DEVELOPMENTAL BIOLOGY **203,** 36–48 (1998)
ARTICLE NO. DB989028

Programmed Cell Death during *Xenopus* Development: A Spatio-temporal Analysis

Carmel Hensey and Jean Gautier[1]

Department of Genetics and Development and Department of Dermatology, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, New York 10032

Programmed cell death (PCD) is an integral part of many developmental processes. In vertebrates little is yet known on the patterns of PCD and its role during the early phases of development, when embryonic tissue layers migrate and pattern formation takes place. We describe the spatio-temporal patterns of cell death during early *Xenopus* development, from fertilization to the tadpole stage (stage 35/36). Cell death was analyzed by a whole-mount *in situ* DNA end-labeling technique (the TUNEL protocol), as well as by serial sections of paraffin-embedded TUNEL-stained embryos. The first cell death was detected during gastrulation, and as development progressed followed highly dynamic and reproducible patterns, strongly suggesting it is an important component of development at these stages. The detection of PCD during neural induction, neural plate patterning, and later during the development of the nervous system highlights the role of PCD throughout neurogenesis. Additionally, high levels of cell death were detected in the developing tail and sensory organs. This is the first detailed description of PCD throughout early development of a vertebrate, and provides the basis for further studies on its role in the patterning and morphogenesis of the embryo.

***Key Words:* *Xenopus* development; programmed cell death; neurulation; nervous system development; whole-mount TUNEL staining.**

INTRODUCTION

Programmed cell death refers to the naturally occurring cell death that is part of the developmental program of an organism (Jacobson *et al.*, 1997). This loss of cells can be fundamental to some developmental processes and serves many functions, such as sculpting or deleting structures, controlling cell numbers, and eliminating abnormal, misplaced, or nonfunctional cells (Ellis *et al.*, 1991; Sanders and Wride, 1995).

The first clear demonstration of cell death being a highly regulated component of development came from lineage studies in the nematode *Caenorhabditis elegans* in which 131 of 1090 cells are fated to die, and these programmed cell deaths occur reproducibly at specific times and positions throughout the cell lineage (Hengartner and Horvitz, 1994). Cell death primarily affects cells in the neuronal lineages; however, some mesodermal cells also undergo programmed cell death (PCD). PCD has also been described during early development in *Drosophila,* where it is widespread but most prominent in the central nervous system (Steller *et al.*, 1994).

In vertebrates, the information is more sparse especially during early development, although cell death has long been recognized to occur throughout embryonic development (Glucksmann, 1951). Cell death has been reported as early as the blastocyst stage, and during gastrulation. Cell death in the core of the mouse blastocyst is believed to be important for cavitation (Coucouvanis and Martin, 1995), and has been detected in the gastrulating chick and mouse embryo (Sanders *et al.*, 1997), as well as in urodele amphibians (Imoh, 1986). In the gastrulating chick embryo, cell death was detected in all cell layers with high numbers of cell deaths occurring in the epiblast of the rostral germinal crescent, while, in the gastrulating mouse and newt embryo, cell death was randomly distributed throughout the embryos. The role of cell death during these early stages of development is not known (Sanders and Wride, 1995).

The majority of studies of PCD in vertebrate development have focused on relatively late stages of morphogenesis, such as limb bud, and nervous system development

[1] To whom correspondence should be addressed. Fax: (212) 305-7391. E-mail: jg130@columbia.edu.

0012-1606/98 $25.00

(Burek and Oppenheim, 1996; Hinchliffe, 1981). This cell death, which occurs in a stereotypic manner at specific times in development, is usually referred to as "programmed cell death," although the genetic basis of this program is not known, unlike the case of invertebrates. The role of this programmed cell death in the formation of digits in the vertebrate limb and in the morphogenic reorganization of organs such as heart and kidney has been clearly demonstrated (Coles *et al.*, 1993; Pexieder, 1975).

Neuronal death plays a major part in the development of the nervous system and is thought to be regulated by a number of different mechanisms, although studies have primarily concentrated on its function in the control of cell number caused by a limited supply of neurotrophic factors: "trophic cell death" (Burek and Oppenheim, 1996; Oppenheim, 1991). Neurons lacking sufficient or correct neurotrophic support undergo programmed cell death, thus eliminating neurons that have not made any connections or have made incorrect ones. The cumulative result of this trophic cell death is that anywhere from 20 to 80% of different neuronal populations are lost as part of this developmental process. Thus PCD has an important role in the establishment of functional patterns of synaptic organization and axonal pathways.

PCD is also detected at earlier stages of neurogenesis when trophic support is not yet a survival determining factor, and cell death is regulated by other, as yet poorly understood, mechanisms. In chicken, PCD is detected during the folding and closure of the neural tube. It has also been shown to have a clear role in sculpting cephalic neural crest cells later in development. Studies in chick have shown that regions from where neural crest cells migrate are separated by stripes where all presumptive neural crest cells undergo apoptosis (Graham *et al.*, 1993; Jeffs *et al.*, 1992). It has been proposed that some cell deaths in the developing brain may occur through phenotypic selection based on the intrinsic properties of cells, in a manner comparable to the killing of T cells by phenotypic specific selection (Blaschke *et al.*, 1996; Voyvodic, 1996).

During metamorphosis in insects and amphibians, high levels of PCD are detected in organs that are remodeled. Studies of cell death in *Xenopus* have focused on cell death during metamorphosis (Tata, 1996), where an extensive program of cell death is initiated resulting in 100% of cells dying in some larval tissues, such as tail and gills. More recently an apoptotic program was identified in *Xenopus* (Anderson *et al.*, 1997; Hensey and Gautier, 1997; Sible *et al.*, 1997; Stack and Newport, 1997). This cell death program is abruptly activated at the onset of gastrulation following DNA damage, or treatment of embryos with inhibitors of transcription, translation, or DNA replication.

Many of these cell deaths occur by apoptosis, a morphological characterization of a particular type of cell death that is distinguished by membrane blebbing, nuclear and cytoplasmic shrinkage, chromatin condensation, and DNA fragmentation (Kerr *et al.*, 1972). The characteristic DNA fragmentation can be exploited as a means of detecting dying cells by labeling of the free DNA ends. This has led to the development of the TUNEL (TdT-mediated dUTP digoxigenin nick end labeling) technique allowing for the *in situ* labeling of cells destined to die by apoptosis (Gavrieli *et al.*, 1992). TUNEL staining is widely accepted as the most specific and sensitive technique for *in situ* detection of apoptosis in cells, whole embryos, or tissue sections.

We have detected substantial levels of cell death during the early stages of *Xenopus* embryonic development. Here we present a detailed spatio-temporal analysis of the naturally occurring cell deaths during early development in *Xenopus*, using TUNEL labeling on whole-mount *in situ* and embryo sections.

MATERIALS AND METHODS

Embryological methods. Albino *Xenopus laevis* were obtained from Xenopus I (Michigan). Embryos were fertilized and cultured by standard methods (Sive *et al.*, 1994) and staged according to Nieuwkoop and Faber (1967).

TUNEL staining. The whole-mount TUNEL staining protocol was previously described (Hensey and Gautier, 1997). Briefly, albino embryos were fixed in MEMFA (100 mM Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 4% formaldehyde), and stored in methanol at −20°C. Embryos were rehydrated in PBT (0.2% Tween 20 in PBS), washed in PBS, and incubated in 150 U/ml terminal deoxynucleotidyltransferase (Gibco BRL), and 0.5 μM digoxigenin–dUTP (Boehringer Mannheim). The reaction was terminated in PBS/1 mM EDTA, at 65°C, followed by washes in PBS. Detection and chromogenic reaction was carried out according to Harland (1991). The embryos were blocked in PBT + 20% goat serum, followed by incubation with anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim). Embryos were extensively washed in PBS and staining developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates. The reaction was visible within 30 min, and embryos were viewed following dehydration in methanol and mounting in benzyl benzoate/benzyl alcohol 2:1. Whole mounts were photographed on a Zeiss Axiophot using a standard 35-mm camera.

Embedding and sectioning. Cleared embryos were transferred to xylene and washed in xylene, 1 × 5 min, 1 × 30 min. This was followed by a 30-min incubation in xylene:paraplast (1:1) at 55°C. Embryos were then transferred to paraplast, and incubated for 3 × 30 min at 55°C. Embryos were positioned in molds, allowed to set overnight, and 10-μm sections were cut. Sections were dewaxed in Hemo-De (Fisher), mounted, and viewed using a Zeiss Axioplan microscope.

RESULTS

Whole-mount TUNEL was used to detect DNA fragmentation which is a characteristic of normal developmental cell death. In our previous studies of cell death in *Xenopus* embryos TUNEL staining correlated with all characteristic apoptotic features, such as DNA laddering, chromatin condensation, caspase activation, and ultrastructural alterations (Hensey and Gautier, 1997).

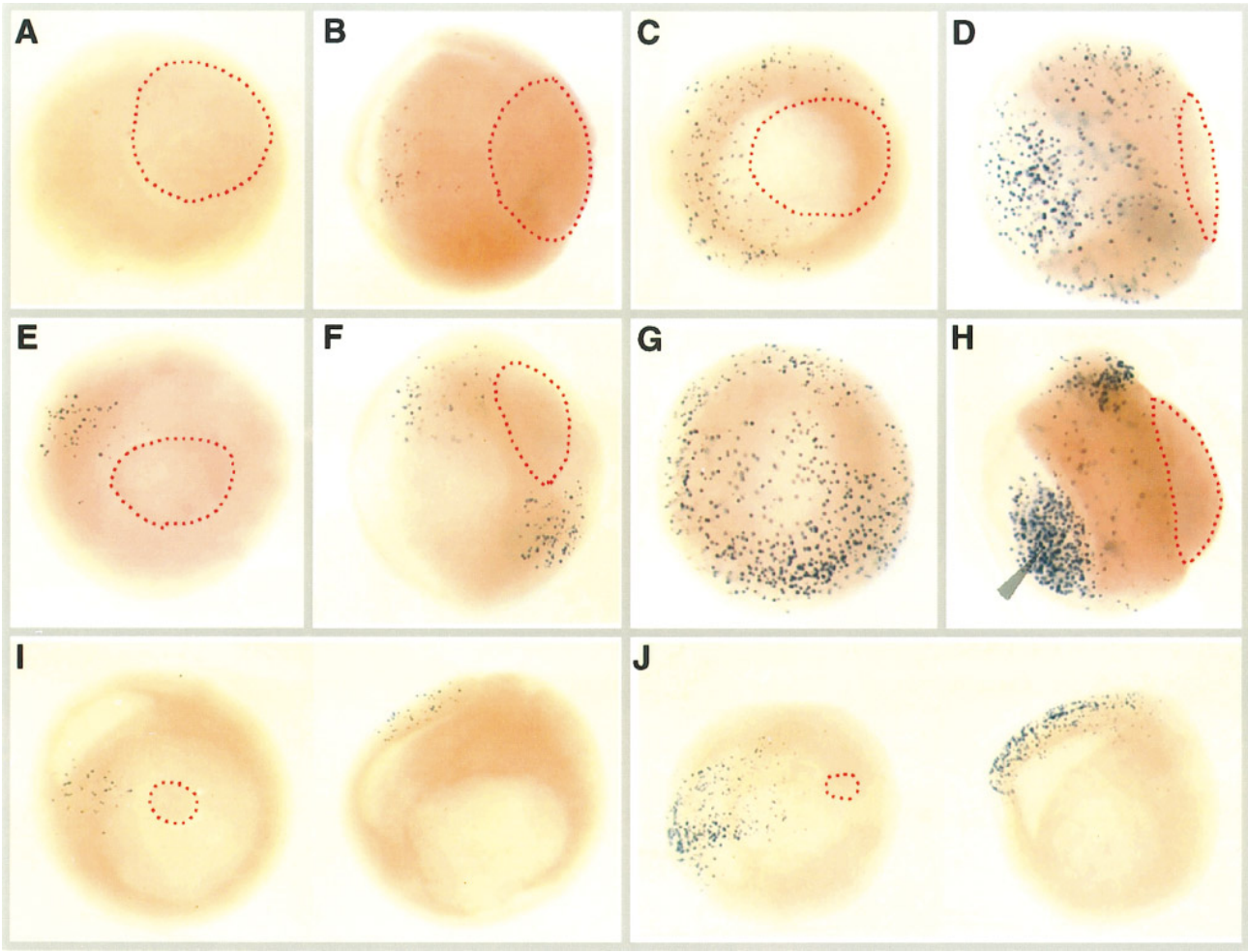

FIG. 1. Whole-mount TUNEL staining reveals dying cells in gastrulation-stage embryos. (A) Stage 10.5, vegetal view, no TUNEL staining was detected in this embryo. (B) Stage 10.5, lateral view, a small patch of TUNEL staining is evident in the equatorial region of the embryo. (C) Stage 10.5, vegetal view, hundreds of TUNEL-positive nuclei were evident in the circumblastoporal region. (D) Stage 10.5, lateral view, TUNEL-stained nuclei covered the surface of the embryo except for the blastopore. (E) Stage 11.5, vegetal view, a patch of TUNEL staining in the equatorial region extends to the edge of the blastopore. (F) Stage 11.5, lateral view, two bilaterally symmetrical patches of TUNEL staining border the blastopore. (G) Stage 11.5, animal view, TUNEL staining was detected in the animal pole and extended vegetally to the edge of the blastopore. (H) Stage 11.5, lateral view, a partially exogastrulating embryo showing an accumulation of TUNEL-positive nuclei in the blastopore (arrowhead). (I) Stage 12.5, animal view (left), lateral view (right). A dorsal median stripe of TUNEL-stained nuclei extended from the blastopore. (J) Stage 12.5, animal view (left), lateral view (right). TUNEL-stained nuclei were detected across the dorsal side of the embryo. Throughout gastrulation TUNEL staining was never detected in the blastopore, indicated by the dotted red line. The embryos shown are representative of the TUNEL staining observed following analysis of 237 embryos of which 67% were stained. Embryos with more than 5 TUNEL-stained nuclei were considered TUNEL positive.

Gastrulation

The earliest TUNEL positive embryos were detected at stage 10.5, the beginning of gastrulation. Apoptotic nuclei were never detected at earlier stages, confirming our previous observations (Hensey and Gautier, 1997). There was considerable variation in the degree of TUNEL staining and patterns observed throughout gastrulation, and a representative sample of the cell death that was detected is shown (Figs. 1A–1J).

Stages 10.5–11.5. A similar range of staining patterns was observed at stages 10.5 and 11.5, with the amount of cell death in a single embryo varying from none at all (Fig. 1A) to cases in which hundreds of TUNEL-positive nuclei were observed (Figs. 1C, 1D, 1G, and 1H). In some cases isolated patches of TUNEL staining appeared randomly

around the equatorial region of the embryo (Fig. 1B). Much of the staining detected was localized to the circumblastoporal region (Fig. 1C) or localized to patches which bordered the blastopore (Fig. 1E), occasionally staining opposite sides of the blastopore in a bilaterally symmetrical manner (Fig. 1F). In the most intensely stained embryos, staining extended across the animal pole and vegetally to the edge of the blastopore (Figs. 1D and 1G). Some TUNEL-positive cells were occasionally observed accumulating in the blastocoel of these more strongly stained embryos (Fig. 1D). Sectioning of stage 11.5 TUNEL-stained embryos revealed that the staining was confined to the outer cell layers of the embryo, namely the ectoderm and underlying mesoderm (data not shown).

In rarer cases, there was a large accumulation of TUNEL-positive cells in the blastocoel (Fig. 1H, arrowhead). This embryo appears to be partially exogastrulating and could be developmentally defective; whether this is the reason for the intense staining in the blastocoel, which was not observed in other embryos, is not clear.

Stages 12–12.5. Late in gastrulation a more distinct staining pattern became apparent. TUNEL-positive cells often formed a dorsal median stripe of varying width in the middle of the future neural plate (Figs. 1I and 1J). In some embryos two stripes extended laterally from the blastopore on the dorsal side and may correspond to the lateral margins of the prospective neural plate (data not shown). Lateral staining of the presumptive mesoderm was occasionally observed (data not shown). Sections of these embryos showed dying cells were mainly located in the presumptive neuroectoderm, as well as in the region of the mesoderm underlying the neuroectoderm (data not shown).

In summary, the amount and distribution of dying cells varied considerably early in gastrulation, while bands of staining across the dorsal region of the embryo, i.e., the future neural plate, were the characteristic feature late in gastrulation. During this period of development staining was never detected in the blastopore (Fig. 1). Overall, cell death was detected in 67% of embryos at this time, stages 10.5–12.5.

Neural Plate Stage

From this stage on the cell deaths we detected occurred reproducibly at specific times and places in the developing embryo, and we believe these distinct patterns of cell death represent PCD. During stage 13 cell death was primarily detected in the dorsal region with varying but distinct patterns seen in the area of the neural plate. Staining was observed in broad bands that extended from the blastopore slit toward the anterior of the embryo medially (Fig. 2C), paramedially (Fig. 2A), or laterally (Fig. 2B). In some cases, the stripes broadened in the anterior direction and seemed to delineate the neural plate (Fig. 2A), with only a narrow dorsal medial stripe showing no staining. In other embryos, a dorsal median stripe extended from the blastopore slit along the entire length of the dorsal side of the embryo (Fig. 2C). Sections of stained embryos showed that PCD was mostly localized to the presumptive neuroectoderm (Fig. 7A), and in some cases staining was also detected in the mesoderm, in the region underlying the neuroectoderm (data not shown). In a few cases, staining seemed to be excluded from the neural plate and was detected entirely within the nonneural ectoderm. Two broad stripes extended from the blastopore slit, just lateral of the plate area for approximately two-thirds the length of the embryo (Fig. 2B). Similar staining patterns were seen in 67% of embryos at this stage, while no cell death was detected in remaining embryos (data not shown).

Through stages 14 and 15 the TUNEL staining became more localized anteriorly and appeared to be concentrated at the anterior edge of the neural plate. By stage 14 a distinct "horseshoe-shaped" pattern of staining was seen in the anterior of the embryo (Fig. 2F). This pattern persisted at stage 15 but was detected more anteroventrally as neurulation begins (Figs. 2E and 2G). Occasionally, a low level of PCD was also detected in the posterior region of stage 15 embryos (Fig. 2E). As was the case with other stages some embryos did not show staining at this time (Fig. 2D), although a similar anterior staining pattern was observed in all TUNEL-positive embryos (52%).

Cell death was concentrated to specific areas of the developing neural plate between stages 13 and 15. Such striking patterns of cell death during early neurogenesis have not been described previously.

Stages 16–17

Three representative examples of the patterns of PCD seen during the neural fold stage are shown (Fig. 3). Unique patterns were detected in the anterior and dorsal regions of embryos, corresponding to the developing nervous system. The varying intensities appear to represent a degree of overlap in staining patterns, with the most intensely stained embryo probably representing a peak in PCD (Fig. 3C), while those showing less staining are likely preceding or following stages (Figs. 3A and 3B). Apoptotic cells were found in defined stripes on each side of the dorsal midline, with the most intense staining corresponding to the primary sensory neurons (Figs. 3A and 3C dorsal view), while a light scattering of staining was detected throughout the neural fold (Fig. 3C). The staining fanned out anteriorly, and was most intense in the future brain region (Fig. 3C, anterior view) as well as in ventral–anterior patches corresponding to the future sensory placodes (Fig. 3B, anterior view). The end on anterior view shows distinct subdivisions of staining, which correspond to the olfactory (Fig. 3C, arrow), and optic placodes (Figs. 3B and 3C, arrowhead). A degree of left–right asymmetry is also evident in the staining (Figs. 3A and 3C), which in some cases was so striking that only one half of the embryo was stained (data not shown). This is the earliest stage at which asymmetries were evident in the cell death patterns, and such staining occurred randomly on either the right, or the left side of the

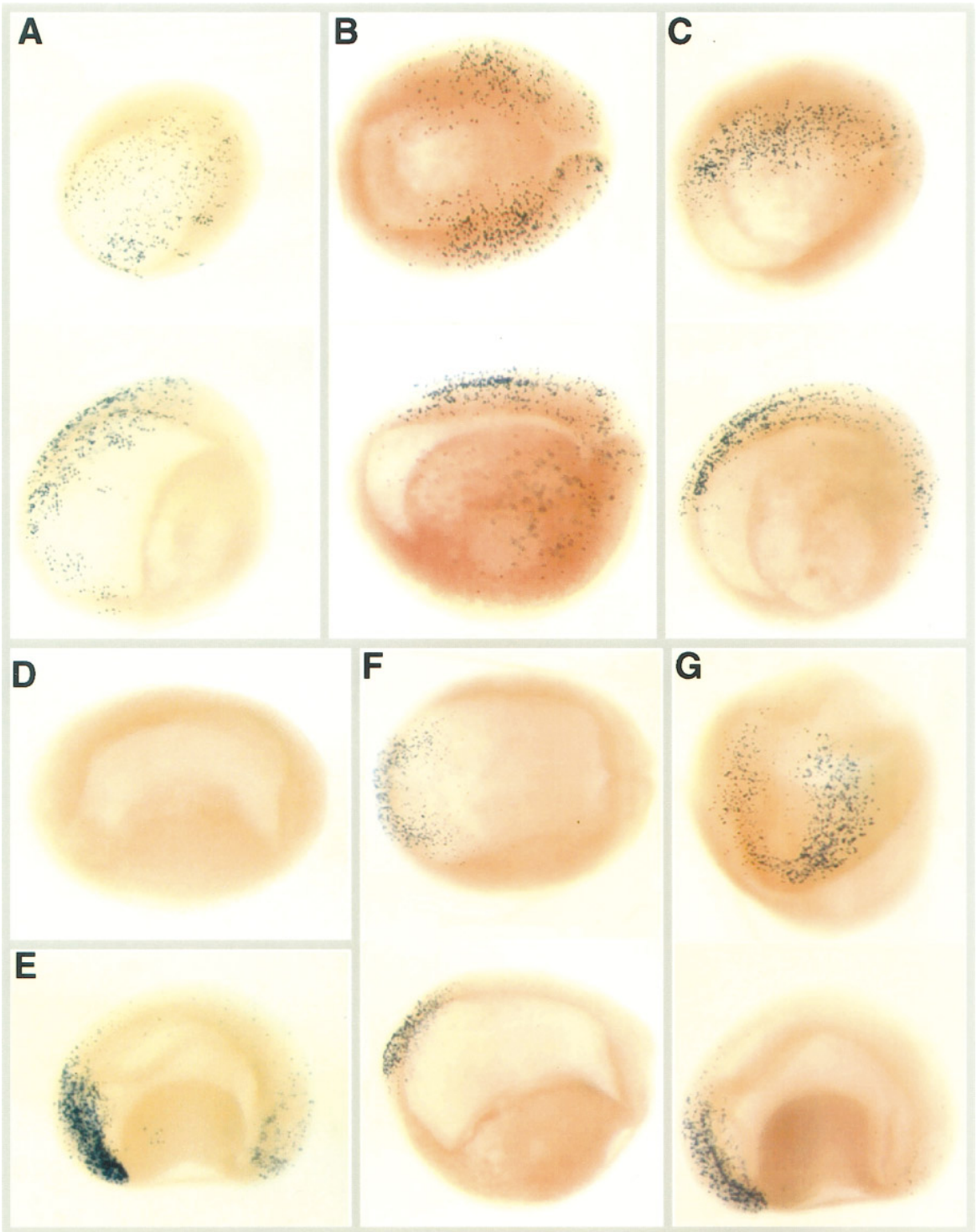

FIG. 2. Programmed cell death as detected by whole-mount TUNEL staining at the neural plate stage. (A) Stage 13, dorsal (top) and lateral (bottom) view. The dorsal side of the embryo is TUNEL stained with a band of staining broadening toward the anterior end of the embryo, delineating the neural plate. (B) Stage 13, dorsal (top) and lateral (bottom) view. Two broad bands of staining extend laterally from the blastopore slit. (C) Stage 13, dorsal (top) and lateral (bottom) view. A broad medial stripe of staining was detected on the dorsal side. (D) Stage 14, lateral view, anterior is left, no TUNEL staining was detected. (E) Stage 15, lateral view, anterior is left. Intense TUNEL staining was detected in the anteriormost portion of the embryo. Light staining was evident posteriorly. (F) Stage 14, dorsal (top) and lateral (bottom) view, anterior is left. A semicircular band of staining in the anteriormost portion of the embryo, corresponding to the anterolateral edge of the neural plate. (G) Stage 15, end on anterior view (top), lateral view (bottom), anterior is left. TUNEL staining formed a horseshoe-shaped band of staining in a more anteroventral position than stage 14. The embryos shown are representative of the staining observed following staining of 58 stage 13 embryos of which 67% were TUNEL positive, and 37 stage 14/15 embryos of which 52% were TUNEL positive. Embryos with more than 5 TUNEL-stained nuclei were considered TUNEL positive.

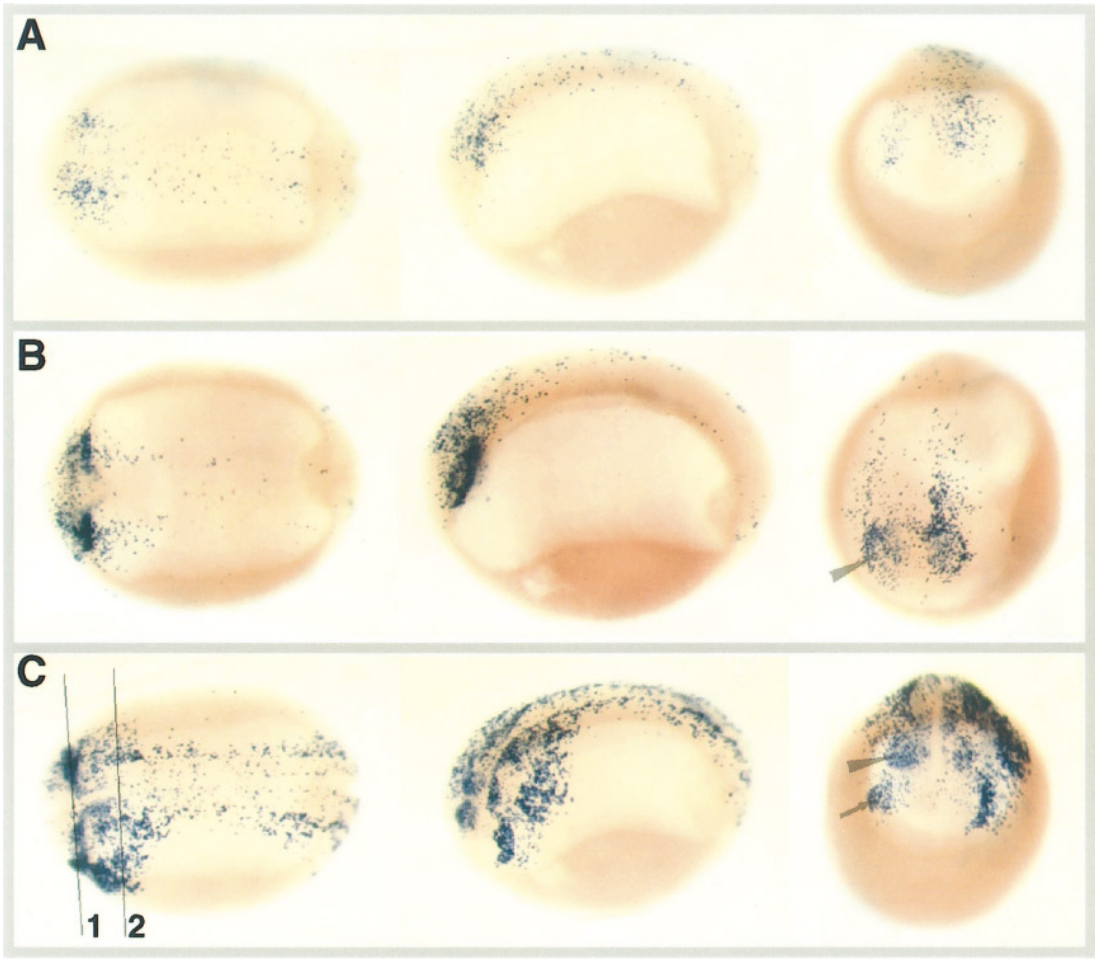

FIG. 3. Programmed cell death as detected by whole-mount TUNEL staining at the neural fold stage. A, B, and C, stage 17. Dorsal, lateral, and end-on anterior views are shown for each embryo (left to right). (A) TUNEL staining was detected in the anteriormost portion of the embryo with a light scattering of staining along the length of the neural fold. (B) Strong TUNEL staining in the anteriormost portion of the embryo, corresponding to the optic placodes (arrowhead). Staining extends from these placodes along the neural fold. (C) Strongest staining was in the anterior embryo with patches of TUNEL staining corresponding to the optic (arrowhead), and olfactory (arrow) placodes, and developing brain. Dying cells were found in defined stripes on each side of the dorsal midline corresponding to the primary sensory neurons. A scattering of dying cells was detected throughout the neural fold. Lines 1 and 2 mark the approximate location of transverse sections shown in Figs. 7B and 7C, respectively. The cell death patterns are representative of the staining observed following staining of 33 embryos of which 64% were TUNEL positive. Embryos with more than 5 TUNEL-stained nuclei were considered TUNEL positive.

embryo. An anterior section of the most intensely stained embryo (Figs. 3C, 1), showed PCD concentrated in two circular patches which appeared to be linked by a thin line of dying cells (Fig. 7B). A section just posterior to this (Fig. 3C, 2), showed staining in the region of the neural crest (Fig. 7C). In more posterior sections staining was scattered along the neural fold (data not shown).

During this stage cell death continued to be localized to the developing nervous system, particularly the developing brain and sensory placodes, in addition to being detected in the population of primary sensory neurons in the developing neural tube. TUNEL staining was detected in 64% of embryos during this developmental stage.

Stages 18–25

At the late neural stage (stages 18–20) the degree of TUNEL staining was greatly reduced compared to previous stages (compare Figs. 3 and 4A, 4B, and 4C). Light staining was detected in the anterior, dorsal, and/or posterior of embryos at this time (Figs. 4A, 4B, and 4C). The strongest staining was restricted to small patches in the anteriormost portion of embryos (Figs. 4A and 4C), and sectioning revealed that this staining was in the neuroepithelium of the developing brain (data not shown). The posterior and dorsal staining was more diffuse with only a scattering of TUNEL positive cells

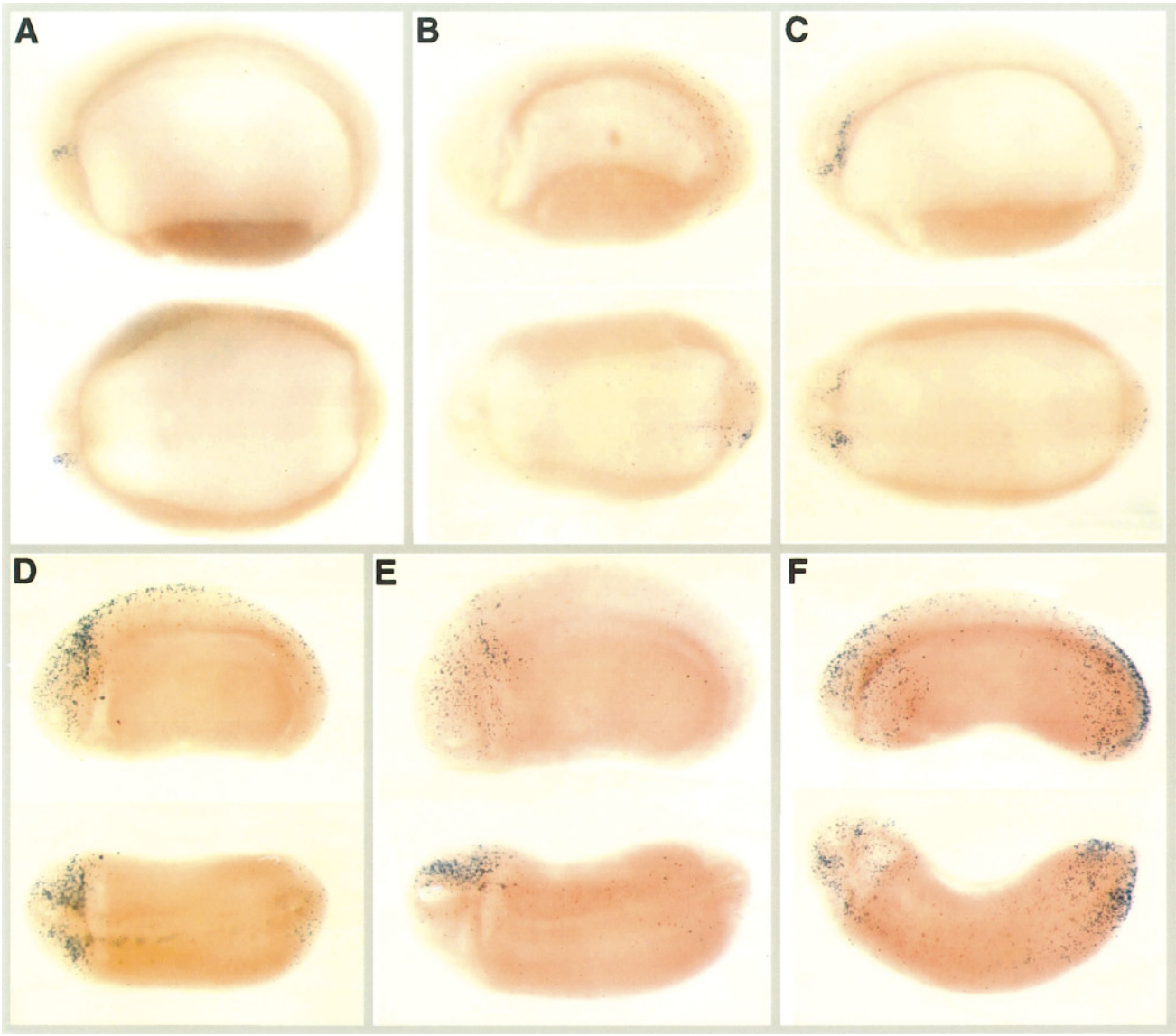

FIG. 4. Programmed cell death as detected by whole-mount TUNEL staining at the late neural stage. Each panel shows a lateral (top), and a dorsal (bottom) view. (A) Stage 18, TUNEL staining in the anteriormost portion of the developing brain. A clear asymmetry is present. (B) Stage 18, TUNEL staining in the posterior of the embryo, along the neural fold. (C) Stage 18, bilaterally symmetrical staining in the anterior developing brain, and faint staining in the posterior of the embryo. (D) Stage 23, bilaterally symmetrical diffuse staining in the developing head possibly corresponding to migrating neural crest cells. (E) Stage 23, diffuse staining in the developing head, showing a distinct right–left asymmetry. (F) Stage 25, diffuse TUNEL staining throughout the head and tailbud. The staining shown is representative of the programmed cell death seen following staining of 89 embryos of which 98% were TUNEL positive. Embryos with more than 5 TUNEL-stained nuclei were considered TUNEL positive.

being detected (Fig. 4B). Left–right asymmetries were also evident in these staining patterns (Fig. 4A).

Between stages 21 and 25 the majority of PCD was localized in the head and varying degrees and patterns of staining were detected (Figs. 4D, 4E, and 4F). The anterior pattern of staining was diffuse (Figs. 4D, 4E, and 4F). The earliest embryos of these series occasionally showed staining in the spinal cord (Fig. 4D). As embryos reach stage 25, dying cells were observed in the posterior quarter in the region where the tail will differentiate (Fig. 4F). Marked left–right asymmetry was observed in a fraction of the embryos at these stages (Fig. 4E). Sectioning revealed the anterior staining possibly corresponded to migrating neural crest cells (data not shown).

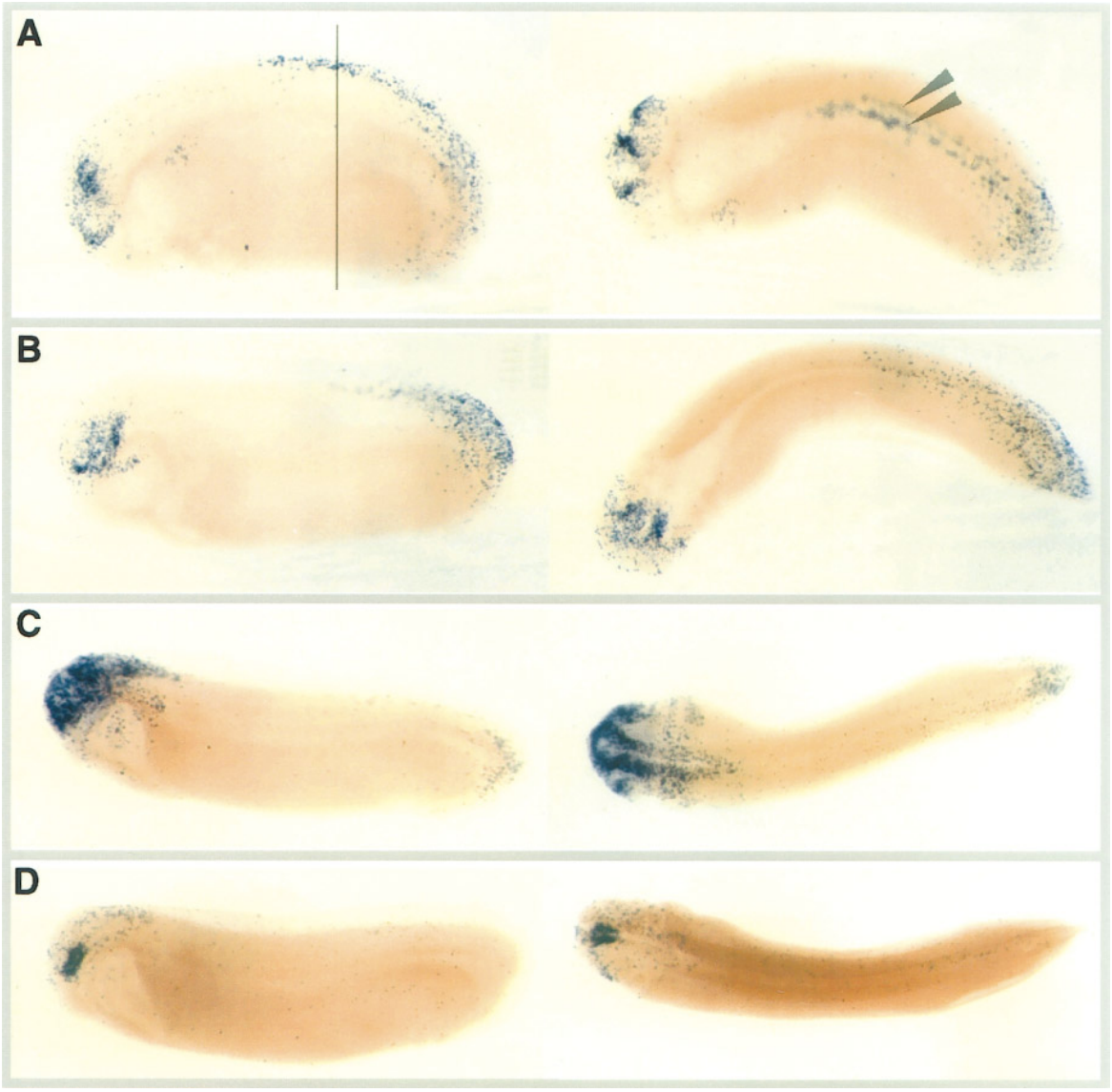

FIG. 5. Programmed cell death as detected by whole-mount TUNEL staining during stages 26–28. Each panel shows a lateral view (left), and a dorsal view (right). (A) Stage 26, the embryo looks younger in the lateral view due to curvature. TUNEL staining is evident in the ventral forebrain and midbrain in addition to the eye vesicle. Staining was also evident in the posterior half of the spinal cord (arrowheads), and in the tailbud. Line indicates the approximate location of the transverse section shown in figure 7D. (B) Stage 26, staining in the midbrain, posterior spinal cord and tailbud. (C) Stages 27–28, staining in the midbrain, hindbrain, and eye vesicles. (D) Stages 27–28, bilaterally symmetrical staining in the midbrain. The patterns of cell death shown are representative of the cell death detected following staining of 36 embryos, all of which were TUNEL positive. Embryos with more than 5 TUNEL-stained nuclei were considered TUNEL positive.

During this period, cell death is considerably reduced compared to earlier stages of neurulation, although this low level of TUNEL staining was detected in 98% of embryos. This may be due to the fact that neural induction has already taken place, and remodeling of the developing CNS has not yet begun.

Stages 26–28

During the tailbud stage programmed cell death was detected throughout the developing brain, in the eyes, spinal cord, and developing tail (Fig. 5), and 100% of embryos were stained at these stages. PCD was detected as

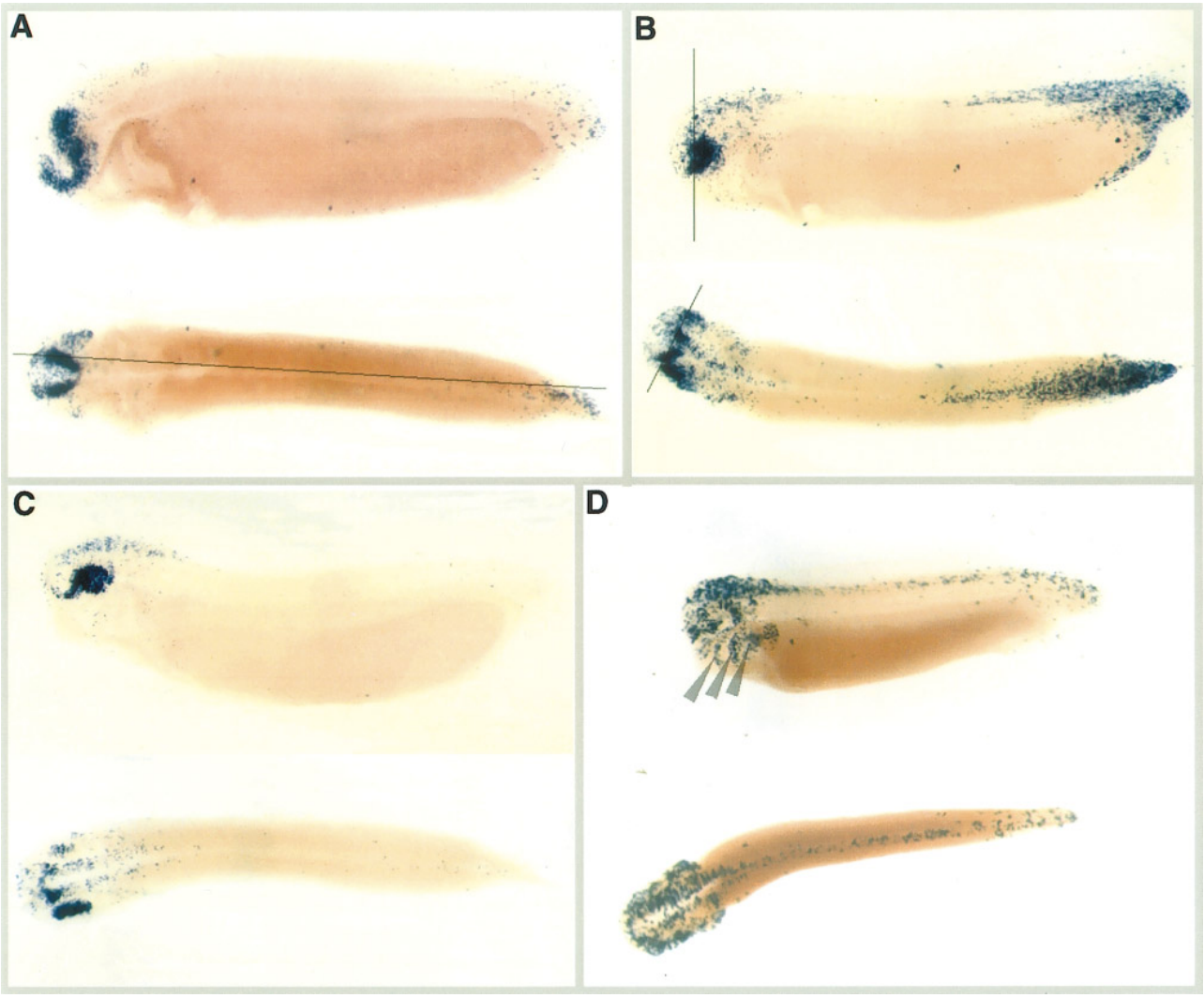

FIG. 6. PCD as detected by whole-mount TUNEL staining during stages 29–35. Each panel shows a lateral (top), and dorsal view (bottom). (A) Bilaterally symmetrical TUNEL staining is detected in the fore- and midbrain; lighter staining is detected in the eye vesicle. Line indicates the approximate location of a sagital section shown in Fig. 7F. (B) TUNEL staining is detected in the brain, eye vesicles, the posterior half of the spinal cord, and the elongating tail. (C) Dying cells were detected in the brain and developing eye. (D) TUNEL staining in the rhombencephalon, the outer ring of the eye vesicle the branchial arches (arrowheads), and the otic vesicle. Staining was also evident along the length of the spinal cord and in the tail tip. The staining is representative of the patterns seen following staining of 92 embryos of which 99% were TUNEL stained. Embryos with more than 5 TUNEL-stained nuclei were considered TUNEL positive.

two bilaterally symmetrical regions in the forebrain (Fig. 5A), and midbrain (Figs. 5A, 5B, and 5D). In other embryos staining extended from the midbrain to the hindbrain (Fig. 5C). Two bilaterally symmetrical stripes of dying cells were detected in some embryos (Fig. 5A, arrowheads). Sectioning revealed that this pattern was due to cell death in the spinal cord (Fig. 7D). The other major site of programmed cell death was the developing tailbud (Figs. 5A–5C). In a number of cases cell death was detected in the optic vesicle (Figs. 5A and 5C).

Stages 29–35

During these stages PCD continued to be concentrated in the central nervous system, and was also detected in the sensory organs, branchial arches, and elongating tail, with 99% of embryos showing TUNEL staining at this time. PCD was most frequently observed in the midbrain (Figs. 6A, 6B, and 6C), with the most extensive PCD being detected in the ventral forebrain and midbrain (Fig. 6A). A parasagital section of a similarly stained embryo is shown,

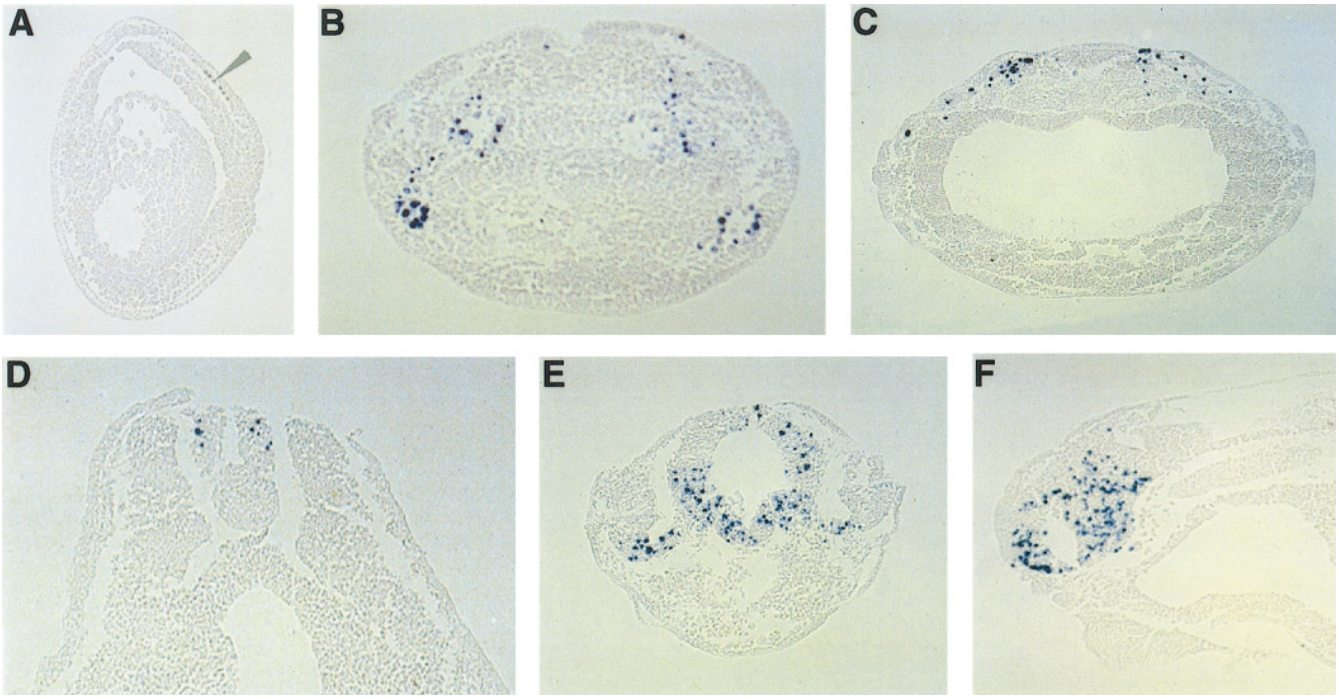

FIG. 7. Sections of TUNEL-stained embryos from stages 13 to 35. (A) Stage 13, arrowhead indicates TUNEL staining in the presumptive neuroectoderm. (B) Stage 17, transverse section (1) indicated in Fig. 3C, shows TUNEL staining in circular patches which correspond to the developing brain and sensory placodes. (C) Stage 17, a transverse section (2) indicated in Fig. 3C, shows TUNEL staining in the region of the neural crest. (D) Stage 26, a transverse section indicated in Fig. 5A, TUNEL staining was detected in both sides of the spinal cord giving rise to two bilaterally symmetrical dorsal stripes, Fig. 5A (arrowheads). (E) Stage 31/32, a transverse section through the head as indicated in Fig. 6B shows TUNEL staining in the brain and optic stalk. Most intense staining is evident in the ventral diencephlon. (F) Stage 29/30, a sagital section of the embryo indicated in Fig. 6A, shows TUNEL staining in the mesencephalon, diencephalon, and telencephalon regions of the brain.

with staining evident in the diencephalon, telencephalon, and mesencephalon (Fig. 7F).

In addition to cell death in the brain, the developing eye and tail also displayed high levels of cell death (Fig. 6B). Anterior transverse sections revealed PCD in the ventral diencephalon of this embryo (Fig. 7E). PCD was evident in all layers of the neuroepithelium and in the optic stalk. A scattering of TUNEL-positive cells was also detected in the dorsal brain (Fig. 7E; right side), although the level of cell death was much lower in this region. In more posterior sections, at the level of the mesencephalon, staining persisted in the ventral half of the brain and was also detected in the developing retina (data not shown). The optic vesicle frequently showed cell death (Figs. 6B, 6C, and 6D), as did the elongating tail (Figs. 6A, 6B, and 6D). The dorsal staining in the posterior half of the embryo is due to PCD in the spinal cord. This band of staining widened posteriorly and TUNEL-positive nuclei were detected throughout the elongating tail tip, in the spinal cord, notochord, and fin (data not shown).

In some cases strong staining was detected in the hindbrain and extended the length of the spinal cord to the tip of the tail (Fig. 6D). The otic vesicle was also TUNEL stained in this embryo. Cell death was also detected in the branchial arches of some embryos (Fig. 6D). Low levels of cell death were detected in some embryos with only a sprinkling of TUNEL-positive cells being detected in the brain and optic vesicle (data not shown).

High levels of cell death were detected in the developing fore-, mid-, and hindbrain between stages 26 and 35. This is the period in development when the brain acquires most of its form and structure (Nieuwkoop and Faber, 1967), and PCD is likely playing a role in the morphogenesis of the brain at this period. Additionally, cell death was frequently observed in the spinal cord, branchial arches, tail, and the developing sensory organs.

DISCUSSION

Spatial Characteristics of Cell Death in Developing Xenopus Embryos

We see differences in the spatial characteristics of dying cells depending on the stage of development and the tissues

analyzed, and dying cells appear to be randomly distributed in some cases or highly patterned in others.

Random cell death. During early and mid gastrulation the distribution of some dying cells appears random (Figs. 1A–1H). Dying cells have been observed during gastrulation in other organisms, where a similar randomness in their distribution was detected. In the gastrulating newt, *Cynops pyrrhogaster*, chicken, and mouse embryos cell deaths showed no defined pattern (Imoh, 1986; Sanders *et al.*, 1997).

Cell death in the region of the developing tail also appeared less defined than the patterns of death we observed in other tissues. In the early tailbud (Figs. 5A and 5B) we see a scattering of TUNEL-positive cells, while later in the elongating tail tip cell death is observed throughout the tissues of the tail region including the spinal chord, notochord, and fin (Fig. 6B).

Patterned cell death. The cell death that was detected in the presumptive and developing nervous system exhibited distinct, reproducible patterns which occurred in a stereotypic manner at specific times in the development. This therefore represents the normally occurring PCD that is an essential part of embryonic development.

During neural induction and early neurulation the spatiotemporal distribution of TUNEL-stained nuclei roughly coincides with the area of the neural plate and the varying patterns we detect within this region suggest that PCD may have an important role during the process of neural induction and patterning of the plate area (Figs. 1 and 2).

At the neural fold stage the distinct stripes of cells along the dorsal side of the embryo (Fig. 3) are coincident with stripes of primary neurons seen with the neural specific marker N-tubulin (Oschwald *et al.*, 1991). At this time cell death also occurs in the developing placodes (Figs. 3B and 3C). As development progressed cell death was localized to specific brain regions and the developing sensory organs (Figs. 5 and 6). We frequently observed cell death in the spinal cord (Figs. 5A, 6B, and 7D).

Most reproducible patterns of PCD were detected in the developing nervous system from the earliest stages of neural induction to remodeling of the CNS at later stages. Additionally, cell death was detected in the developing eye, otic vesicle, and the spinal cord.

Asymmetries. Cell death patterns were very dynamic and this is exemplified by the common occurrence of left–right asymmetries (Figs. 3A and 3C, anterior views, and Figs. 4E and 6C), a phenomenon which was first detected at the neural fold stage. Asymmetries have been reported during cell death in the *Drosophila* nervous system (Steller *et al.*, 1994).

Dynamics. It is striking that during gastrulation and neurulation no cell death was detected in a fraction of embryos. The rapid clearance of dead cells from the embryo may be one reason for such an effect. Cells that undergo cell death are usually degraded rapidly, and a clearance time of 1 h or less has been observed (Jacobson *et al.*, 1997). Thus embryos showing no staining may be passing through similar waves of cell death, but due to the rapid clearance of dead cells it was not detected. Analysis of clearance times in vertebrate embryos has proved difficult, and *Xenopus* may provide a useful system to estimate these clearance times using vital fluorescent dyes to detect dying cells *in vivo*.

During gastrulation the absence of cell death in some embryos could also be explained if the function of cell death is the elimination of damaged cells. However, during neurulation, where the patterns of cell death detected are highly reproducible, the lack of staining in some embryos cannot be explained as an absence of damaged cells. Other reasons for the presence of TUNEL-negative embryos might be a variability in the staging of embryos, or variations in the timing of cell death with respect to the age of the embryo.

Regulation of Cell Death during Development

There are probably different mechanisms regulating cell death depending on the stage in development when it occurs. We can only speculate as to the mechanisms involved based on what is already known about the causes of cell death during development in other systems, and the regulation of cell death pathways in general.

Cell death during gastrulation may be due to the detection and subsequent elimination of damaged cells. During the period of rapid cleavage which precedes gastrulation there are no cell cycle checkpoints which might allow for the generation of cells with incomplete sets of DNA, chromosome abnormalities, or chromosome breaks (Kimelman *et al.*, 1987). Gastrulation follows the midblastula transition (MBT), and the onset of zygotic transcription, at which point the cell cycle lengthens and checkpoints are activated (Frederick and Andrews, 1994; Graham and Morgan, 1966; Newport and Kirschner, 1982). Our previous studies have shown that *Xenopus* embryos do not activate a cell death pathway before stage 10.5, so it is conceivable that during gastrulation, at stages 10.5–11.5, any damaged cells that have accumulated since fertilization are removed. Circular patches of dying cells, such as those observed in Figs. 1B and 1E, could originate from a single damaged cell, and it is likely that some embryos would have more damaged cells that others, thus explaining some of the variations in amount of cell death we observed.

In addition, cell death at this time could also be the consequence of the mechanical stress that cells are subjected to at this stage. Both presumptive neuroectodermal and mesodermal cells are undergoing movements: extension, convergence, and involution that generate mechanical stress which might be detrimental to some cells. The fact that we never observe cell death in the blastopore, a region that does not undergo such extensive movements during gastrulation, supports this hypothesis. Mechanical stress-induced apoptosis has been reported (Cheng *et al.*, 1995).

Studies of cell death in the developing nervous system have focused on the role of neurotrophic factors in regulat-

ing neuronal survival. However, we observe cell death in the developing nervous system at stages in development prior to axonogenesis or the establishment of synaptic connections; therefore, this cell death cannot be regulated by target-derived signals. Regulation of neuronal survival at this stage is poorly understood and multiple mechanisms may be involved in the regulation of cell survival at this time. It has been suggested that cell death may reflect cell-specific phenotype recognition mechanisms (Voyvodic, 1996). Additionally, neuronal death might be integrated with cell cycle regulation and differentiation (Ross, 1996). During the later stages target-derived trophic support could be playing a role in regulating neuronal numbers.

We can only speculate as to the genes involved in regulating these cell death pathways. It is interesting to note that some of the growth factors implicated in these early neuronal patterning events, such as the bone morphogenetic protein 4 (BMP-4), have also been shown to play a role in PCD during development (Ganan *et al.*, 1996). Interestingly, there is an area of overlap between the area of cell death in the developing nervous system and the BMP-4 expression pattern in *Xenopus* (Hemmati-Brivanlou and Thomsen, 1995), reinforcing the idea that this gene may play a role in the execution of this morphogenetic cell death program.

Another gene whose expression pattern overlaps in a striking fashion with the cell death pattern observed at the neural fold stage is Pax-6 (Fig. 3B) (Hirsch and Harris, 1997). Pax-6 expression follows eye development, and is also expressed in cells fated to form part of the brain, suggesting a role for cell death in the developing eye placode and brain. Analysis of Pax-6 mutant mice revealed defects in PCD during development, suggesting a role for Pax-6 in the regulation of PCD (Grindley *et al.*, 1995).

Functions of Cell Death during Xenopus Development

Nothing is known about the function of cell death during the earliest stages of neural induction and neural plate formation. Cell death could be utilized to phenotypically select cells within a population that did not receive the proper signals, therefore strengthening boundaries between presumptive territories. During early vertebrate development the cells of the ectoderm choose between two possible fates, neural and epidermal (Hemmati-Brivanlou and Melton, 1997), and cell death could provide a means of early phenotypic selection to "sharpen" boundaries between neural and nonneural tissues. It is possible that ectodermal cells receiving intermediate levels of neuralizing factors could be considered "phenotypically ambiguous" and be eliminated.

The high levels and reproducible patterns of cell death that were detected during neural induction, neurogenesis, and CNS development strongly suggest that PCD may play an important role during early embryogenesis in *Xenopus.* Among the vertebrate model systems *Xenopus* will be very useful for studying the role of PCD in early development, particularly during early neurogenesis.

ACKNOWLEDGMENTS

We thank Dr. C Stern for comments on the manuscript. C.H. is a U.S. Army Postdoctoral Fellow (DAMD 17-97-2-7075). J.G. is an Irma T. Hirschl Scholar and is supported by a Career Development Award from the U.S. Army (DAMD 17-97-1-7071).

REFERENCES

Anderson, J. A., Lewellyn, A. L., and Maller, J. L. (1997). Ionizing radiation induces apoptosis and elevates cyclin A1-Cdk2 activity before but not after the midblastula transition in *Xenopus. Mol. Biol. Cell* **8,** 1195–1206.

Blaschke, A. J., Staley, K., and Chun, J. (1996). Widespread programmed cell death in proliferative and postmitotic Regions of the fetal cerebral cortex. *Development* **122,** 1165–1174.

Burek, M. J., and Oppenheim, R. W. (1996). Programmed cell death in the developing nervous system. *Brain Pathol.* **6,** 427–446.

Cheng, W., Baosheng, L., Kajstura, J., Li, P., Wolin, M. S., Sonnenblick, E. H., Hintze, T. H., Olivetti, G., and Anversa, P. (1995). Stretch-induced programmed cell death. *J. Clin. Invest.* **96,** 2247–2259.

Coles, H. S., Burne, J. F., and Raff, M. C. (1993). Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development* **118,** 777–784.

Coucouvanis, E., and Martin, G. R. (1995). Signals for death and survival: A two-step mechanism for cavitation in the vertebrate embryo. *Cell* **83,** 279–287.

Ellis, R. E., Yuan, J. Y., and Horvitz, H. R. (1991). Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* **7,** 663–698.

Frederick, D. L., and Andrews, M. T. (1994). Cell cycle remodeling requires cell–cell interactions in developing *Xenopus* embryos. *J Exp Zool* **270,** 410–416.

Ganan, Y., Macias, D., Duterque-Coquillaud, M., Ros, M. A., and Hurle, J. M. (1996). Role of TGF beta s and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. *Development* **122,** 2349–2357.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992). Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119,** 493–501.

Glucksmann, A. (1951). Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* **26,** 59–86.

Graham, A., Heyman, I., and Lumsden, A. (1993). Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. *Development* **119,** 233–245.

Graham, C. F., and Morgan, R. W. (1966). Changes in the cell cycle during early amphibian development. *Dev. Biol.* **14,** 439–460.

Grindley, J. C., Davidson, D. R., and Hill, R. E. (1995). The role of Pax-6 in eye and nasal development. *Development* **121,** 1433–1442.

Harland, R. M. (1991). *In situ* hybridization: An improved whole-mount method for *Xenopus* embryos. *In* "Methods in Cellular Biology" (B. K. Kay and H. B. Peng, Eds.), Vol. 36, pp. 685–695. Academic Press, San Diego.

Hemmati-Brivanlou, A., and Melton, D. (1997). Vertebrate neural induction. *Annu. Rev. Neurosci.* **20,** 43–60.

Hemmati-Brivanlou, A., and Thomsen, G. H. (1995). Ventral mesodermal patterning in Xenopus embryos: expression patterns and activities of BMP-2 and BMP-4. *Dev. Genet.* **17,** 78–89.

Hengartner, M. O., and Horvitz, H. R. (1994). Programmed cell death in *Caenorhabditis elegans. Curr. Opin. Genet Dev.* **4,** 581–586.

Hensey, C., and Gautier, J. (1997). A developmental timer that regulates apoptosis at the onset of gastrulation. *Mech. Dev.* **69,** 183–195.

Hinchliffe, J. R. (1981). Cell death in embryogenesis. *In* "Cell Death in Biology and Pathology" (I. D. Bowen and R. A. Lockshin, Eds.), pp. 35–78. Chapman and Hall, London.

Hirsch, N., and Harris, W. A. (1997). *Xenopus* Pax-6 and retinal development. *J. Neurobiol.* **32,** 45–61.

Imoh, H. (1986). Cell death during normal gastrulation in the newt, Cynops pyrrhogaster. *Cell Differ.* **19,** 35–42.

Jacobson, M. D., Weil, M., and Raff, M. C. (1997). Programmed cell death in animal development. *Cell* **88,** 347–354.

Jeffs, P., Jaques, K., and Osmond, M. (1992). Cell death in cranial neural crest development. *Anat. Embryol. (Berlin)* **185,** 583–588.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26,** 239–257.

Kimelman, D., Kirschner, M., and Scherson, T. (1987). The events of the midblastula transition in *Xenopus* are regulated by changes in the cell cycle. *Cell* **48,** 399–407.

Newport, J., and Kirschner, M. (1982). A major developmental transition in early *Xenopus* embryos: I. characterization and timing of cellular changes at the midblastula stage. *Cell* **30,** 675–686.

Nieuwkoop, P. D., and Faber, J. (1967). "Normal Table of *Xenopus laevis* (Daudin)." North Holland, Amsterdam.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14,** 453–501.

Oschwald, R., Richter, K., and Grunz, H. (1991). Localization of a nervous system-specific class II beta-tubulin gene in *Xenopus laevis* embryos by whole-mount in situ hybridization. *Int. J. Dev. Biol.* **35,** 399–405.

Pexieder, T. (1975). Cell death in the morphogenesis and teratogenesis of the heart. *Adv. Anat. Embryol. Cell Biol.* **51,** 3–99.

Ross, M. E. (1996). Cell division and the nervous system: regulating the cycle from neural differentiation to death. *Trends Neurosci.* **19,** 62–68.

Sanders, E. J., Torkkeli, P. H., and French, A. S. (1997). Patterns of cell death during gastrulation in chick and mouse embryos. *Anat. Embryol. (Berlin)* **195,** 147–154.

Sanders, E. J., and Wride, M. A. (1995). Programmed cell death in development. *Int. Rev. Cytol.* **163,** 105–173.

Sible, J. C., Anderson, J. A., Lewellyn, A. L., and Maller, J. L. (1997). Zygotic transcription is required to block a maternal program of apoptosis in Xenopus embryos. *Dev. Biol.* **189,** 335–346.

Sive, H. L., Grainger, R. M., and Harland, R. M. (1994). "Early Development of *Xenopus laevis.*" Cold Spring Harbor, NY.

Stack, J. H., and Newport, J. W. (1997). Developmentally regulated activation of apoptosis early in Xenopus gastrulation results in cyclin A degradation during interphase of the cell cycle. *Development* **124,** 3185–3195.

Steller, H., Abrams, J. M., Grether, M. E., and White, K. (1994). Programmed cell death in *Drosophila. Philos. Trans. R. Soc. London Ser. B Biol. Sci.* **345,** 247–250.

Tata, J. R. (1996). Metamorphosis: An exquisite model for hormonal regulation of post- embryonic development. *Biochem. Soc. Symp.* **62,** 123–136.

Voyvodic, J. T. (1996). Cell death in cortical development: How much? Why? So what? *Neuron* **16,** 693–696.

Received for publication May 11, 1998
Revised July 1, 1998
Accepted July 15, 1998