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DIO-1 is a gene involved in onset of apoptosis *in vitro*, whose misexpression disrupts limb development

(transcription factors/interdigital webs)

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ABSTRACT **The *DIO-1* (*death inducer-obliterator-1*) gene, identified by differential display PCR in pre-B WOL-1 cells undergoing apoptosis, encodes a putative transcription factor whose protein has two Zn finger motifs, nuclear localization signals, and transcriptional activation domains, expressed in the limb interdigitating webs during development. When overexpressed, *DIO-1* translocates to the nucleus and activates apoptosis *in vitro*. Nuclear translocation as well as induction of apoptosis are lost after deletion of the nuclear localization sequences. *DIO-1* apoptotic induction is prevented by caspase inhibitors and Bcl-2 overexpression. The *in vivo* role of *DIO-1* was studied by misexpressing *DIO-1* during chicken limb development. The most frequently observed phenotype was an arrest in limb outgrowth, an effect that correlates with the inhibition of mesodermal and ectodermal genes involved in this process. Our data demonstrate the ability of *DIO-1* to trigger apoptotic processes *in vitro* and suggest a role for this gene in cell death during development.**

Apoptosis is a major form of cell death, characterized morphologically by chromatin condensation, nuclear disruption, and formation of cytosol containing apoptotic bodies. It is an efficient mechanism for eliminating unwanted cells and is of central importance for development and homeostasis in metazoan animals (1). Many different signals within or from outside the cell have been shown to influence the decision between life and death (2). Most are controlled through triggering of specific receptors, which leads to activation of specific mediators; they may then act to suppress or promote activation of the death program. It is hence not surprising that initiation of apoptosis is precisely regulated.

A common meeting point for cell death signals is the cytoplasm, where the caspases exert their function and are blocked by their inhibitors (3–6). Very little is known as to how these signals are transmitted to the nucleus. A caspase-activated DNase (CAD) and its inhibitor (ICAD) have recently been identified in the cytoplasmic fraction of a mouse lymphoma cell line. Caspase pathway activation by different stimuli cleaves ICAD, allowing CAD to enter the nucleus and degrade chromosomal DNA (7, 8). In addition to the caspases, inducible gene products also appear to be required for apoptotic death in some cell types (9). Evidence for this was derived from experiments in which cell death could be suppressed by the inhibition of RNA or protein synthesis in cells that should otherwise die (10), suggesting that gene transcription and RNA translation are required for death to occur in these cells. Indeed, transcriptional activation of specific genes is absolutely required for physiological apoptosis in both insect and vertebrate embryos (10, 11). Several transcriptional regulators are known to control apoptosis, among which p53 (12), Nur77 (13), the glucocorticoid receptor (14), STAT1 (15), c-myc (12, 16), c-jun (17), and NF-κB (18) have been identified to date.

Much of the natural cell death that occurs during insect and vertebrate development appears to be mediated by the transcriptional activation of killer genes. Although no such genes have yet been identified in vertebrates, recent studies in the fly *Drosophila melanogaster* have uncovered three components of the genetic program controlling programmed cell death (PCD), *hid*, *grim,* and *reaper*, whose transcriptional activation precedes, induces, and is necessary for PCD by apoptosis (19–21). The three genes map to a single genetic complex and function as death switches that are regulated at the level of transcription. Their ectopic activation triggers apoptosis in otherwise viable cells, and their inactivation prevents apoptosis of cells that would normally undergo PCD. We and others have recently demonstrated that the expression of one of these genes, *grim*, activates apoptosis in mammalian cells, implying conservation during metazoan evolution of both the gene and the mechanisms required to trigger cell death (9).

The developing limb is perhaps one of the best-suited model systems for the study of this process, because fine tuning is required between cell proliferation and cell apoptosis to allow proper limb modeling, a process subject to intervention without endangering embryo viability. Whilst much has recently been learned regarding factors involved in cell proliferation, less is known about the mechanisms implicated in programmed cell death during development. It has recently been shown that inhibition of NF-κB translocation by viral overexpression of a transdominant-negative IκB leads to perturbation of limb outgrowth (22). We have now tested *in vivo* the effect of a, to our knowledge, novel gene, *DIO-1* (*death inducer-obliterator-1*), identified by differential display PCR in pre-B cells undergoing apoptosis. Its mRNA and protein are present at very low levels in the cytoplasm. Once an appropriate apoptotic signal is detected, the protein translocates to the nucleus and up-regulation is observed at both transcript and protein levels. When overexpressed, it induces apoptosis in cell lines growing *in vitro*, which is prevented by blocking caspase activity. The protein encoded, DIO-1, is expressed in the limb interdigitating membranes during development. *DIO-1* expression in distal proliferating mesodermal cells of the developing chicken limb bud prevents limb outgrowth, an effect that correlates with inhibition of mesodermal and ectodermal genes involved in limb outgrowth. These data demonstrate the ability of *DIO-1* to trigger apoptotic processes *in vitro*, as well as the

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Abbreviations: AER, apical ectodermal ridge; E2, 17β-estradiol; NLS, nuclear localization signal.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AJ238332).

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utility of limb development as a model system to characterize genes involved in apoptosis.

MATERIALS AND METHODS

Cloning of *DIO-1*. Differential display experiments were performed by using an RNAmap kit (GenHunter, Brookline, MA) according to the manufacturer's specifications. Briefly, 200 ng of total cytoplasmic RNA (after DNase treatment with the MessageClean Kit; GenHunter) isolated from WOL-1 cells at 0, 2, 4, and 8 h after IL-7 withdrawal were reverse transcribed with oligo(dT) primers (T_{12}MN) in the presence of Moloney murine leukemia virus reverse transcriptase. They were amplified with several combinations of 5′ decamer arbitrary primers and the T_{12}MN used for reverse transcription in the presence of [^{35}S]dATP (1,200 Ci/mmol). Amplified products were resolved in an 8-M urea/6% polyacrylamide DNA sequencing gel and analyzed by autoradiography. Bands of interest were isolated, reamplified, cloned in the pCR-Script SK(+) vector (Stratagene), and used for Northern analysis and sequencing. *DIO-1* cDNA was obtained from WOL-1 cDNA by 5′ rapid amplification of cDNA ends (RACE) by using a Marathon cDNA Amplification Kit (CLONTECH), with the 3′ primer L282 (5′-AGGTGTACCTTGTACAGCAGTGAAAC-3′). The resulting 2.6-kbp band was excised from the gel and cloned in the TA-type vector pGEM-T (Promega). Resulting clones were sequence analyzed for orientation, and the oriented sense with respect to the T7 promoter was called DIO-1pGEM-T. To confirm the ORF sequence obtained, a cDNA library from mouse brain cloned in λZAP II (Stratagene) was screened by probing with the RACE clone; the same probe was used to screen a human fetal kidney cDNA library (CLONTECH) from which the human *DIO-1* homologue was cloned.

Cells and Transfections. WOL-1 cells were derived from adult BALB/c mouse bone marrow. WOL-1 is an untransformed IL-7-dependent stroma cell- independent pre-BI cell line, capable of reconstituting irradiated severe combined immunodeficient mice. Cells were cultured in Iscove's modified Dulbecco's medium supplemented with penicillin (100 units/ml)/streptomycin (100 μg/ml)/1 mM sodium pyruvate/nonessential amino acids/50 μM 2-mercaptoethanol/2 mM L-glutamine/10% FCS/IL-7 (3% supernatant from a murine IL-7-producing cell line). The Ba/F3 and FL5.12 cell lines were maintained in RPMI medium 1640 with 10% FCS and 5% supernatant of a murine IL-3-producing cell line, whereas A20 and WEHI-231 grew in the same medium without IL-3. The FL5.12hBcl-2 stable cell line was cultured in 1 mg/ml G-418 (Calbiochem). MEF(10.1)Val5MycER cells were cultured at 39°C in phenol red-free DMEM containing 10% FCS. Where indicated, 1 μM 17β-estradiol (E2) was added to activate the MycER fusion protein after 24 h FCS starvation (12). WOL-1, A20, Ba/F3, and FL5.12 cell lines were cultured at 37°C, and all cell lines were maintained in a humidified atmosphere with 5% CO_2.

Transient DNA transfection was performed by electroporation. For each transfection, 2×10^6 log phase cells were collected by centrifugation and resuspended in 200 μl of RPMI medium 1640 without FCS. After addition of 10 μg of plasmid DNA (1 mg/ml), samples were gently shaken and electroporated in a 0.4-cm electrode gap gene pulser cuvette at 960 μF and 320 V with a GenePulser (Bio-Rad). Samples were diluted with 6 ml of the same medium supplemented with 10% FCS and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were analyzed for cell-cycle staining by FACS at 48 h after electroporation.

Northern Blot Analysis. Total cytoplasmic RNA was prepared as described (23). RNA (10 μg) was Northern blotted by using a ^{32}P-labeled *DIO-1* riboprobe made by DIO-1pGEM-T digestion with *Bgl*II and *in vitro* transcribed from SP6 by using the Riboprobe *In Vitro* Transcription System (Promega). Hybridization was performed in 50% formamide at 65°C; washes were in 0.1× SSC + 0.1% SDS at 80°C. Blots were exposed on Kodak X-Omat AR film at −70°C with two intensifying screens.

Antibody Production and Western Blot. We synthesized a peptide corresponding to amino acids 58–72 of murine *DIO-1* with an additional N-terminal cysteine (CSLRRSGRQPKRTERV); it was coupled to maleimide-activated keyhole limpet hemocyanin and the purified conjugate injected into New Zealand White rabbits. Polyclonal antibody was affinity purified on a peptide-thiopropyl Sepharose column. For Western blot, cells were collected at different times after IL-7 removal from culture medium; 5×10^5 cells were lysed with RIPA buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS), and the total extract separated in 8% SDS/PAGE, transferred and incubated with the affinity-purified polyclonal anti-*DIO-1* antibody (1:100 dilution in TBS-1% nonfat dry milk). Protein-loading equivalence was confirmed by Ponceau S staining.

***In Situ* Hybridization and Histology.** Whole-mount *in situ* hybridization was as described (24) with minor modifications (25). The *DIO-1* digoxigenin probe was made by *Bgl*II digestion of the DIO-1pGEM-T and transcription from the SP6 promoter. The probe used for *Lhx-2* (700 bp) encompasses the homeobox and the second LIM (*lin-11, ISl-1, mec-3*) domain. The remaining probes have been described elsewhere and include *Msx-1* (26), *Fgf-8* (27), and *NF-κB* (22). To visualize cartilage, embryos were fixed in trichloroacetic acid after viral infection, stained with 0.1% alcian green, and dehydrated/cleared in methyl salicylate.

Virus Production and Injection Protocols. Chicken embryos (from MacIntyre Poultry, San Diego, CA, or SPAFAS, Preston, CT) were infected with a virus containing the *DIO-1* ORF. Virus preparation and injections were as previously described (28). After injection, embryos were incubated at 37°C and fixed at different time points for *in situ* hybridization or phenotypic analysis.

RESULTS

Isolation of *DIO-1* cDNA: Protein Structure and Sequence Relationships. To search for genes implicated in apoptosis, we used the differential display PCR (DDRT-PCR) technique (29) using mRNA obtained from the WOL-1 pre-B cell line as a target. WOL-1 was derived from BALB/c adult bone marrow; it grows exponentially in the presence of IL-7 and undergoes apoptosis on IL-7 withdrawal. The DDRT-PCR technique gave rise to several positive bands, 10 of which were initially identified as undergoing up- or down-regulation during apoptotic death and were therefore considered candidates for subsequent analysis. They were further amplified, sequenced, and compared with known gene sequences by using the National Center for Biotechnology Information BLAST program (30). Of these, one band (DIO-1) revealed that the nucleotide sequence was a gene that showed no significant identity to any known gene or translated products in the databases. To confirm the sequence obtained by rapid amplification of cDNA ends (RACE), a murine cDNA library was screened by using a labeled *DIO-1* probe. Five positive clones were identified and characterized by restriction mapping and sequencing. Analysis of the cDNA revealed inserts identical in sequence to the ORF cloned by RACE. The longest ORF corresponds to a 614-aa protein (Fig. 1*A*) and shows a Kozak consensus sequence before the ATG (considered as the +1 position) known to be crucial for initiation of translation (31). It also comprises a putative nuclear localization signal (NLS) and transcriptional activation domain in the N-terminal region, two central Zn finger motifs, and a lysine-rich carboxyl terminus. Having analyzed the domains of the putative pro-

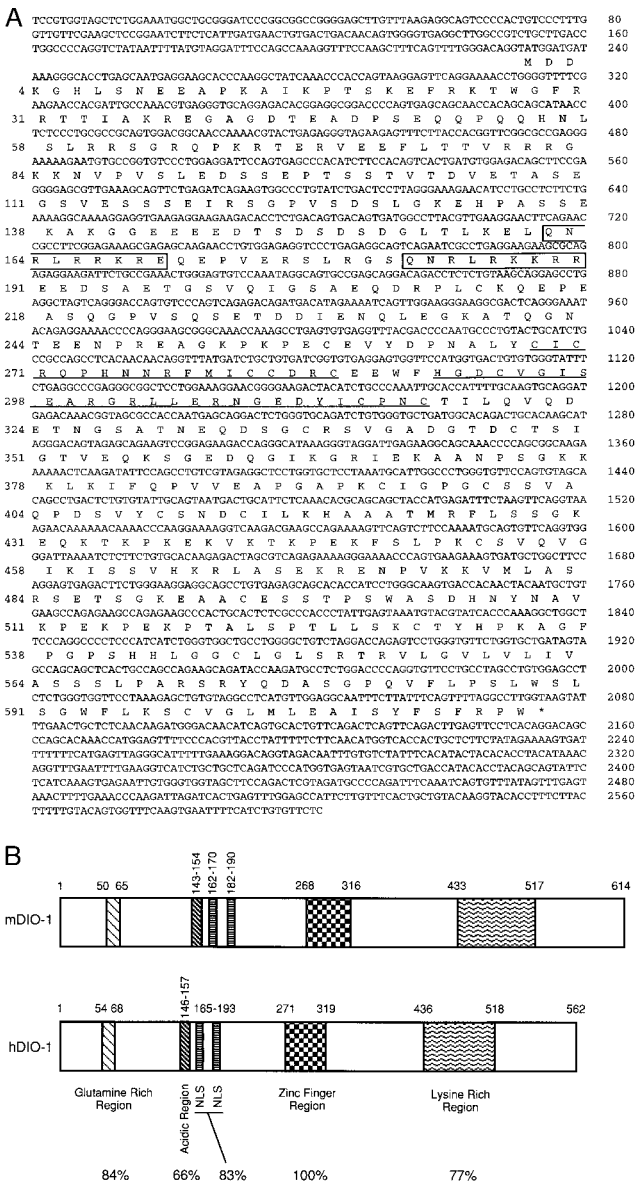

FIG. 1. Nucleotide and predicted amino acid sequences of murine and human *DIO-1*. (*A*) The bipartite NLS sequence is boxed, and the zinc finger motifs are underlined. (*B*) Schematic representation of the predicted ORF of murine and human *DIO-1*. The start and end positions of the amino acids defining the motifs are numbered above. Percentages indicate degree of identity between human and mouse for each domain. Overall similarity is 74%.

tein, we sought to determine their degree of conservation in closely related species to assess their function. A human cDNA library was screened by using the murine clone as probe; positive clones were sequenced and showed strong similarity to the murine gene in the ORF, with a high degree of structural and compositional conservation (Fig. 1*B*).

***DIO-1* Is Present in All Tissues and Its Levels Are Up-Regulated During Apoptosis.** To study *DIO-1* gene regulation during apoptosis, *DIO-1* expression pattern was examined by Northern blot analysis. Various tissues were analyzed to determine *DIO-1* transcript distribution, and two 9.5- and 5.4-Kb mRNA species were detected in all tissues tested (Fig. 2*A*). Southern blot analysis of genomic DNA showed that *DIO-1* is a single-copy gene in both mouse and human. RNA samples were isolated from several cell lines in exponential growth or undergoing apoptosis as a result of various experimental treatments. In the exponential growth phase, WOL-1 cells express low levels of *DIO-1* mRNA, which increase after induction of apoptosis (Fig. 2*B*). *DIO-1* is up-regulated in IL-7-deprived cells or those treated with IFN-γ or dexamethasone, but not in cells treated with etoposide, UV irradiation, or in those undergoing p53-induced cell death (Fig. 2*B*). It is also up-regulated in anti-IgM-treated WEHI-231 cells. In MEF(10.1)Val5MycER cells, up-regulation is observed in the absence of serum after addition of E2, but not before or at 32°C (even in the presence of E2 or serum). Up-regulation of *DIO-1* mRNA levels in cells undergoing apoptosis was confirmed in Western blot by using a polyclonal anti-*DIO-1* antibody raised against a synthetic peptide comprising amino acids 58–72. In cell extracts derived from WOL-1 cells undergoing IL-7 deprivation-induced apoptosis, a 67-kDa band was up-regulated 2 hr after induction (Fig. 2*C*), but not after etoposide-induced cell death (data not shown). In all cases in which up-regulation of the *DIO-1* transcript or of the DIO-1 protein itself was detected, there was a clear peak in the kinetic levels of *DIO-1* up-regulation before any signs of cell death were detectable.

***DIO-1*-Induced Apoptosis Is Inhibited by Bcl-2 and Z-VAD and Lost by Deletion of the NLS.** The role of *DIO-1* in the apoptotic process was evaluated by transient transfection of the gene into several cell lines and examination of cell death kinetics. Transfection of a *DIO-1* expression plasmid into Ba/F3 cells results in a dramatic loss of cell viability at 48 hr after transfection (Fig. 3). All cells displayed morphological alterations characteristic of apoptosis, becoming rounded, condensed, and finally dying. This effect was specific in that transfection of Ba/F3 with an empty vector had no effect on cell survival. To verify the generality of this observation, *DIO-1* constructs were transfected into A20 or FL5.12 cells (Fig. 3). In both cases, apoptosis was induced after kinetics similar to those observed for Ba/F3. Transfection into MEF(10.1)Val5MycER cells gave rise to apoptotic morphology as assessed by 4′6′-diamidino-2-phenylindole staining; using the *DIO-1*-specific antibody, we found that endogenous *DIO-1* is located in the cytoplasm of MEF(10.1)Val5MycER cells in exponential growth. When apoptosis is triggered in these cells by addition of E2 at 39°C in the absence of serum, *DIO-1* is translocated to the nucleus (not shown). This translocation appears to be critical for activation of the apoptotic pathway, as deletion of the NLS renders the DIO-1 protein unable to translocate to the nucleus, thus impairing its ability to trigger apoptosis (Fig. 3). When *DIO-1* was transfected into stable FL5.12 cells overexpressing human Bcl-2, cells were resistant to apoptosis, showing that Bcl-2 coexpression inhibits *DIO-1* death-promoting activity, as has also been described for other systems (32). We also incubated *DIO-1*-transfected FL5.12 cells alone or in the presence of the caspase inhibitor Z-VAD-fmk. After 48-h expression in the presence of IL-3, the apoptosis induced by *DIO-1* was completely blocked because of caspase inhibition, an observation that again clearly suggests that the death pathway induced by this gene requires caspase activity. Finally, extensive efforts to derive stable *DIO-1* transfectants in these three cell lines were unsuccessful, suggesting the lethality of *DIO-1* expression in these cells. All together, these results show that *DIO-1* overexpression results in the activation of a cell death program analogous to that operative in other systems, and that activation of the death pathway requires *DIO-1* translocation to the nucleus, where it probably performs a function compatible with its structure as a transcription factor.

Alteration of Limb Development by *DIO-1* Overexpression. Using whole-mount *in situ* hybridization, we also studied *DIO-1* expression throughout mouse development, which is expressed in the most distal limb cells on developmental day 10.5 (Fig. 4) and close to or within the limb interdigitating webs on day 12.5 (Fig. 4 *Inset*). Based on the *in vitro* effects and *in vivo* expression pattern described above, as well as on the presence of *DIO-1* transcripts in the limb cells undergoing

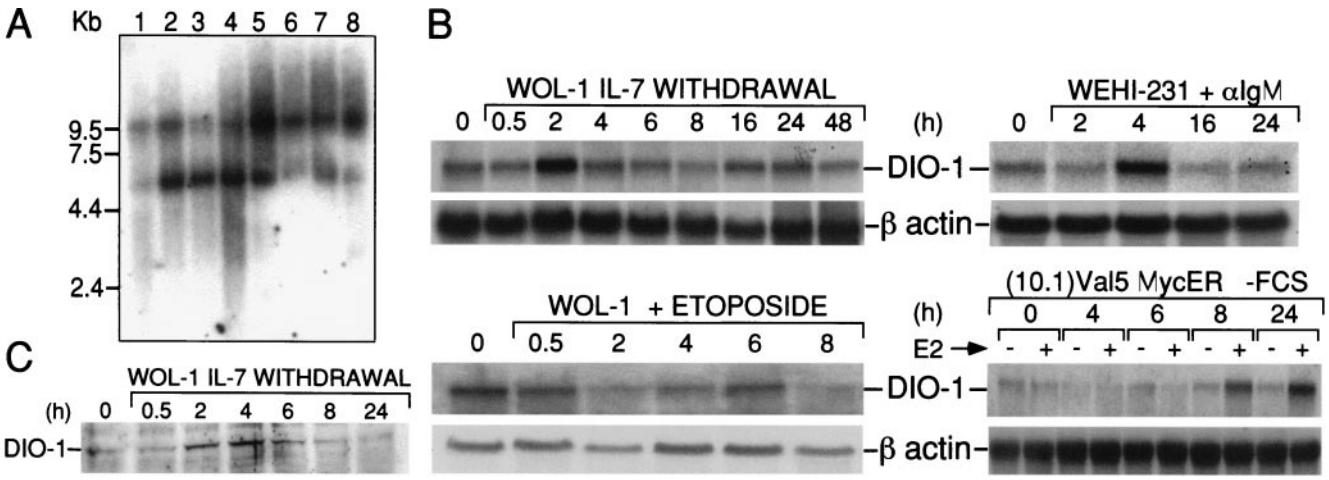

FIG. 2. *DIO-1* is differentially expressed under several apoptotic conditions and induces apoptosis when overexpressed. (*A*) *DIO-1* expression was analyzed in murine tissues by hybridization with the *DIO-1* riboprobe of a mouse MTN blot (CLONTECH). Molecular size markers are indicated on the left. Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis. (*B*) Northern blots containing 10 μg per lane of total cytoplasmic RNA from the indicated cell lines, treated with several apoptotic stimuli at different time points, were hybridized to the *DIO-1* riboprobe. The blots were reprobed with an actin probe for normalization of the amounts loaded. (*C*) Western blot analysis of WOL-1 cells driven to apoptosis by IL-7 starvation. The position of the *DIO-1* gene product is indicated.

apoptotic cell death, we hypothesized that *DIO-1* may influence the control of cell proliferation and death during vertebrate limb development (see ref. 33 for a review on vertebrate limb outgrowth).

Retroviral technology was used to misexpress *DIO-1* in the chicken limb. A replication-competent retroviral vector containing the *DIO-1* ORF was injected into limb primordia at stages 10–23. The consequences of *DIO-1* expression were analyzed in embryo limb buds throughout development. At 60–72 h after injection, infected limb buds failed to develop a normal apical ectodermal ridge (AER, the pseudo-stratified epithelium located at the tip of the limb, required for normal limb bud outgrowth) (Fig. 5 *A* and *B*). Maximal interference with limb outgrowth was observed when embryos were injected at stages 13–17. In 35% of the experiments performed at this stage, truncation occurred in the most distal elements, showing absence of digits, carpals, and metacarpals (Fig. 5 *C* and *D*). In the majority of cases (65%), however, reduction in size and malformation of the tibia and fibula are observed (Fig. 5 *E* and *F*). Misexpression before stage 13 caused alteration, but not truncation, in 12% of the cases; misexpression of *DIO-1* after stage 18 reduced malformation frequency to 40%, and no truncations were observed. These data indicate that to affect the phenotype of the developing limb bud, *DIO-1* must be expressed in a permissive environment and is not the consequence of nonspecific toxic effects. Finally, we analyzed the caspase activity level in the developing limbs. The maximal effects of *DIO-1* appear to correlate with maximum caspase activity (not shown), reinforcing the view that execution of the death program by *DIO-1* is caspase dependent, although *DIO-1* overexpression precedes caspase activity.

Because misexpression of *DIO-1* can perturb AER formation, we would expect this process to be preceded by changes in gene expression, in both the ectoderm and the underlying limb bud mesoderm. *In situ* hybridization of embryos infected with the *RCAS-DIO-1* construct by using riboprobes for me-

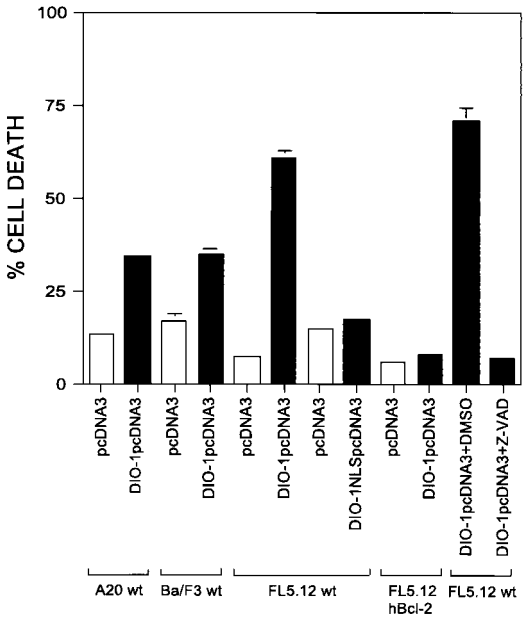

FIG. 3. *DIO-1* induces apoptosis *in vitro*. The *DIO-1* ORF was cloned into the pcDNA3 mammalian expression vector (Invitrogen). Both empty vector and the *DIO-1* construct were transiently transfected by electroporation into A20 and Ba/F3 cell lines. After 48-hr expression, the cells were permeabilized and stained with propidium iodide and cell cycle analyzed by FACS. FL5.12 wild-type and stably transfected hBcl-2 cells were transiently transfected as before. DIO-1NLSpcDNA3 encodes a mutant protein lacking amino acids 162–192, which is therefore unable to translocate to the nucleus. Where indicated, the general caspase inhibitor Z-VAD-fmk (50 μM final concentration; Bachem) was added immediately after transfection.

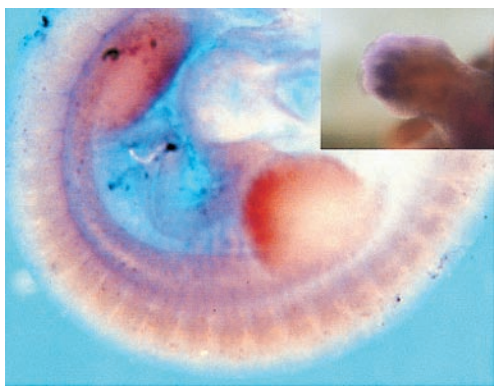

FIG. 4. Whole-mount *in situ* hybridization showing *DIO-1* expression pattern during murine development. Staining is shown of a 10.5-day mouse embryo and a 12.5-day mouse embryo limb (*Inset*).

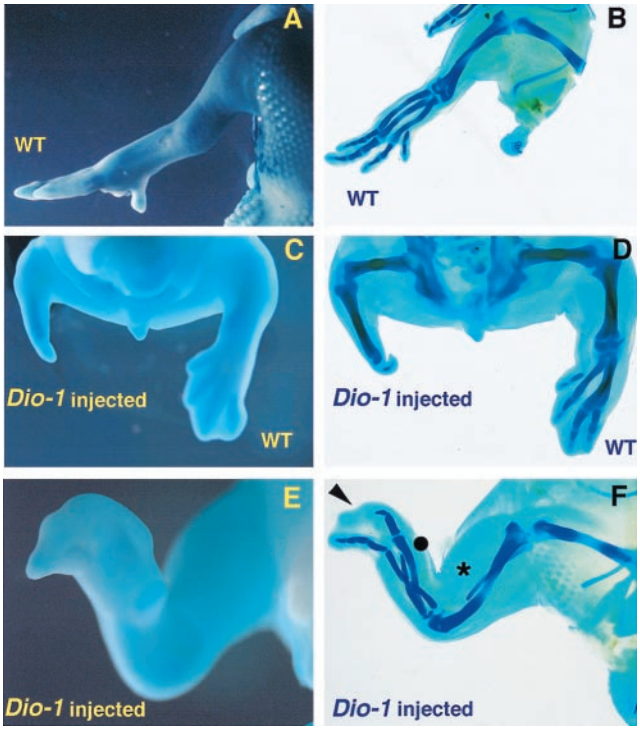

FIG. 5. *DIO-1* overexpression inhibits chicken limb outgrowth. A retroviral vector containing the *RCAS-DIO-1* construct was injected into limb primordia of stage 8–12 chicken embryos. Embryos were examined at different stages after infection. (*A*) Whole-mount preparation showing the hind limb of a wild-type embryo. (*B*) Alcian green staining of the same limb to visualize the normal cartilage pattern. (*C*) An infected embryo 6 days after injection, showing extensive truncation of the distal elements of the leg. (*D*) The same embryo after cartilage staining. Note the complete absence of elements distal to the tibia–fibula joint. (*E* and *F*) Whole- mount and cartilage staining of an embryo 8 days after infection with the *RCAS-DIO-1* construct. The infected limb is distorted and reduced in size, exhibiting an absence, reduction or malformation of phalanges, tarsals, and metatarsals (dot). In a few cases, the fibula was reduced in size (asterisk).

sodermal genes involved in limb outgrowth, such as *Msx-1* (Fig. 6*A*), *Lhx*-2 (Fig. 6*C*), and *NF-κB* (Fig. 6*D*), showed down-

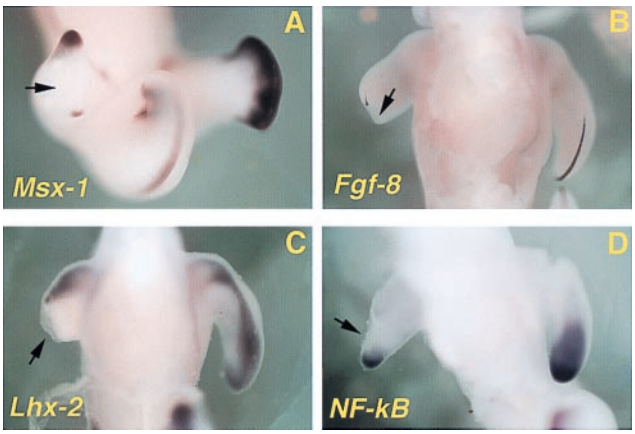

FIG. 6. *DIO-1* overexpression alters gene expression in the developing chicken limb bud. Misexpression of the *RCAS-DIO-1* construct leads to arrested limb outgrowth, preceded by changes in the expression of genes involved in outgrowth of the limb. Note the reduced size of the infected limb buds (left limb buds in all cases). Transcripts for *Msx-1* (*A*) *Fgf-8;* (*B*) *Lhx-2;* (*C*) and *NF-κB* (*D*) are strongly down-regulated (arrows) in the injected limb buds (compare with the normal expression pattern in the uninjected limb bud, right limb bud in all cases).

regulation in their transcript levels. Transcripts for ectodermal genes involved in limb outgrowth, such as *Fgf-8*, are also absent or down-regulated (Fig. 6*B*). It is not known whether *DIO-1* misexpression is directly responsible for the down-regulation of ectodermal gene markers (i.e., *Fgf-8*), or if this is a consequence of the previously altered mesodermal gene expression. The combination of these results indicates that *DIO-1* may be important during limb development, and its apoptotic function may be a driving force in sculpting the final structure.

DISCUSSION

The apoptotic pathway is still elusive and, in many cases, depends on the outcome of the balance between levels of survival and apoptotic genes, at either the transcriptional or translational level. Here we report the cloning and characterization of a gene involved in apoptosis, which is up-regulated under certain apoptotic conditions that do not involve p53-mediated cell death. This up-regulation is observed quite early in cell death kinetics and always before any of the classical characteristics of apoptosis, i.e., DNA laddering, haploid $subG_0/G_1$ cell-cycle peak, or alteration of cell membrane polarity, can be detected. This indicates that *DIO-1* acts very early in the apoptotic cascade and suggests a key role in the control of the initiating triggering mechanism. This gene is a putative transcription factor, based on its sequence analysis, which may have a role in regulating the cell death process at the transcriptional level. Other genes with similar characteristics have been reported, including p53 (34), c-myc (16), or members of the glucocorticoid receptor family (35). Our studies demonstrate that *DIO-1* overexpression induces massive cell death, which can be blocked through overexpression of hBcl-2, known to inhibit caspase activity (36–39). This indicates that *DIO-1* is upstream of the caspase cascade and that the induction of apoptosis driven by this gene proceeds through the main apoptotic route described so far. The Z-VAD-fmk blockade of *DIO-1*-induced apoptosis supports these conclusions.

The apoptotic mechanism activated by *DIO-1* requires its translocation to the nucleus; this finding, as well as its sequence and differential localization in living and apoptotic cells, can be used to draw inferences on its mode of function. *DIO-1* may thus be associated in the cytoplasm to a protein that prevents its entry into the nucleus, to which it must presumably be translocated to activate downstream mechanisms that initiate a caspase-executed apoptotic pathway. Such a mechanism is used by the nuclear transcription factor NF-κB, which is maintained as a complex with IκB in the cytoplasm until a given stimulus activates a caspase, leading to IκB phosphorylation, ubiquitination, and degradation, releasing the NF-κB proteins to traverse to the nucleus and activate gene transcription. A similar mechanism has been proposed for control of caspase-activated DNase and its translocation to the nucleus (7, 8).

To understand the *in vivo* role of this gene, we used the developing limb as a model. This system, which has been thoroughly characterized by developmental biologists, uses interference with limb outgrowth through modification of the gene expression pattern to analyze genes implicated in cell death. Here we have demonstrated that *DIO-1* affects chicken limb formation by general disruption of growth.

Limb buds infected with *DIO-1* constructs were reduced in size and failed to develop a defined AER. Severely truncated limbs with deformed and/or absent zeugopodal elements (most commonly the radius) and missing digits were also observed. After budding, continued limb outgrowth depends on correct AER formation. The reduced limb buds observed after *DIO-1* expression closely resemble the limb buds obtained after AER removal. All together, our experiments suggest that *DIO-1* overexpression perturbs maintenance of

AER function and hence limb outgrowth. Interestingly, the phenotypic changes caused by *DIO-1* misexpression kinetics, which result in altered limb development, appear to require the presence of the caspase activity required for elimination of interdigiting webs. *Fgf-8*, an AER-restricted gene involved in initiation and maintenance of limb outgrowth, is down-regulated or absent in infected limb buds, as are transcripts for *Msx-1* and *Lhx-2* genes involved in limb outgrowth whose expression is regulated by NF-κB. It thus appears that allowing DIO-1 protein translocation to the nucleus, down-regulation is observed for both mesodermal- and ectodermal-specific gene expression. It is unclear, however, whether this down-regulation occurs primarily in the mesoderm and indirectly in the AER or, alternatively, whether down-regulation of gene expression occurs initially in the AER and subsequently in the mesoderm. Identification of the mechanisms of action of *DIO-1* may be instrumental in answering this question and may provide another missing link in the identification of the mechanism that controls cell proliferation and cell death. In contrast to the consequences of *DIO-1* overexpression, ectopic NF-κB expression does not lead to significant morphological perturbations (data not shown). In sum, it appears that some proteins, such as NF-κB, control the cell proliferation and outgrowth of the vertebrate limb, whereas others, such as *DIO-1*, promote cell death. The integral process of limb outgrowth would thus require a balanced interaction between both forces.

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