of E1A expression are blocked, allowing its growth-promoting properties to be realized (White et al., 1991; Rao et al., 1992). E1A produces two major mRNAs of 13S and 12S that encode proteins of 289 and 243 residues, respectively, and that are identical except for a central conserved region, termed CR3, in the 289-residue product that transactivates expression of other early viral units (E2, E3, E4; Kimelman et al., 1985). p53 is required for E1A-induced apoptosis in cells infected by an E1B-defective Ad5 mutant expressing the 12S E1A mRNA (Debbas and White, 1993; Lowe and Ruley, 1993; Lowe et al., 1994; Teodoro et al., 1995; Querido et al., 1997). However, the alternatively spliced E1A 13S transcript can also induce apoptosis independently of p53 and appears to rely on CR3 and additional early viral genes to do so (Teodoro et al., 1995). Identification of E4 as the requisite early region providing these cooperating transcripts has come from mapping experiments conducted in p53-null SAOS-2 cells infected with various mutant viruses (Marcellus et al., 1996). More recent studies suggest the contribution of the E4orf4 protein and possibly, E4orf6<sup>2</sup>. The ability of E4 proteins to contribute to viral cytotoxicity may relate to viral particle transmission during infection, in which accumulation of progeny virus inside apoptotic bodies permits the spreading of the virus to neighboring cells while evading host immune surveillance (Teodoro and Branton, 1997).

In the present study, we report that one of the E4 adenoviral proteins, E4orf4, is sufficient by itself to induce p53-independent cell death. Conditional expression of this 14-kD product in rodent cells triggers an apoptotic pathway that may involve a novel signaling event. In contrast to induction of apoptosis that involves E1A and E1A-dependent

activation of caspase-3 (Boulakia et al., 1996; Sabbatini et al., 1997), we detect no involvement of the zVAD-fmk-inhibitable caspases in the mechanism of apoptosis caused by the solitary delivery of Ad2 E4orf4.

## Materials and Methods

### Cell Culture and Inducible Expression System

CHO fibroblasts, CHO LR73, and p53-null mouse embryo fibroblasts, HyA4 (Lowe et al., 1994), were maintained in  $\alpha$ MEM supplemented with 10% fetal bovine serum and streptomycin sulfate/penicillin (100 U/ml). Cells were grown in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. To establish the CHO-rtTA-orf4 cell lines, the reverse tetracycline (rtTA) transactivator plasmid (pUHD17-1) was first cotransfected in CHO cells together with the pCDNA3 vector encoding the neomycin resistance gene. Stable transfectants were isolated after a 15-d selection period in the presence of G-418 (800  $\mu$ g/ml), and screened on the basis of their responsiveness to doxycycline, as previously described (Gossen et al., 1995). Clone CHO-13 was selected for its low basal and highly doxycycline-induced transactivation activity. A hemagglutinin (HA) epitope tag (Chen et al., 1996) was placed at the 5' end of Ad2 E4orf4 coding sequence by standard PCR using the sense and antisense oligos, 5' GGGGTACC ATG GCG TAC CCA TAC GAT GTT CCA GAT TAC GCT ATG GTT CTT CCA GCT CTT CC 3' and 5' CTA CTG TAC GGA GTG CGCC 3', respectively. The resulting DNA fragment was sequenced and then cloned into the SacII/XbaI sites of PUHD10-3, containing the hCMV minimal promoter with heptamerized tet operators (Gossen and Bujard, 1992). Studies confirmed that the HA tag did not interfere with the ability of orf4 to induce cytotoxicity. HA-orf4/PUHD10-3 plasmid was cotransfected together with pJK-puro, encoding the puromycin resistance gene into CHO-13 inducible clonal cell line. Stable transfectants were isolated after a 15-d selection period in the presence of puromycin (2  $\mu$ g/ml) and then screened for induction of orf4 protein after addition of doxycycline at 1  $\mu$ g/ml (Sigma Chemical Co., St. Louis, MO). Two clones, named CHO-orf4-7 and CHO-orf4-4, were selected on the basis of their high doxycycline-induced HA-orf4 protein expression. The percentage of cells expressing HA-orf4 was evaluated after induction in the presence of doxycycline for varying periods, by immunolocalization studies (50% for clone CHO-orf4-7, and 38% for clone CHO-orf4-4, respectively).

### Transient Transfection and Luciferase Assay

CHO LR73 and HyA4 cells were seeded at a density of 150,000 cells per well in 24-well plates and then cotransfected by the calcium phosphate method with 0.2  $\mu$ g of the reporter plasmid pRSV-luc, together with 2.0  $\mu$ g of expression vector containing either E4orf4 or E4orf2 coding sequences (Öhman et al., 1993) cloned into pCDNA3 as described<sup>2</sup>, and 3.8  $\mu$ g of sheared salmon sperm DNA, or 3.8  $\mu$ g of expression vector containing Bcl-2 (Nguyen et al., 1994) or Bcl-X<sub>L</sub> sequences (Ng et al., 1997). 48–72 h after transfection, cells were lysed, and then aliquots were assayed for luciferase activity as previously described (Lavoie et al., 1996a). Data are representative for at least three independent experiments performed in duplicate and are expressed as relative luciferase units, which were calculated relative to the luciferase activity in cells cotransfected with the corresponding empty vectors set to 1 U. For screening of the CHO-rtTA clonal cell lines, cells were transfected with 0.2  $\mu$ g of pTRE-luc/pUHD10-3 (Gossen and Bujard, 1992) and 4.8  $\mu$ g of sheared salmon sperm DNA. 24 h after transfection, doxycycline was added to the culture medium at 1  $\mu$ g/ml for 36 h and then the luciferase activity was measured.

### Cell Viability and DNA Degradation Assay

CHO-orf4 cell lines were seeded in 12-well plates and incubated in the presence of 1  $\mu$ g/ml doxycycline for various periods of time. Nonadherent and adherent cells were collected, and then aliquots were mixed with an equal volume of 0.4% trypan blue (GIBCO BRL, Gaithersburg, MD). The percentage of cells that picked up the dye was determined and considered to be dead cells. Data are representative of at least three independent culture dishes per time point and these are corrected for the percentage of cells expressing orf4 as revealed by immunofluorescence on parallel cultures induced with doxycycline. Measurement of internucleosomal DNA cleavage was achieved in CHO-orf4-7 cells incubated in the pres-

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ence of 1  $\mu\text{g/ml}$  doxycycline or 1.0  $\mu\text{g/ml}$  anti-human Fas antibody (clone CH-11; Upstate Biotechnology Inc., Lake Placid, NY) for various periods of time. Where required, zVAD-fmk was added simultaneously with the apoptotic triggering signal at a final concentration of 50  $\mu\text{M}$  (Enzyme System Products, Dublin, CA). Adherent and nonadherent cells from a 60-mm Petri dish were collected, washed in PBS, and resuspended gently in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% Triton X-100). After a 30-min incubation at room temperature, the samples were centrifuged at 12,000 rpm for 30 min. 150  $\mu\text{l}$  of 5 M NaCl and 500  $\mu\text{l}$  of 100% ethanol were added to the supernatant, and DNA was then precipitated overnight at  $-20^\circ\text{C}$ . Samples were centrifuged at 12,000 rpm for 30 min, and then pellets were resuspended in Tris/EDTA (TE). Extracted nucleic acids were treated with RNaseA at  $37^\circ\text{C}$  for 1 h and then analyzed on 1% agarose gels stained with ethidium bromide.

### Immunoblotting and Immunofluorescence Analysis

Cells were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol, 0.05% bromophenol blue, 1 mM phenylmethylsulfonyl fluoride), and then equal amounts of protein from whole cell lysates were separated by SDS-PAGE in 12–14% acrylamide gels. Proteins were detected immunologically after electrotransfer onto nitrocellulose membranes as described previously (Lavoie et al., 1995). Blots were developed with either mouse anti-HA HA.11 (BAbCO, Richmond, CA), rabbit polyclonal CPP32 antibody raised against the recombinant p17 subunit of CPP32 (Boulakia et al., 1996), provided by D.W. Nicholson (Merck-Frosst Center for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada), or rabbit polyclonal poly(ADP-ribosyl) polymerase (PARP) antibody 422, raised against the automodification domain of bovine PARP as described (Duriez et al., 1997), provided by G. Poirier (Centre Hospitalier de l'Université Laval Research Center, Sainte-Foy, Quebec, Canada). Horseradish peroxidase-linked goat anti-rabbit or anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), revealed by the enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL), was used to detect the antigen-antibody complexes. Protein concentrations were measured using the Bio-Rad (Hercules, CA) assay. For immunolocalization of HA-orf4, cells were plated on glass coverslips and then incubated in the presence of doxycycline for various periods of time. Cells were washed in PBS containing 1 mM  $\text{MgCl}_2$  and then fixed in 3.7% formaldehyde/PBS for 20 min. Permeabilization of cells was performed in 0.2% Triton X-100/PBS for 5 min, and then mouse anti-HA HA.11, followed by Texas red-conjugated anti-mouse immunoglobulin G (Molecular Probes, Inc., Eugene, OR) were used to detect HA-orf4. The nuclear morphology of cells was analyzed by staining of DNA with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Inc.). Annexin V binding assays

were performed by incubating the cells at  $37^\circ\text{C}$  in the presence of fluorescein-conjugated human annexin V at a final concentration of 0.1  $\mu\text{g/ml}$  for 15 min. Cells were then fixed in binding buffer (10 mM Hepes, NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , 1.8 mM  $\text{CaCl}_2$ , 3.7% formaldehyde, 0.1  $\mu\text{g/ml}$  annexin V) for 10 min in the dark and then visualized. Mitochondrial membrane potential was monitored by incubating cells grown on coverslips in culture medium containing 137 mM KCl to abolish the plasma membrane potential, and 1  $\mu\text{g/ml}$  tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes, Inc.) at  $37^\circ\text{C}$  for 10 min. Coverslips were rinsed in warm dye-free culture medium containing 137 mM KCl and then mounted in a living cell chamber made of 0.7-mm-thick rubber containing the same medium, as previously described (Chen, 1989). In parallel experiments, cells were preincubated in the presence of 10  $\mu\text{M}$  CCCP at  $37^\circ\text{C}$  for 20 min to abolish the mitochondrial membrane potential before *in vivo* staining, as a control for the specificity of the dye (data not shown).

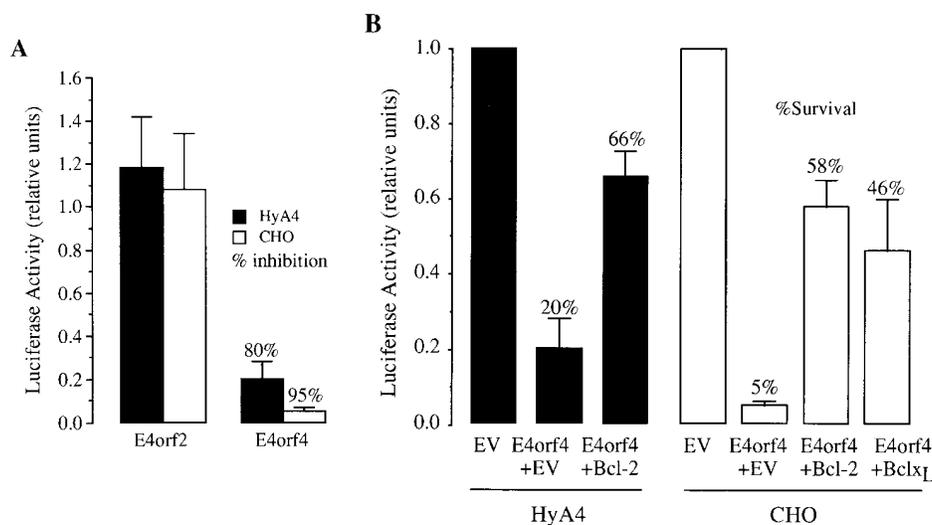
### Electron Microscopy

CHO-orf4-7 cells were incubated in the presence of 1  $\mu\text{g/ml}$  doxycycline and 50  $\mu\text{M}$  zVAD-fmk for 36 h. Nonadherent and adherent cells were collected, washed in sucrose buffer, and then fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4, for 90 min at room temperature. Postfixation was performed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at  $4^\circ\text{C}$ , followed by en bloc staining with 2% uranyl acetate for 30 min. Samples were dehydrated and embedded in Epon (Polysciences, Inc., Warrington, PA). Thin sections (75-nm) were analyzed by transmission electron microscopy using an electron microscope (model 410; Philips Electron Optics, Eindhoven, The Netherlands).

### Results

#### *p53-independent E4orf4 Cytotoxicity Can Be Significantly Overcome by Coexpression of Bcl-2 Family Members*

Cytotoxicity assays using a panel of E4-mutated adenoviruses identified E4orf4 as an important contributor to p53-independent apoptosis triggered by 13S E1A<sup>2</sup>. To directly assess the effect of this protein without interference from other viral products, an indirect measure for E4orf4-dependent cytotoxicity was established by transiently ex-



**Figure 1.** (A) Ectopic expression of E4orf4 triggers a decrease in the transcription of a luciferase reporter, regardless of the p53 status. p53-null mouse embryo fibroblasts (HyA4), or wt p53<sup>+</sup> fibroblasts (CHO LR73) were cotransfected with pRSV-luc, together with vectors containing E4orf4 or E4orf2 sequences, or the corresponding empty vector (EV). 3 d after the transfection, cells were lysed and then luciferase expression was measured. The relative luciferase activity was calculated relative to the luciferase expression measured in cells transfected with pRSV-luc and the EV, set to 1 U. Data are representative for at least three independent experiments. (B) E4orf4 cytotoxicity is partially overcome by antiapoptotic Bcl-2 family members. HyA4 or CHO LR73 cells were cotransfected with pRSV-luc, together with a combination of E4orf4 and Bcl-2, E4orf4 and BclxL, or E4orf4 and EV in a ratio of 0.2:2.0:4.0  $\mu\text{g}$  of expression plasmids. Luciferase activities were measured 3 d after transfections and are expressed relative to the luciferase activity measured in cells transfected with pRSV-luc/E4orf4/EV set to 1 U. Error bars were calculated based on at least three independent experiments.

apoptotic Bcl-2 family members. HyA4 or CHO LR73 cells were cotransfected with pRSV-luc, together with a combination of E4orf4 and Bcl-2, E4orf4 and BclxL, or E4orf4 and EV in a ratio of 0.2:2.0:4.0  $\mu\text{g}$  of expression plasmids. Luciferase activities were measured 3 d after transfections and are expressed relative to the luciferase activity measured in cells transfected with pRSV-luc/E4orf4/EV set to 1 U. Error bars were calculated based on at least three independent experiments.

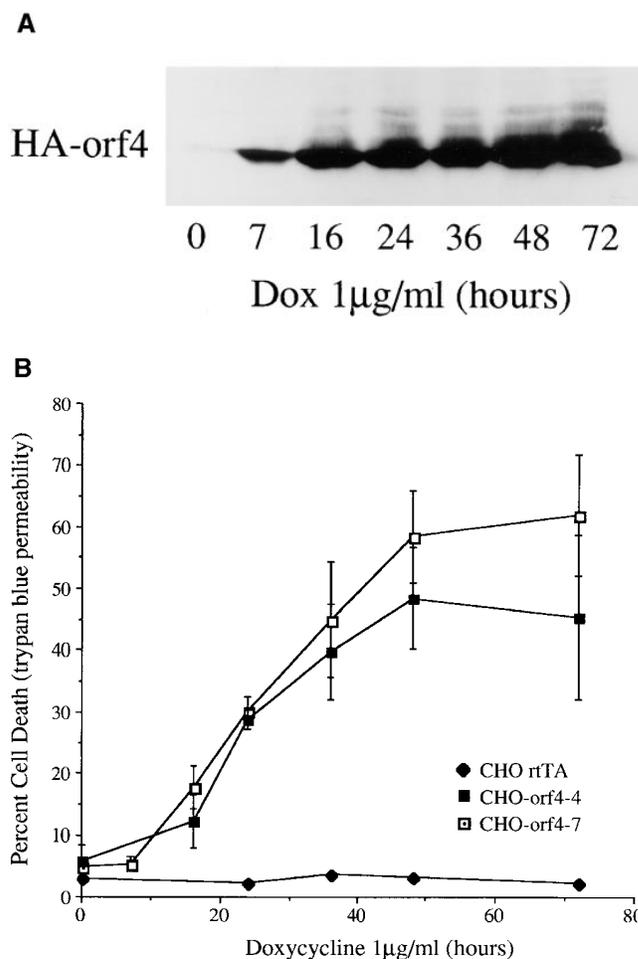
pressing E4orf4 together with a luciferase reporter in p53-null mouse embryo fibroblasts (HyA4) by standard cotransfection (Fig. 1 A). By 3 d after transfection, E4orf4 produced an 80% decrease in luciferase activity compared to luciferase expression measured in cells transfected either with the corresponding empty vector or with another E4 product, E4orf2. Expression of E4orf4 in CHO LR73 cells, which are p53<sup>+</sup>, also produced a similar decrease in luciferase activity (Fig. 1 A), suggesting that E4orf4 is potentially cytotoxic regardless of the p53 status.

As a first step to investigate the nature of the E4orf4-dependent loss of luciferase expression, the transient transfection assays were conducted in the presence of the dominant suppressors of apoptosis, Bcl-2 or Bcl-X<sub>L</sub>. HyA4 and CHO LR73 cells were cotransfected with E4orf4, the luciferase reporter, and either Bcl-2 or Bcl-X<sub>L</sub>. The results showed that the presence of Bcl-2 or Bcl-X<sub>L</sub> significantly reversed the decrease in luciferase activity mediated by expression of E4orf4, as compared to the luciferase activity measured in cells transfected with E4orf4 and the corresponding Bcl-2/Bcl-X<sub>L</sub>-deleted control vector (Fig. 1 B), suggesting that E4orf4-dependent loss of luciferase activity likely resulted from induced cellular apoptosis.

### Conditional Expression of E4orf4 in Rodent Cells Causes a Cell Death Process That Is Characterized by Typical Apoptotic Hallmark Features

To better characterize E4orf4 cytotoxicity and to determine whether its expression is sufficient to induce cell death, we established a rtTA-inducible system in CHO LR73 cells (CHO-orf4). An HA-tagged version of Ad2 E4orf4 was used in this system to facilitate the detection of the viral product. Clonal inducible CHO-orf4 cell lines were isolated as described (refer to Materials and Methods). HA-orf4 protein was induced to a significant level in CHO-orf4-7 cells within 7 h of exposure to doxycycline and increased further to reach a maximum level of expression per milligram total cell protein at 48 h after treatment (Fig. 2 A). Induction of HA-orf4 resulted in early cell lifting and a progressive increase in cell death, with the onset of membrane permeability to trypan blue occurring at ~16 h after induction, and reaching 40–50% of the cell population expressing HA-orf4 within 48 h of doxycycline exposure (Fig. 2 B).

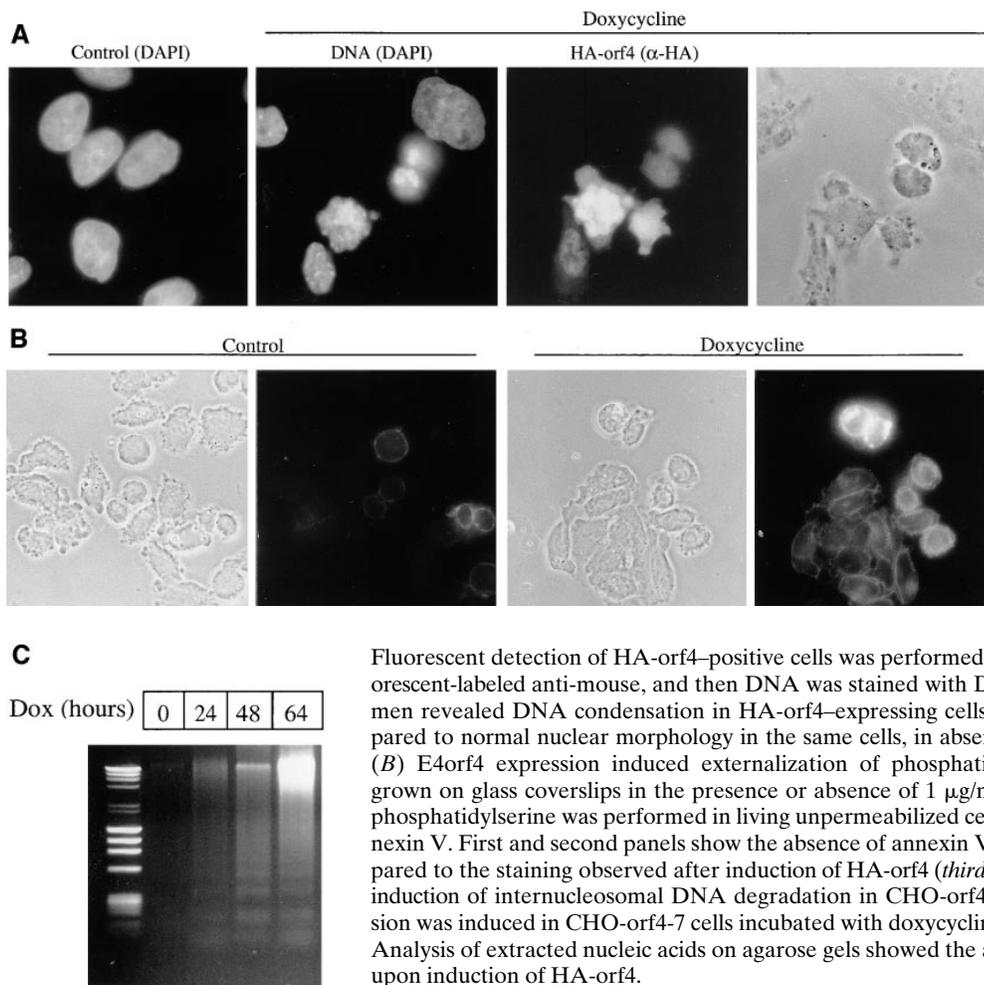
The nuclear morphology of HA-orf4-expressing cells was analyzed by DNA staining of doxycycline-treated CHO-orf4-7 using DAPI, combined with immunolocalization of HA-orf4 in the same cells using anti-HA antibody. DNA staining of control CHO-orf4-7 cells that did not express HA-orf4 showed a diffuse staining pattern and a regular nuclear morphology (Fig. 3 A, *first panel*). In contrast, induction of HA-orf4 expression in the same cell line incubated in the presence of doxycycline for 24 h produced striking changes in the nuclear morphology, as well as in the whole cell structure. HA-orf4-expressing cells dramatically shrank and showed an irregular nuclear morphology, characterized by intense staining of condensed chromatin (Fig. 3 A, *second panel*). Fragmentation of the nucleus was also observed in cells expressing high levels of HA-orf4. These typical apoptotic features occurred exclusively in the presence of HA-orf4, since some cells in the population that had been exposed to doxycycline, and which did not show



**Figure 2.** (A) Time course induction of HA-orf4 expression in CHO-orf4-7 clonal cell line. An rtTA-inducible system was established using an HA-tagged version of Ad2 E4orf4 in CHO LR73 cells, as described in Materials and Methods. CHO-orf4-7 clonal cells were incubated in the presence of doxycycline at 1  $\mu$ /ml for the indicated periods of time. Equal amounts of whole cell lysates were separated by SDS-PAGE on a 14% polyacrylamide gel, and then HA-orf4 was detected by Western blots using an anti-HA antibody. (B) Time course of E4orf4 killing in CHO-orf4 clones. Positive cell lines CHO-orf4-4 and CHO-orf4-7, as well as a control CHO-rtTA cell line, were incubated in the presence of 1  $\mu$ /ml doxycycline. At the indicated periods of time, nonadherent and adherent cells were harvested, and then equal aliquots of cell suspensions were mixed with an equal volume of trypan blue. The percent of cell death was calculated from the amount of cells incorporating the dye relative to the total amount of cells. Data are corrected for the total amount of cells expressing HA-orf4 determined by immunostaining of the protein and are representative of at least three different Petri dishes.

expression of the protein, also did not show abnormal DNA staining (Fig. 3 A, *third panel*). Interestingly, immunostaining of HA-orf4 revealed that, although the protein was diffusively distributed throughout the whole cell, a stronger staining was detected at the nuclear level, suggesting that HA-orf4 may be preferentially localized in the nucleus in this cell system (Fig. 3 A, *third panel*).

Another important event during apoptosis is the acquisition of plasma membrane changes that allow phagocytes to recognize and engulf these cells before they rupture.



**Figure 3.** Induction of E4orf4 expression triggers an apoptotic cell death process. (A) HA-orf4 expression induced DNA condensation. Cells grown on glass coverslips were incubated with 1  $\mu$ g/ml doxycycline for 24 h.

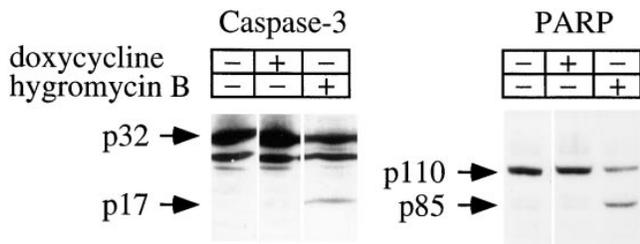
Fluorescent detection of HA-orf4-positive cells was performed using an anti-HA followed by a fluorescently labeled anti-mouse, and then DNA was stained with DAPI. Double staining of the specimen revealed DNA condensation in HA-orf4-expressing cells (*second and third panels*) as compared to normal nuclear morphology in the same cells, in absence of HA-orf4 (*–dox, first panel*). (B) E4orf4 expression induced externalization of phosphatidylserine. CHO-orf4-7 cells were grown on glass coverslips in the presence or absence of 1  $\mu$ g/ml doxycycline for 16 h. Staining of phosphatidylserine was performed in living unpermeabilized cells using fluorescein-conjugated annexin V. First and second panels show the absence of annexin V binding in untreated cells, as compared to the staining observed after induction of HA-orf4 (*third and fourth panels*). (C) Time-course induction of internucleosomal DNA degradation in CHO-orf4-expressing cells. HA-orf4 expression was induced in CHO-orf4-7 cells incubated with doxycycline for the indicated periods of time. Analysis of extracted nucleic acids on agarose gels showed the appearance of typical DNA ladders upon induction of HA-orf4.

Early redistribution of phosphatidylserine to the outer plasma membrane leaflet, for example, has been described as a general feature of apoptosis, regardless of the initiating stimulus (Martin et al., 1995). Induction of HA-orf4 in CHO-orf4-7 cells also led to externalization of phosphatidylserine. When fluorescein-conjugated annexin V, a phosphatidylserine binding protein, was used as a probe to detect the presence of the phospholipid, a dramatic increase in staining of nonpermeabilized cells was detected after incubation of CHO-orf4-7 cells with doxycycline for 16 h (Fig. 3 B, *third and fourth panels*). In contrast, annexin V staining was barely detectable in most cells in the absence of HA-orf4 (Fig. 3 B, *first and second panels*). As described in other systems, externalization of phosphatidylserine occurred soon after HA-orf4 induction and preceded cellular death.

Finally, isolation of low molecular weight DNA from CHO-orf4-7 cells incubated with doxycycline for various periods of time revealed the presence of oligonucleosomal DNA ladders, which became detectable after 24 h but reached a significant intensity 64 h after induction of HA-orf4 (Fig. 3 C). As expected, such apoptotic DNA degradation was observed at relatively late times after HA-orf4 expression, as compared to changes such as DNA condensation and externalization of phosphatidylserine.

### *E4orf4-induced Apoptosis Is Not Inhibited by $\alpha$ VAD-fmk*

To initially characterize the signaling pathway implicated in E4orf4-mediated apoptosis, activation of caspase-3 (CPP32/apopain/Yama), an ICE-like cysteine protease that has been shown to perform a central role in many apoptosis systems (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995; Casciola et al., 1996; Schlegel et al., 1996), was monitored. CHO-orf4-7 cells were incubated in the presence of either doxycycline, to induce HA-orf4-mediated apoptosis, or hygromycin B, to induce apoptosis (Chen et al., 1995) in the absence of the viral protein. Immunoblotting of total cell extracts was performed using an antibody raised against the recombinant p17 subunit of caspase-3 (Boulakia et al., 1996), or the 422-anti-PARP antibody, which recognizes the full-length PARP and the 85-kD cleaved product observed after apoptotic stimuli (Duriez et al., 1997). Processing of procaspase-3 upon an apoptotic signal yields two subunits of 17 and 12 kD, which assemble to form the active tetrameric holoenzyme (Nicholson et al., 1995). Treatment of CHO-orf4-7 cells with hygromycin B for 48 h triggered processing of procaspase-3, as revealed by the appearance of the 17-kD subunit, as well as cleavage of PARP, a substrate for active caspase-3 (Nicholson et al., 1995; Tewari et al., 1995) (Fig. 4). In contrast, no



**Figure 4.** Absence of procaspase-3 processing and PARP cleavage in cells undergoing E4orf4-mediated apoptosis. CHO-orf4-7 cells were incubated in the presence of 1  $\mu$ g/ml doxycycline, or 400  $\mu$ g/ml hygromycin B, for 48 h. Equal amounts of whole cell lysates were separated by SDS-PAGE on a 12% polyacrylamide gel, and pro-CPP32 (p32), as well as the cleaved subunit p17, were detected by Western blot using an antibody raised against the recombinant p17 subunit of CPP32. PARP was detected in the same extracts by immunoblotting with anti-PARP antibody 422.

17-kD band was detectable in CHO-orf4-7 after a 48-h incubation in the presence of doxycycline (Fig. 4), when HA-orf4 expression was maximal (Fig. 2 A). Consistent with this, no PARP cleavage was observed. A detailed kinetic analysis performed at various times after induction of HA-orf4 failed to reveal at any time processing of procaspase-3 or cleavage of PARP in cells undergoing HA-orf4-mediated cell death (data not shown).

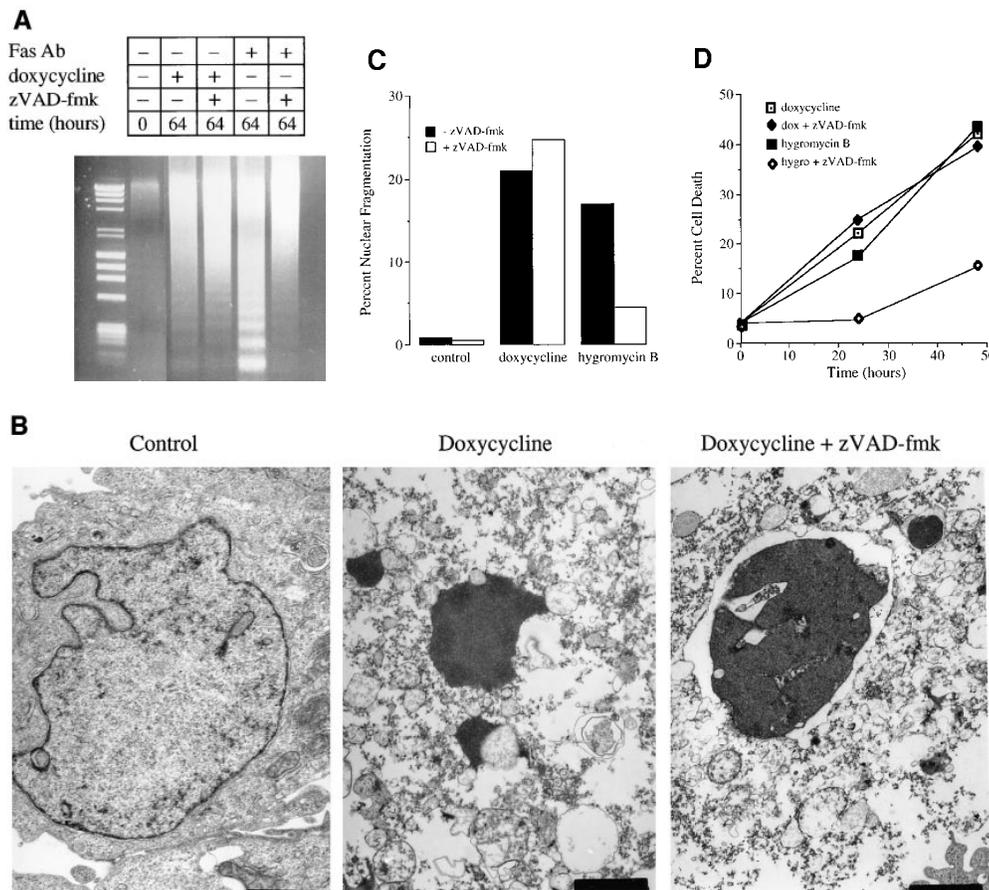
The requirement of caspase activities for orf4-induced apoptosis was further assessed by examining the effect of zVAD-fmk on orf4-mediated apoptosis. zVAD-fmk is a cell-permeable inhibitor of all caspases examined to date and blocks or retards cellular apoptosis that is caused by a diverse set of signals (Polverino and Patterson, 1997) including cell death in response to ligation of Fas (Slee et al., 1996; Xiang et al., 1996). As expected therefore, zVAD-fmk had an inhibitory effect on the appearance oligonucleosomal ladders in CHO-orf4-7 cells that had not received doxycycline, but rather had been treated with Fas antibody for as long as 64 h (Fig. 5 A). When present during induction of E4orf4 in these cells, however, no such inhibition by zVAD-fmk was observed (Fig. 5 A). Similarly, zVAD-fmk did not prevent the typical ultrastructural changes in cells undergoing HA-orf4-mediated apoptosis. Analysis of doxycycline-treated CHO-orf4-7 cells by electron microscopy showed that chromatin condensation and cytoplasmic vacuolation still occurred in the presence of the protease inhibitor (Fig. 5 B), whereas these morphological changes were inhibited in Fas-treated cells in the presence of the inhibitor (data not shown). To exclude the possibility of a transient protective effect of zVAD-fmk at earlier times, as suggested in a recent report (Sabbatini et al., 1997), early changes in nuclear morphology, detectable by DAPI staining of DNA, were analyzed after incubation of CHO-orf4-7 cells with doxycycline for 16 h. Although zVAD-fmk prevented the appearance of apoptotic figures in a population of cells treated with hygromycin B during 16 h, no protective effect was observed in cells expressing HA-orf4 (Fig. 5 C). Also, zVAD-fmk had no effect on the time-course induction of orf4-mediated cell death, whereas the kinetics of hygromycin B-induced death were significantly delayed (Fig. 5 D).

### **E4orf4-mediated Cell Death Is Accompanied By a Loss in Mitochondrial Membrane Potential**

A common biochemical transformation in cells undergoing apoptosis is a loss in the mitochondrial electrochemical potential as a consequence of permeability transitions at the inner membrane of the organelle (Kroemer et al., 1995; Zamzami et al., 1996). Moreover, these mitochondrial transitions, which can be assayed in whole cells using potential-sensitive dyes, occur in cells undergoing apoptosis in the absence of caspase activation (Xiang et al., 1996). Thus, mitochondria were stained in living CHO-orf4-7 cells using a derivative of rhodamine, a fluorescent lipophilic cation that accumulates selectively in mitochondria in the presence of an intact mitochondrial membrane potential, in accordance with the Nernst potential (Chen, 1989). The results showed that, in the absence of HA-orf4 expression, dye accumulated in mitochondria of all cells, leading to a typical granular staining surrounding the nucleus (Fig. 6, *Control*). However, induction of HA-orf4 expression triggered a dramatic decrease in staining over the same incubation period with the dye, an effect that was maintained in the presence of zVAD-fmk. The residual staining was rather diffuse and delocalized, similar to that observed after treatment of these cells with hygromycin B (Fig. 6).

### **Discussion**

Until recently, studies on oncogenesis have focused on the regulation of signal transduction pathways leading to cell proliferation (Stanbridge and Nowell, 1990; Lavoie et al., 1996b). However, the requirement of negative control, including growth arrest and programmed cell death, in the course of cellular maturation/differentiation and maintenance of appropriate cell numbers, is now recognized as an equally important process (Cohen, 1991; Levine, 1993; Oltvai and Korsmeyer, 1994; Thompson, 1995). Evidence has accumulated that defects in both the genes that regulate cell growth and the genes that control cell death can cooperate in neoplasia and its progression towards more malignant phenotypes. Considering that p53 remains the most frequent target for genetic alterations identified in human cancers, understanding the molecular basis of p53-independent apoptosis is thus essential. Because E1A can induce both p53-dependent and -independent apoptosis, it provides an interesting model to study the various regulatory pathways implicated in triggering the commitment of a cell to apoptosis, as well as the players responsible for the execution of the processes. Induction of p53-independent cell death induced by the E1A 13S product relies at least in part on the ability of the protein to transactivate one or more of the viral E4 products (Marcellus et al., 1996), suggesting that E4 proteins, individually or in cooperation, contribute to the death process. Additional mapping studies<sup>2</sup> identified E4orf4 as one of the candidate E4 products. Here, we have documented the ability of E4orf4 to independently induce cytotoxicity by a mechanism that involves many of the classical hallmark features of apoptosis. However, the results obtained suggest that E4orf4-mediated apoptosis may not require activation of the known caspases. This latter conclusion is based on the failure to detect processing of procaspase-3, an event com-



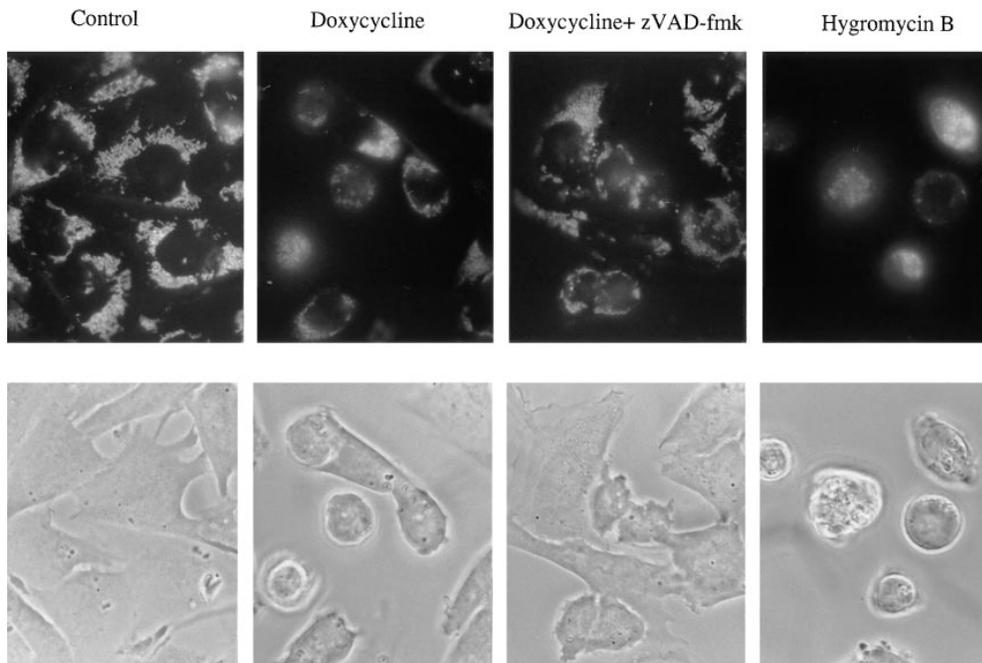
**Figure 5.** zVAD-fmk does not prevent E4orf4-induced apoptosis. (A) Internucleosomal DNA degradation was analyzed in doxycycline-treated CHO-orf4-cells, in the presence or absence of 50  $\mu$ M zVAD-fmk, as compared to cells treated with 1  $\mu$ g/ml Fas Ab in the same conditions. Analysis of the extracted nucleic acids showed that whereas the presence of zVAD-fmk significantly attenuated the DNA ladders induced by treatment with Fas Ab, it did not prevent E4orf4-mediated DNA degradation. (B) The ultrastructure of CHO-orf4-7 cells was analyzed by electron microscopy in untreated cells (*first panel*), as compared to cells incubated with doxycycline for 36 h, in the presence (*third panel*) or absence (*second panel*) of 50  $\mu$ M zVAD-fmk. Condensation of the chromatin as well as cytoplasmic vacuolation is still detectable in the presence of the ICE-like protease inhibitor. (C) CHO-orf4-7 cells grown on glass coverslips were incubated in the presence

of either 1  $\mu$ g/ml doxycycline, or 400  $\mu$ g/ml hygromycin B for 16 h, in the presence or absence of 50  $\mu$ M zVAD-fmk. Immunodetection of HA-orf4 and DNA staining was then performed by double immunofluorescence. Cells were counted, and the percentage of cells arboring an abnormal nuclear morphology is shown. At least 300 cells were counted for each condition, and data related to doxycycline-treated cells were corrected for the number of cells expressing HA-orf4. (D) CHO-orf4-7 cells were incubated in the presence of either 1  $\mu$ g/ml doxycycline, or 400  $\mu$ g/ml hygromycin B for 16 h, in the presence or absence of 50  $\mu$ M zVAD-fmk. At the indicated times, nonadherent and adherent cells were harvested and pooled, and then equal aliquots of cell suspension were mixed with an equal volume of trypan blue. The percent cell death was calculated from the amount of cells incorporating the dye relative to the total amount of cells. Data are corrected for the total amount of cells expressing HA-orf4 determined by immunostaining of the protein and are representative of at least three different Petri dishes.

mon to many apoptosis signaling pathways (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Schlegel et al., 1996; Tewari et al., 1995; Casciola et al., 1996), and more importantly, by the failure of the wide spectrum caspase inhibitor, zVAD-fmk, to block E4orf4 cytotoxicity in cell cultures. Furthermore, the kinetics of orf4-induced apoptosis were unaffected by zVAD-fmk, whereas apoptosis induced by hygromycin B was delayed, implying that the failure of zVAD-fmk to influence orf4-mediated killing was not because of ineffective inhibition of caspases. Due to the absence of a P4 determinant, zVAD-fmk has been designed to inhibit all ICE-like caspases (Zhu et al., 1995). It cannot be ruled out that an as yet unidentified caspase is induced by orf4 and must have unusual properties. p53-independent apoptosis induced by the 13S E1A product in the context of a virus that contains an intact E4 region is associated with processing and activation of procaspase-3 (Boulakia et al., 1996), suggesting either that this E1A product contributes to more than one apoptosis signaling event within the p53<sup>-/-</sup> context, or that additional E4 genes are required.

Whatever the case, our results suggest a model in which the 13S E1A product is capable of inducing p53-independent apoptosis because one or more E4 products, including E4orf4, contributes to an apoptosis-inducing function that would otherwise require a cellular p53 downstream event.

The proapoptotic member of the Bcl-2 family, Bax (Oltvai and Korsmeyer, 1994), exhibits several of the properties that might be expected of such a p53 downstream event that can be substituted by E4orf4. First, evidence has been obtained that *BAX* is a target of p53 transcriptional activation (Miyashita et al., 1994; Selvakumaran et al., 1994; Miyashita and Reed, 1995). Second, although Bax expression induces activation of procaspase-3, it is also capable of inducing all of the apoptotic manifestations that are also observed for E4orf4 when expressed in the presence of zVAD-fmk (Xiang et al., 1996). Finally, Bcl-X<sub>L</sub>, a heterodimerizing partner of Bax, is also capable of functioning at a step downstream of the caspases, protecting cells against the loss of mitochondrial membrane potential. This is consistent with our finding that Bcl-2/Bcl-X<sub>L</sub> protects cells against



**Figure 6.** E4orf4 expression induces a loss in the mitochondrial membrane potential. CHO-orf4-7 cells grown on glass coverslips were either treated with 1  $\mu\text{g}/\text{ml}$  doxycycline, or 400  $\mu\text{g}/\text{ml}$  hygromycin B for 16 h, in the presence or absence of 50  $\mu\text{M}$  zVAD-fmk. Cells were then incubated with 1  $\mu\text{g}/\text{ml}$  TMRE for 10 min and then visualized by immunofluorescence microscopy using a living cell chamber. The first panel shows untreated cells arboring a normal mitochondrial membrane potential. Induction of apoptosis using either HA-orf4 expression (*second and third panels*) or hygromycin B (*third panel*) resulted in a dramatic decrease of the TMRE staining, indicating a loss of the mitochondrial membrane potential.

E4orf4 cytotoxicity. It remains to be determined, however, if E4orf4 is simply acting like a partially functional analogue of Bax. Sequence analysis of E4orf4, for example, does not reveal the presence of strong similarities to the structural hallmarks of the Bax family, notably the BH3 domain (Yang and Korsmeyer, 1996). The one known function of E4orf4 relates to its ability to bind the heterotrimeric form of protein phosphatase (PP)2A and to increase the activity of the holoenzyme (Kleinberger and Shenk, 1993), but whether or not this increase in PP2A is sufficient to explain the apoptosis inducing properties of E4orf4 is not yet known. It is additionally relevant, however, that regulators of the Bcl-2 family setpoint are subject to phosphorylation/dephosphorylation events. These include Bad (Zha et al., 1996), as well as Bcl-2 itself (Haldar et al., 1995; Strack et al., 1996; Chang et al., 1997). Another possibility, therefore, is that E4orf4 links PP2A to these potential targets at sites where the Bcl-2 family proteins function downstream of the caspases (Boise and Thompson, 1997). Finally, it may be that E4orf4 is multifunctional, despite its relatively small size (14-kD), and that its role in apoptosis is different than its effect on PP2A. Future characterization of E4orf4 interaction(s) with cellular proteins should provide insights into the signaling pathways involved in mediating E4orf4 p53-independent apoptosis.

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