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Research Article

c-Fos but not v-Fos protein induces programmed cell death of v-myb-transformed monoblasts

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Abstract. c-Fos and v-Fos belong to a group of proteins forming the transcription factor AP-1 that is important for regulation of proliferation, differentiation and programmed cell death in multiple cell types. In this study, we examined the role of c-Fos and v-Fos proteins in v-myb-transformed BM2 monoblasts. We show that while the v-Fos protein prolongs the G0G1 phase of the BM2 cell cycle, c-Fos leaves the cell cycle unaffected and, rather, induces programmed cell death. The apoptosis-promoting activity of the c-Fos protein is markedly enhanced in cells cultivated under serum-free conditions. c-Fos-induced apoptosis of BM2 cells occurred in the presence of Bcl-2 and was not dependent on the transcription activation function of the c-Fos protein. No differentiation-promoting activity of the Fos proteins was observed. The effects of Fos proteins on BM2 cells differ from those induced by Jun proteins, suggesting differential roles of individual components of the AP-1 transcription factor in regulation of essential cellular processes.

Key words. Fos; Jun; AP-1; v-Myb; apoptosis; differentiation; proliferation; cell cycle.

Oncogene activation is a significant event in cancer development. Model systems that enable direct comparison of the effects of proto-oncogenes and oncogenes on the phenotype of a cell can yield underlying information about their function and roles in cellular transformation. In this study, we compared the effects of cellular and viral fos genes ectopically expressed in a v-myb-transformed monoblastic cell line BM2.

c-Fos and v-Fos proteins as well as Fra-1, Fra-2 and FosB are members of the Fos protein family [for a review see ref. 1]. c-Fos, like other products of immediate early genes, is quickly and efficiently synthesized upon stimulation with various growth and stress factors thus participating in conversion of an extracellular stimulus to a specific cellular response. Based on the type of the signal and intracellular factors, c-Fos proteins can efficiently affect cellular proliferation, differentiation and programmed cell death [for a review see ref. 2]. c-Fos forms heterodimers with members of Jun, ATF/CREB and Maf protein families, thus forming the AP-1 transcription factor [for a review see ref. 3]. AP-1 binds to the 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) sequence TGAG(C)TCA and its variants depending on the specific composition of the AP-1 subunits [for a review see ref. 1]. Transcription of genes containing TRE in their promoters can be both up- and down-regulated by AP-1. Since the phorbol ester TPA can induce monocytic differentiation of several leukemic cell lines,

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such as HL-60, THP-1, U-937 and BM2, AP-1 is supposed to be engaged in this effect [4, 5]. In addition, increased levels of Fos protein were detected in maturing monocytic cells treated with various extracellular inducers such as tumor necrosis factor, lipopolysaccharide and interleukin-6 [6]. The crucial role of the c-Fos protein in regulation of cell differentiation is well demonstrated in osteoclast maturation [7, 8]. c-Fos protein also takes part in the regulation of programmed cell death. A pro-apoptotic effect of c-Fos protein has been described in a Syrian hamster embryonic cell line, a human colorectal carcinoma cell line, human keratinocytes and photoreceptor cells [9–11]. On the other hand, the c-Fos protein is capable of protecting certain cell types from UV light-induced apoptosis [12, 13].

Transforming variants of the cellular c-fos proto-oncogene were detected in two independent murine osteosarcoma-causing RNA viruses: FBJ-MuSV and FBR-MuSV [14, 15] and in an avian nephroblastoma virus NK24 [16]. The v-Fos^{FBJ} protein differs from the cellular c-Fos protein by five point mutations and by substitution of 48 C-terminal amino acids with 49 different amino acids due to a frameshift mutation [17].

Another protein that is significantly engaged in regulation of differentiation, proliferation and apoptosis of hematopoietic cells is the Myb protein. c-Myb is highly expressed in immature hematopoietic cells of all lineages, and its expression decreases as cells differentiate [18]. Deregulated c-myb expression has been found in most leukemias and lymphomas with an immature phenotype [19]. In addition, the c-myb gene is amplified in a number of human colon and pancreatic carcinomas [20–22]. A transforming variant of c-myb, v-myb, has been transduced by two acutely transforming avian retroviruses: avian myeloblastosis virus (AMV) and E26 leukemia virus [23–25]. The v-Myb of AMV causes acute monoblastic leukemia in chickens and transforms myelomonocytic cells in vitro [26].

In our previous study, we described the effects of elevated levels of c-Jun and v-Jun proteins on the morphology and physiology of v-myb-transformed chicken monoblasts (BM2) [27]. In this study, we used the same model to explore the effects of c-Fos and v-Fos proteins. Since both Jun and Fos proteins are components of the AP-1 transcription factor, this system provides a unique tool to determine the individual functions of the major members of the AP-1 protein family as well as to compare the functions of their cellular and viral variants. We show that the effects of these functionally related proteins on cellular fate are significantly different.

Materials and methods

Plasmid construction

Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, Mass.). The FBJ murine sarcoma provirus was cut out of the plasmid pFBJ-2 [28] (kindly provided by C. van Beveren) using KpnI and subcloned into pUC18 to make pUC18-v-FOS. This construct was cut with AccI and recessed ends were filled in with the Klenow fragment of DNA polymerase I. v-fos cDNA was cloned into SA CLA 12 NCO [29], excised with ClaI and cloned into the unique ClaI site of NEO-MAV-CLA [30] thus forming the MAV-NEO-FOS plasmid. v-fos cDNA was cut out of the plasmid MAV-NEO-FOS using XbaI. The XbaI-resistant fragment harboring v-fos cDNA was cloned into the XbaI site of the vector pMT-IRES-CD4 [31] forming the pMT-vFOS-CD4 plasmid. c-fos cDNA was cut out of the plasmid pTZ19R [32] using NcoI and SmaI, recessed termini were filled using the Klenow fragment of DNA polymerase I and cloned into the Klenow-filled XbaI site of the vector pMT-IRES-CD4 [31] forming the pMT-cFOS-CD4 plasmid.

Cell transfection and cultivation

Conditions of cultivation of BM2 cells have been described elsewhere [33]. For stable transfection, Fugene 6 reagent (Roche Diagnostics, Mannheim, Germany) was used. Three microliters of Fugene 6 reagent well added to a sterile microtube containing 97 µl of serum-free OPTI-MEM medium (GIBCO BRL/Life Technologies, Gaithersburg, Md.) and incubated for 5 min at room temperature. Then, pMT-vFOS-CD4 (or pMT-cFOS-CD4) plasmid DNA (2 µg) mixed with pSV2Neo (0.5 µg) was added and incubated for 15 min at room temperature. Next, this solution was used for transfection of 2×10^6 exponentially growing BM2 cells. The next day, G418 (500 µg/ml) and 3 ml of DMEM including chicken and fetal calf sera were added. Stable G418-resistant transfectants that appeared within 2 weeks of cultivation underwent negative and positive selection with anti-CD4-coated paramagnetic beads (Dynal, Oslo, Norway) as described [31] and were cloned by limiting dilution.

Immunoblotting

Sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) and immunoblotting were performed as described elsewhere [33]. The blots were probed using either a polyclonal antiserum directed against the highly conserved DNA-binding domain of c-Fos/v-Fos (sc-253; Santa Cruz Biotechnology, Santa Cruz, Calif.), and v-Fos (F 7799; Sigma, St. Louis, Mo.), or a monoclonal antiserum specific for avian Bcl-2 (610538; BD Transduction Laboratories, San Diego, Calif.). To control for sample loading, the blots were probed with a PCNA-specific antibody kindly provided by N. H.Waseem [34]. The blots

were developed with either a goat anti-rabbit or goat anti-mouse secondary antibody, both conjugated to alkaline phosphatase as recommended by the manufacturer (Promega, Madison, Wis.).

Proliferation, viability and cell cycle analyses

For growth curves, 2×10^5/ml BM2, BM2vFOS and BM2cFOS cells were treated with zinc chloride (1.5×10^{-4} M) for 5 days. Viable cells determined by eosin dye exclusion were counted daily using a hemocytometer.

For cell cycle analyses, the cells were cultivated in the presence of zinc chloride, then washed twice in 2 vol of phosphate-buffered saline (PBS), resuspended in 0.5 ml of PBS, fixed in 4 ml of 70% ethanol and stored at 4 °C for 24 h. Fixed cells were then centrifuged, washed with PBS and stained in 0.5 ml of Vindelov solution [35] for 30 min at 37 °C. Cells were light-protected until their DNA content was measured on a FACSCalibur system (Becton Dickinson, San Jose, Calif.). At least 10,000 cells were analyzed and the percentage of cells in each phase of the cell cycle was determined using ModFit 2.0 software (Verity Software House, Topsham, Me.). Cell debris was excluded by appropriate raising of the forward-scatter threshold. Single cells were identified and gated by pulse-code processing of the area and the width of the fluorescence signal.

Transcription activation assay

For transient transfection of BM2CD4, BM2vFOS and BM2cFOS cells, 5 µg of the p3TPluc plasmid containing three AP-1-binding sites [36] was used together with 5 µg of the cmv-βgal plasmid as an internal control of transfection. The plasmids and 5×10^6 exponentially growing BM2CD4, BM2vFOS or BM2cFOS cells were transferred into 4 µl of regular medium containing sera and 1.25% dimethylsulfoxide (DMSO) in a sterile electroporation cuvette, and electroporation was performed as described [37]. Electroporated cells were transferred to a 10-cm dish containing 10 ml of growth medium with 1.25% DMSO. Next day, the cells were washed with regular medium to remove DMSO, and zinc chloride (1.5×10^{-4} M) was added for 24 h. Harvested cells were washed with PBS and processed for luciferase and β-galactosidase assays as described before [37]. Relative light units were normalized for transfection efficiency using β-galactosidase activity as an internal control.

Analysis of mitochondrial membrane potential ($\Delta\Psi$m)

BM2, BM2vFOS and BM2cFOS cells (1×10^6) were cultivated in 2 ml of serum-free medium and treated with zinc chloride (1×10^{-4} M) for 24 h. The variation of $\Delta\Psi$m was studied using tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes, Eugene, Ore.). Cells were washed twice with Hank's balanced salt solution without calcium and magnesium ions (HBSS), resuspended in TMRE (100 nM) in HBSS (approximately 1×10^6 cells/ml) and incubated for 20 min at room temperature in the dark. At the end of the incubation period, cells were washed in HBSS, resuspended in a total volume of 500 µl and at least 30,000 of cells were analyzed using a FACSCalibur flow cytometer. Data were evaluated as the percentage of cells with negative TMRE fluorescence from a population with scatter characteristics of viable cells. CellQuest 3.0 software (Beckton Dickinson, San Jose, Calif.) was used for data analysis.

DNA fragmentation test

BM2, BM2vFOS and BM2cFOS cells (2×10^5/ml) were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 24 h. DNA was isolated from harvested cells using the Invisorb Apoptosis Detection Kit (Invitek, Berlin, Germany), resolved by electrophoresis in a 1.5% TAE agarose gel and stained with ethidium bromide.

Evaluation of cellular and nuclear morphology of cells undergoing programmed cell death

For analysis of morphology by light microscopy, BM2CD4, BM2vFOS and BM2cFOS cells (2×10^5/ml) were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 24 h. BM2CD4 cells treated with camptothecin (3 µM) for 6 h were used as a control. Cells were washed with PBS, centrifuged to the slides, stained using the Diff-Quik staining set (DADE AG, Düdingen, Switzerland) and analyzed by light microscopy.

For analysis of morphology using fluorescence miscroscopy, the same cells were cultivated in 2 ml of serum-free medium and treated with zinc chloride (1×10^{-4} M) for 24 h. Cells were washed with PBS and fixed in 5 ml of an ice-cold methanol:acetic acid (3:1) mixture and stored at −20 °C overnight. Next day, the cells were centrifuged, resuspended in 100 µl of methanol:acetic acid mixture and dropped onto the slide. The nuclei were stained with 10 µl of propidium iodide (20 µg/ml) and analyzed using fluorescence microscopy.

For analysis of c-Fos induced apoptosis in the cells with blocked protein synthesis BM2CD4, BM2vFOS and BM2cFOS cells (2×10^5/ml) were treated with zinc chloride (1.5×10^{-4} M) for 2 h before 1 µg/ml cycloheximide (CHX; Sigma) was added for the next 16 h. Harvested cells were fixed and stained for fluorescence microscopy as described above.

Results

Derivation of BM2 cells inducibly expressing c-fos and v-fos

v-myb-transformed BM2 monoblasts were transfected with the chicken and viral fos genes under the control of

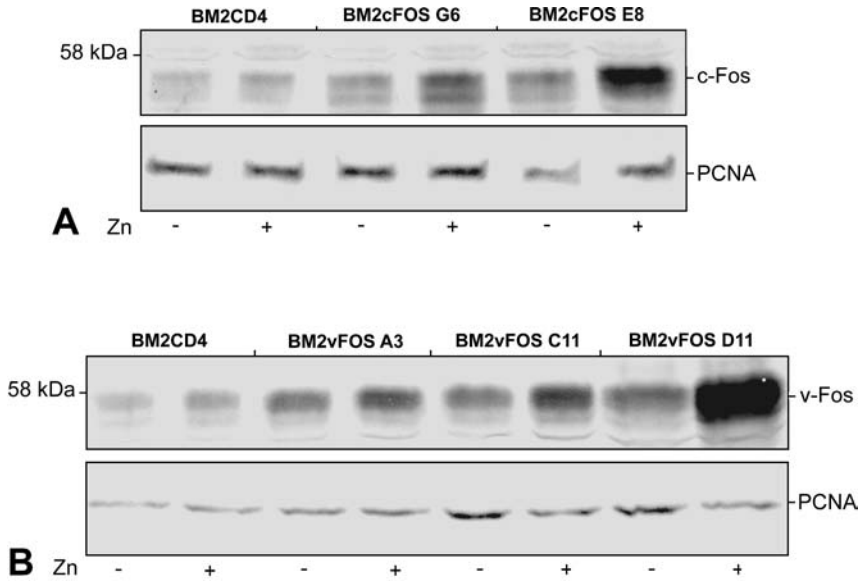

Figure 1. Expression of c-Fos (*A*) and v-Fos (*B*) in v-Myb-transformed monoblasts. A total of 2×10^6 empty vector-transfected BM2 cells (BM2CD4) and pMT-cFOS-CD4 or pMT-vFOS-CD4-transfected BM2 cells (independent clones of BM2cFOS and BM2vFOS, respectively) were either treated with zinc chloride (+Zn) or left untreated (–Zn) for 24 h. Proteins extracted from harvested cells were resolved in 10% SDS-PAGE and analyzed by immunoblotting using anti-Fos polyclonal antibody (sc-253; Santa Cruz Biotechnology). To control for sample loading, the same blots were probed with a PCNA-specific antibody.

a human metallothionein II_A promoter. Two independent clones of stable BM2 transfectants inducibly expressing exogenous c-fos (BM2cFOS) and four independent clones of stable BM2 transfectants expressing v-fos (BM2vFOS) were purified. Inducible fos-expression in these clones upon treatment with zinc chloride was confirmed by immunoblotting (fig. 1). Empty vector-transfected BM2CD4 cells [38] were used as a negative control.

Exogenous Fos proteins enhance AP-1 activity in BM2cFOS and BM2vFOS cells

To test the functional status of Fos proteins in BM2cFOS and BM2vFOS cells we determined their ability to participate in the formation of functional AP-1. The cells were transiently transfected with a reporter plasmid containing three AP-1-binding sites upstream of the TATA box and the luc gene, and treated with zinc chloride to induce the metallothionein promoter. Luciferase activity in the cell extracts was compared with the luciferase activity in extracts of similarly treated BM2CD4 cells. Transactivation by AP-1 was increased about tenfold in zinc-treated BM2vFOS and about fourfold in BM2cFOS cells in comparison with zinc-treated BM2CD4 cells (fig. 2). Similar results were also obtained in the other clones of *fos*-expressing BM2 cells documenting that exogenous c-Fos and v-Fos proteins are capable of enhancing the formation of functional AP-1 complexes and up-regulate expression of a model reporter gene in BM2cFOS and BM2vFOS cells.

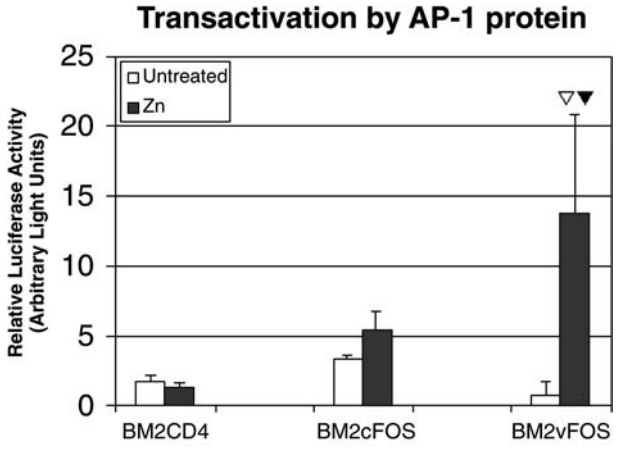

Figure 2. c-Fos and v-Fos proteins up-regulate transactivation by AP-1 protein in BM2cFOS and BM2vFOS cells. A total of 5×10^6 exponentially growing BM2cFOS#E8, BM2vFOS#D11 and BM2 CD4 cells were transfected with 5 µg of p3TPlux plasmid containing three copies of the AP-1-binding site upstream of the TATA box and cDNA coding for luciferase [36] together with 5 µg of cmv-gal internal control plasmid by electroporation. Electroporated cells were treated with zinc chloride (1.5×10^{-4} M) for 1 day. Luciferase and β-galactosidase activities in harvested cells were analyzed as described in Materials and methods. Data show the mean value of luciferase activity from three independent experiments. Error bars indicate standard deviations. ▽ denotes a significant difference ($p < 0.05$) from untreated BM2CD4 cells and ▼ denotes a significant difference ($p < 0.05$) from Zn-treated BM2CD4 cells as determined by ANOVA software.

c-Fos and v-Fos proteins down-regulate growth rate of BM2 cells

To evaluate the effects of Fos proteins on growth of BM2 cells, the same number of BM2cFOS, BM2vFOS and control BM2 cells were seeded and cultivated in the presence or absence of zinc chloride for 5 days. Viable cells were counted daily. We found that both c-Fos and v-Fos protein down-regulated the number of viable BM2 cells (fig. 3). The growth arrest induced by c-Fos was apparent even in uninduced BM2cFOS cells of both clones tested, presumably from leaking metallothionein promoter, this effect was further enhanced upon induction with zinc chloride. Similarly, the v-Fos protein also interfered with proliferation of BM2vFOS cells but this effect was dependent on zinc induction (fig. 3). Zinc-induced fos expression was stable during the experiment (not shown). These results suggest that c-Fos and v-Fos proteins act as growth suppressors of BM2 cells.

c-Fos and v-Fos proteins affect different phases of the BM2 cell cycle

The profiles of BM2cFOS and BM2vFOS growth curves suggested that Fos proteins might control the BM2 cell cycle. To explore this effect in more detail, BM2cFOS, BM2vFOS and control BM2 cells were treated or untreated with zinc chloride for 24 h and the DNA content in individual cells was measured by flow cytometry. c-Fos protein induced a statistically significant ($p < 0.05$) increase in the number of cells containing subdiploid DNA (table 1), suggesting that the frequency of apoptosis is up-regulated in BM2cFOS cells. Interestingly, we did not find any cells with subdiploid DNA content in control BM2 and v-fos-expressing BM2vFOS cells, suggesting that it is the c-Fos protein that can induce DNA fragmentation in BM2 cells. No other phases of the cell cycle were affected by c-Fos. In contrast, a statistically significant ($p < 0.05$) G0G1 phase arrest resulting in an increased frequency of G0G1 and a decreased frequency of

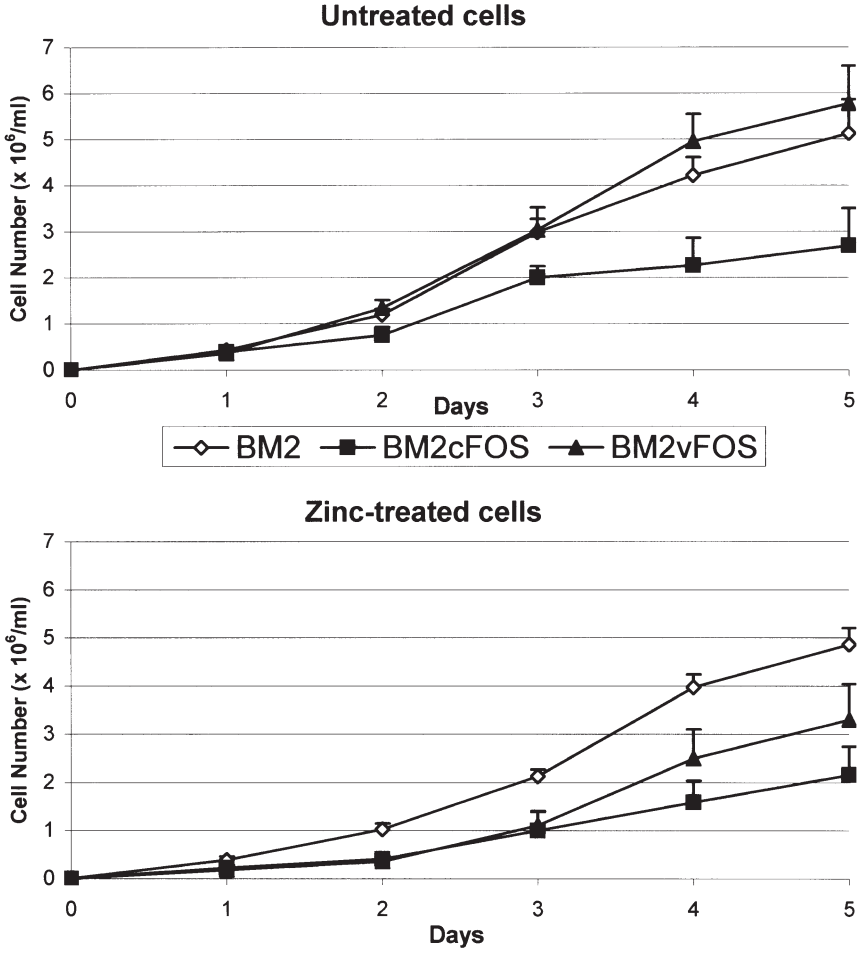

Figure 3. c-Fos and v-Fos proteins block proliferation of BM2 cells. A total of 1×10^6 BM2cFOS#E8, BM2vFOS#D11 and BM2 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 5 days. The number of viable cells determined by eosin dye exclusion was counted daily using a hemocytometer. The data represent mean values from three independent experiments. Error bars indicate standard deviations.

S-phase cells was detected in v-fos-expressing BM2 vFOS cells. v-Fos protein increased the frequency of G0G1 cells from 57% in BM2 controls to 65% in BM2vFOS cells and decreased the frequency of S phase cells from 28% in BM2 controls to 21% in BM2vFOS cells. Similar results were obtained in all independent clones tested. These results suggest that v-Fos protein prolongs the G0G1 phase in BM2 cells, while c-Fos, rather, participates in induction of programmed cell death.

c-Fos but not v-Fos protein decreases viability of BM2 cells

To evaluate the cytotoxicity of over-expressed Fos proteins, we counted viable and dead BM2vFOS, BM2cFOS and BM2 cells treated with zinc chloride for 5 days using eosin dye exclusion by light microscopy. We found BM2cFOS cells to be less viable than BM2vFOS and control BM2 cells cultivated both in the absence and presence of zinc inducer. Even a low, uninduced level of c-Fos protein clearly interfered with the viability of BM2cFOS cells, maintaining a frequency of dying cells at an average level of 16% during the 5 days of treatment in comparison with 4% in the BM2 control (fig. 4). Upon induction with zinc chloride, the frequency of dead BM2cFOS cells increased to 26% during 3 days of cultivation. In contrast, the v-Fos protein slightly but reproducibly improved the viability of BM2 cells. These results suggest that v-Fos and c-Fos proteins differentially regulate the viability of BM2 cells. The increased cytotoxicity of the c-Fos protein correlates well with the presence of fragmented DNA in zinc-treated BM2cFOS cells (table 1).

c-Fos but not v-Fos induces programmed cell death of BM2 cells

Camptothecin is an apoptosis-inducing agent that inhibits topoisomerase 1 [39] causing either chromatin condensation of BM2CD4 cells or their shrinkage to dense bodies (fig. 5, lower panel). Interestingly, the morphology of a significant fraction of BM2 cells expressing c-fos for 24 h was similar to the morphology of camptothecin-treated BM2CD4 cells (fig. 5). However, this effect did not occur in v-fos- or CD4-expressing BM2 cells. This suggests that c-Fos in contrast to v-Fos protein may be engaged in the control of programmed cell death.

The elevated number of BM2cFOS cells containing subdiploid DNA and exhibiting apoptotic morphology prompted us to investigate further the role of Fos proteins in programmed cell death of BM2 cells. First, we examined the proportionality of c-fos expression and the level of DNA fragmentation in BM2cFOS cells. We exposed BM2cFOS, BM2vFOS and control BM2 cells to increasing concentrations of zinc inducer for 24 h and determined both the frequency of cells containing a subdiploid amount of DNA using flow cytometry and the level of fos expression by immunoblotting. The number of cells with a sub-G0G1 DNA content grew proportionally to the amount of c-Fos protein in BM2cFOS cells, reaching 20% at the highest zinc chloride concentration (180 µM) (fig. 6). In contrast, no significant increase in the sub-G0G1 cell fraction was detected in similarly treated BM2vFOS and control BM2 cells. These results support our conclusion that the c-Fos protein induces programmed cell death of BM2 cells.

Next, to confirm that the sub-G0G1 DNA-containing cell fraction indeed represents the cells undergoing apoptosis, DNA-laddering tests were performed in two independent clones of BM2cFOS, BM2vFOS as well as BM2 control cells treated with zinc chloride for 24 h. DNA purified from BM2cFOS cells of both clones and resolved by agarose gel electrophoresis exhibited a typical pattern of an apoptotic ladder (fig. 7). No DNA ladder was detected in BM2vFOS clones or control BM2 cells. These results document that the ability to induce programmed cell death in BM2 cells is a unique feature of the c-Fos but not v-Fos protein and this effect is not clone-specific.

Various cell types can initiate the process of apoptosis in response to a lack of growth factors. To analyze the morphology of starving BM2 cells and their fos-expressing derivatives, the cells were cultivated in the presence or absence of the sera for 24 h, stained with propidium iodide and analyzed by fluorescence microscopy. We noted chromatin condensation and nuclear fragmentation mark-

Table 1. v-Fos protein prolongs the G0G1 phase of BM2 cells, while c-Fos increases the number of cells with a sub-diploid DNA content.

Cell Type	Cell cycle phases			
	subG0G1 (%)	G0G1 (%)	S (%)	G2/M (%)
BM2	0.70 (0.191)	56.90 (5.98)	28.40 (4.6)	14.71 (2.67)
BM2cFOS	*9.03 (2.585)*	55.37 (5.52)	28.58 (4.16)	16.05 (2.41)
BM2vFOS	0.02 (0.019)	*65.09 (5.61)*	*21.34 (3.76)*	13.62 (3.09)

A total of 1×10^6 BM2cFOS#E8, BM2vFOS#D11 and BM2 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 1 day. The cells were fixed, stained with propidium iodide, and the DNA content of at least 10,000 individual cells was analyzed by FACS. Numbers represent the frequency of cells in various phases of the cell cycle (%) ± SD. Data obtained from ten independent experiments were processed using Student's t test. Statistically significant differences ($p < 0.05$) are italicized.

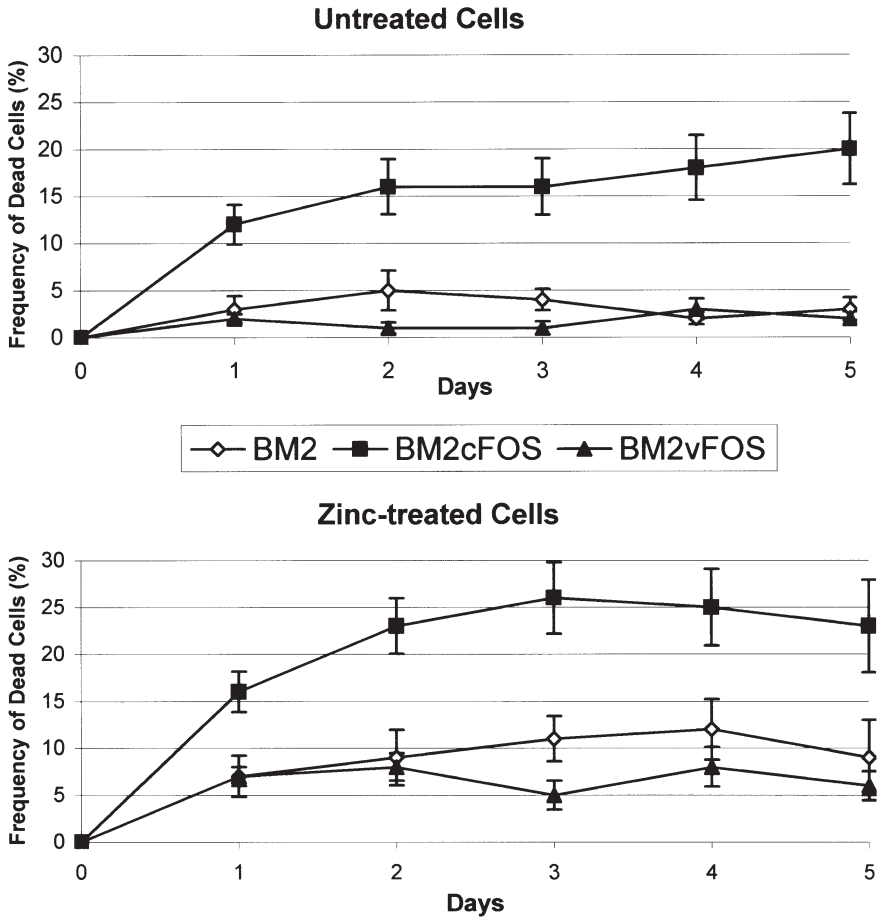

Figure 4. c-Fos but not v-Fos protein decreases viability of BM2 cells. A total of 1×10^6 BM2cFOS#E8, BM2vFOS#D11 and BM2 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 5 days. The number of living and dead cells was determined by eosin dye exclusion. The curves represent mean values of the frequency of dead cells from three independent experiments. At least 300 cells were analyzed in each sample. Error bars indicate standard deviations.

ing apoptosis in zinc-treated BM2cFOS cells cultivated in serum-free medium while this effect was not apparent in BM2vFOS and BM2CD4 cells (fig. 8). In the same cells cultivated under standard serum conditions, the frequency of c-Fos-induced apoptosis was less dramatic (not shown).

Mitochondria play a major role in apoptosis triggered by many stimuli. They integrate death signals through Bcl-2 family members and coordinate caspase activation through the release of cytochrome c as a result of the outer mitochondrial membrane becoming permeable [40]. Therefore, we wished to test whether c-Fos can affect bcl-2 expression and mitochondrial membrane potential in BM2 cells. We cultivated BM2cFOS, BM2vFOS and control BM2CD4 cells in serum-free medium in the presence or absence of zinc chloride for 24 h. First, we determined the level of bcl-2 expression in these cells by SDS-PAGE followed by immunoblotting. The relative amount of Bcl-2 protein in BM2cFOS, BM2vFOS and control BM2CD4 cells did not change upon treatment with zinc inducer, suggesting that bcl-2 expression is not under the control of Fos proteins (not shown). Second, we measured the mitochondrial membrane potential ($\Delta\Psi$m) of the same cells by flow cytometry. The number of cells with a collapsed mitochondrial membrane potential was significantly increased in the population of zinc-treated BM2cFOS cells (23%) in comparison with BM2vFOS (7%) and control BM2CD4 cells (9%) (fig. 9). An increased frequency of cells lacking a mitochondrial membrane potential was noted even upon 12 h of zinc induction (data not shown). These results document that the mechanism of c-Fos-induced apoptosis in BM2cFOS cells is based on regulation of the potential of the mitochondrial membrane and this effect is not mediated by Bcl-2 protein. In contrast, v-Fos protein failed to affect the mitochondrial membrane potential in BM2vFOS cells and is unable to induce programmed cell death.

To examine possible mechanisms mediating c-Fos-induced apoptosis, we determined whether it is dependent

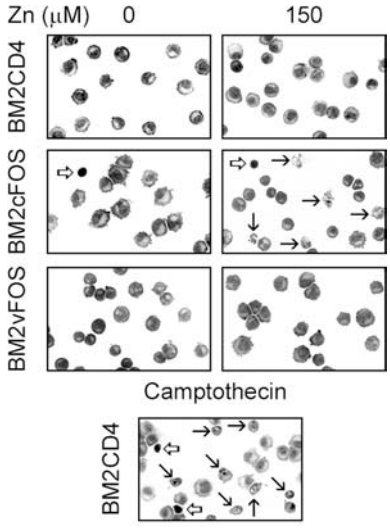

Figure 5. c-Fos- but not v-Fos-expressing BM2 cells exhibit apoptotic morphology. BM2CD4, BM2cFOS#E8 and BM2vFOS#D11 cells were exposed to zinc chloride (Zn) or left untreated for 24 h. As a positive control, BM2CD4 cells were treated with camptothecin for 6 h (3 μM). The cells were washed in PBS, cytocentrifuged, fixed and stained with DiffQuik and analyzed by light microscopy. Arrows indicate the cells exhibiting apoptotic morphology (→, cells with fragmented nuclei; ⇒, cells with condensed morphology).

on synthesis of new proteins within c-Fos-expressing BM2 cells. If the c-Fos-activated death program is blocked by the macromolecular synthesis inhibitor CHX, expression of the c-Fos target genes should be responsible for this effect. The concentration of CHX used, 1 μg/ml, inhibits protein synthesis in preneoplastic hamster cells by 90% within 30 min [9] and it is also efficient in BM2 cells (not shown). We treated BM2, BM2cFOS and BM2vFOS cells with zinc chloride for 2 h to allow synthesis of Fos proteins before addition of CHX for 16 h. Harvested cells were stained with propidium iodide and the frequency of cells with fragmented nuclei was determined by fluorescence microscopy. We found that the frequency of BM2cFOS cells containing fragmented nuclei was not affected by the presence of CHX (fig. 10). This suggests that c-Fos-induced apoptosis does not require expression of new proteins.

Fos proteins do not induce differentiation of BM2 cells

We described earlier that the Fos partner in AP-1, the Jun protein, stimulates differentiation of BM2 cells [27]. To test whether the Fos proteins possess similar activity, we examined the morphology of BM2cFOS and BM2vFOS cells exposed to long-term induction with zinc chloride by phase-contrast microscopy, phagocytosis assay and by immunofluorescence analysis of the differentiation markers Mo-1 [41] and MEP17 [42]. However, in contrast to

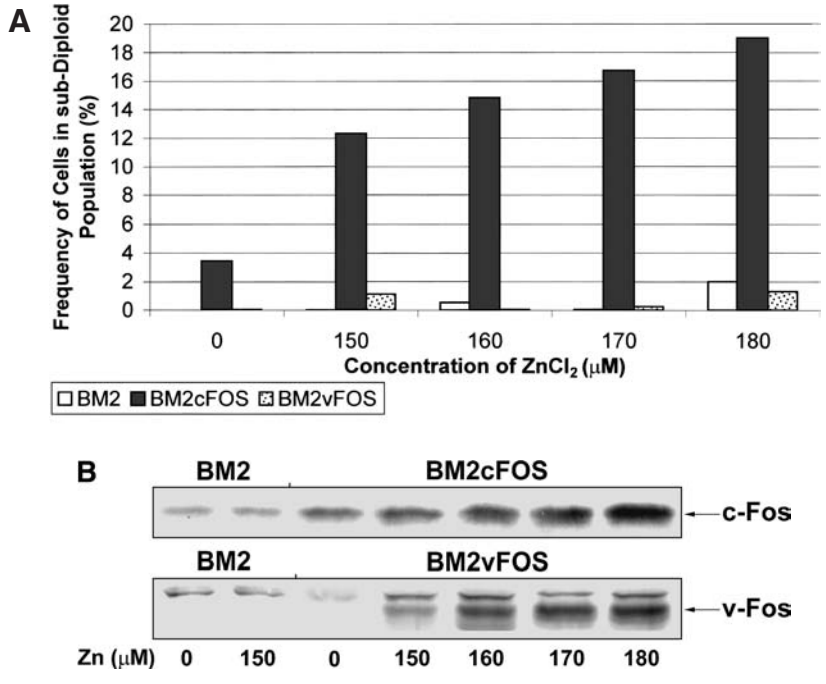

Figure 6. Frequency of BM2cFOS cells containing fragmented DNA increases proportionally to the amount of c-Fos protein. A total of 1×10^6 BM2cFOS#E8, BM2vFOS#D11 and BM2 cells were treated with zinc chloride at the indicated concentrations for 1 day. (*A*) The DNA content of at least 10,000 fixed and propidium iodide-stained cells was analyzed by FACS. The bars represent the frequency of cells containing sub-diploid DNA. (*B*) The level of c-Fos and v-Fos proteins in the same samples was determined by SDS-PAGE followed by immunoblotting using anti-Fos antibody (sc-253; Santa Cruz Biotechnology, for c-Fos; F 7799, Sigma, for v-Fos).

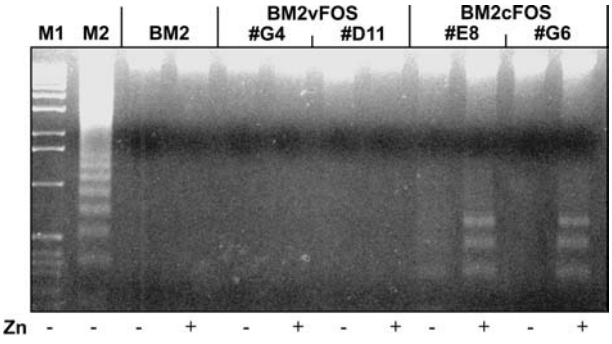

Figure 7. c-Fos but not v-Fos protein causes DNA fragmentation in BM2 cells. Two independent BM2cFOS (E8 and G6) and BM2vFOS (G4 and D11) clones as well as control BM2CD4 cells were cultivated in the presence or absence of 1.5×10^{-4} M zinc chloride (Zn) for 24 h. Cellular DNA was isolated, resolved by agarose electrophoresis and visualized by ethidium bromide staining. The 1-kb DNA ladder (GIBCO BRL/Life Technologies) was loaded in line M1; fragmented DNA resulting from Fas-mediated apoptosis of Jurkat cells was loaded in line M2.

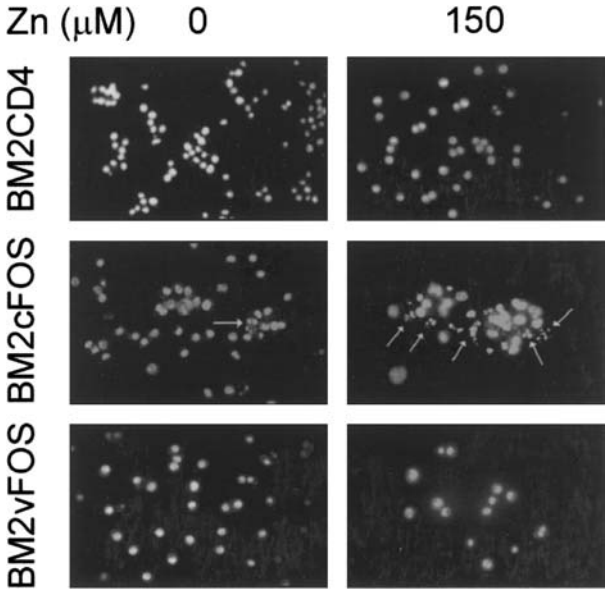

Figure 8. c-Fos but not v-Fos protein causes fragmentation of nuclei of BM2 cells. BM2cFOS#E8, BM2vFOS#D11 and BM2CD4 cells (1×10^6) were cultivated in serum-free medium in the presence or absence of zinc chloride (Zn) for 24 h. The cells were fixed, stained with propidium iodide and their nuclear morphology was examined by fluorescence microscopy. Arrows indicate fragmented nuclei.

Jun proteins, none of these assays showed differentiation-inducing effects of c-Fos and v-Fos proteins in BM2 cells (not shown). This suggests that Fos and Jun proteins play different roles in BM2 cells. While c-Fos induces programmed cell death, c-Jun rather stimulates differentiation of BM2 cells.

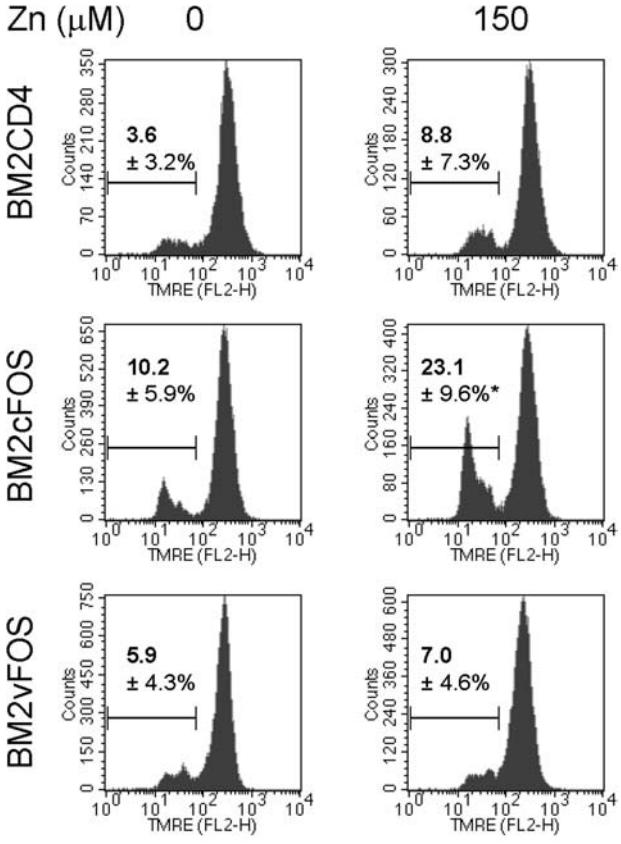

Figure 9. c-Fos but not v-Fos protein induces mitochondrial membrane depolarization. BM2cFOS#E8, BM2vFOS#D11 and BM2CD4 cells (1×10^6) were cultivated in serum-free medium in the presence or absence of zinc chloride (Zn) for 24 h. The frequency of cells with a depolarized mitochondrial membrane was detected by flow cytometry using TMRE probe. Illustrated histograms represent typical results. Percentages in the histograms represent the means ± SD of cells with lost $\Delta\psi$m from four independent experiments. The asterisk denotes a significant difference ($p < 0.05$) from the other experimental groups determined by χ^2 software.

Discussion

AP-1 was one of the first identified mammalian transcription factors. Its involvement in regulation of cellular processes, including cell proliferation, differentiation, programmed cell death and transformation, is documented in a considerable number of studies [for reviews, see refs. 1–3, 43–46]. The effects of AP-1 are cell type specific and depend on the composition of the AP-1 complex. In this study, we compared the effects of ectopically expressed c-Fos and v-Fos proteins on the BM2 line of v-myb-transformed chicken monoblasts. This work supplements our previous data focused on the effects of Jun proteins on BM2 cells [27].

The human metallothionein II_A gene promoter was used to inducibly express c-fos and v-fos cDNAs in BM2 cells. This promoter is heavy metal inducible but it can also

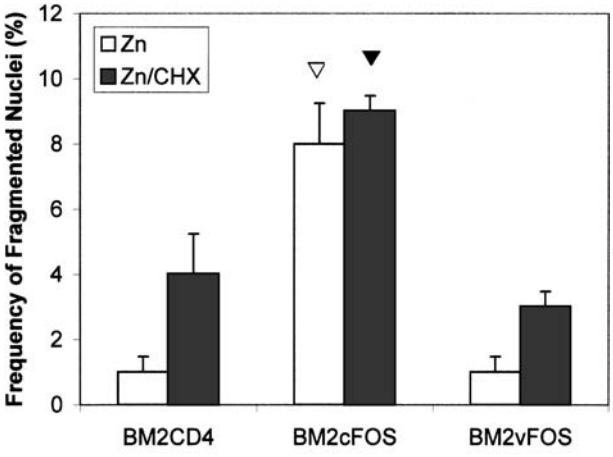

Figure 10. CHX does not inhibit c-Fos-induced apoptosis. BM2cFOS#E8, BM2vFOS#D11 and BM2CD4 cells (2×10^5/ml) were treated with zinc chloride (Zn) for 2 h. Then, CHX was added at a concentration of 1 µg/ml for an additional 16 h. Harvested cells were fixed, stained with propidium iodide and their nuclear morphology was examined by fluorescence microscopy. The fraction of cells with fragmented nuclei was determined; ▽ denotes a significant difference ($p < 0.05$) from zinc-treated BM2CD4 cells and ▼ denotes a significant difference ($p < 0.05$) from BM2CD4 cells treated with zinc chloride and CHX as determined by Student's t test. At least 300 cells were analyzed in each sample. Error bars indicate standard deviations.

respond to other stress factors [47]. A basal level of c-fos expression was repeatedly detected in uninduced BM2cFOS cells of both independent clones. This presumably results from the fact that the human metallothionein promoter is leaky [48, 49]. Interestingly, v-fos expression from the same promoter in BM2vFOS cells was strictly dependent on the presence of zinc inducer. This difference may result from a positive feedback controlling expression from the metallothionein promoter by c-Fos protein that was found cytotoxic for BM2cFOS cells. Basal uninduced c-fos expression causing stress to BM2cFOS cells may further up-regulate the promoter. In contrast, the v-Fos expression that is not cytotoxic to BM2vFOS cells leaves the basal promoter activity unaffected, thus no v-Fos protein was found in uninduced BM2vFOS cells.

Although the c-Fos protein was described as a positive regulator of cell growth in most model cell lines, there is also evidence that Fos proteins can act as negative regulators of cell growth causing growth arrest in the G1 phase of the cell cycle [50, 51]. The results of our work also document growth-suppressing activity of c-Fos as well as v-Fos protein in BM2 cells. The extent of growth suppression correlates positively with the intracellular level of the Fos proteins (not shown). The mechanisms by which c-Fos and v-Fos proteins affect proliferation of BM2 cells are not the same. While v-Fos extends the duration of the G0G1 phase of the cell cycle, c-Fos leaves the cell cycle unaffected and, rather, decreases viability of BM2 cells.

Recently, we described a differentiation-promoting effect of c-Jun protein in BM2 cells [27]. Interestingly, no similar effect was detected in BM2 cells over-expressing c-fos or v-fos genes. This phenomenon may result from a differential subunit composition of the AP-1 complex in BM2 cells expressing jun and fos. The composition of the AP-1 transcription factor determines its DNA-binding specificity and transcription activation function. Jun proteins can form homodimers, and therefore complexes of c-Jun/c-Jun should be the predominant form of AP-1 in BM2 cells over-expressing c-jun. In contrast, Fos proteins cannot form homodimers, thus the complexes of Fos proteins with other endogenous partners (Jun, ATF/CREB and Maf/Nrl proteins) may be formed preferentially in BM2 cells over-expressing the fos genes. In addition, formation of AP-1 complexes containing preferentially c-Fos (v-Fos, respectively) to the detriment of other members of the Fos family, such as Fra-1, Fra-2 and FosB, can be expected in BM2vFOS and BM2cFOS cells. Binding of Jun/Jun homodimers and Fos/Jun heterodimers to DNA causes distinct DNA bending that may result in highly specific protein-protein interactions between AP-1 factors and other promoter-bound transcription complexes [52, 53]. Fos/Jun heterodimers and Jun/Jun homodimers can even recognize and regulate different promoters [54]. Our results confirm that composition of the AP-1 transcription factor plays a decisive role in regulation of cellular fate.

Because the level of c-fos transcription markedly increases at late stages of monocytic differentiation of WEHI-3B cells, c-Fos was suggested to play a role in some macrophage specific functions [18]. The data presented in this study and in our previous paper [27] demonstrate that, instead, the c-Jun protein induces macrophage-specific functions such as cell adhesion, phagocytosis, non-specific esterase activity and the ability to produce oxygen radicals in BM2 cells while c-Fos can regulate programmed cell death. We believe that high level of c-fos RNA found in macrophages and fetal liver cells but not in immature cell lines [18] indicates their commitment to apoptosis.

The v-myb oncogene of AMV is constitutively expressed in BM2 cells. The transforming function of v-Myb oncoprotein partially results from its ability to activate transcription of specific target genes [55]. One of the Myb target genes that has been identified is bcl-*2* coding for the anti-apoptotic Bcl-2 protein [56, 57]. Interestingly, the c-Fos protein possessed an efficient dose-dependent pro-apoptotic effect in BM2 cells causing an apoptotic cellular morphology, DNA fragmentation and a decrease of the mitochondrial membrane potential. These effects were not dependent on the level of Bcl-2 protein, suggesting that c-Fos-induced apoptosis of BM2 monoblasts is insensitive to Bcl-2. A similar effect was also described for B cells [58].

Nontransformed monocytic cells undergo programmed cell death after growth factor deprivation [59]. c-Fos-dependent induction of programmed cell death after growth factor withdrawal has been described in several cell lines [60–62]. BM2 cells can survive and even grow in serum-free conditions for at least 5 days (data not shown). Thus, we tested the effect of c-Fos and v-Fos proteins on survival of BM2 cells after growth factor depletion. Our results show that the c-Fos but not the v-Fos protein can restore the sensitivity of BM2 cells to growth factor deprivation. The essential difference between these related proteins is the unique ability of the c-Fos protein to act as both repressor and activator of transcription, while the v-Fos protein exhibits only transactivation ability [63–65]. Our findings thus support conclusions by Preston et al. [9], that it is rather the transrepression than transactivation activity of c-Fos that is responsible for the programmed cell death. The transrepression activity of c-Fos is presumably preferred under serum-free conditions, causing extensive apoptosis of BM2cFOS cells. The mechanism regulating the c-Fos function may be based on phosphorylation. The C-terminus of the c-Fos protein can be phosphorylated by mitogen-activated protein kinase (MAP kinase) and its downstream substrate 90-kDa ribosomal S6 kinase (RSK). This modification is essential for the efficiency of transactivation by c-Fos [54]. Both kinases can be stimulated by addition of serum [66, 67]. Thus, induction of c-fos expression in BM2cFOS cells cultivated under serum-free conditions may result in accumulation of hypophosphorylated c-Fos protein causing programmed cell death. Because the C-terminal region is truncated in the v-Fos protein, it cannot be regulated by MAP kinase and RSK as c-Fos is, causing failure of its transrepression function [64–69]. The hypothesis that c-Fos functions as a transcriptional repressor in order to activate programmed cell death is further supported by the fact that the protein synthesis inhibitor CHX did not block c-Fos-induced apoptosis of BM2cFOS cells.

v-Myb shares proliferation-promoting and apoptosis-suppressing activities with c-Myb but it does not permit the activation of genes required for terminal differentiation of its cellular progenitor [57, 70, 71]. This study and our previous paper [27] document that transcription factors Fos and Jun that form the AP-1 complex can significantly affect the function of the v-Myb oncoprotein. Both Fos and Jun are able to suppress proliferation of v-myb-transformed BM2 monoblasts. On the other hand, their roles in the regulation of differentiation and apoptosis are different: while the Jun protein induces differentiation, c-Fos stimulates apoptosis of undifferentiated BM2 cells. Thus, the balance between Fos and Jun proteins can be crucial for determination of the fate of the cell.

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