Ionizing Radiation Induces Apoptosis and Elevates Cyclin A1-Cdk2 Activity Before but not after the Midblastula Transition in *Xenopus*

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After the twelfth cell division in *Xenopus* embryos, zygotic gene transcription is activated, cells become motile, and cell division becomes asynchronous. This developmental change is termed the midblastula transition. High doses of γ -irradiation (γ -IR) before the midblastula transition induced apoptotic cell death and increased the levels of cyclin A1 and cyclin A1-Cdk2 activity. The addition of recombinant cyclin A1-Cdk2 induced the formation of apoptotic nuclei in *Xenopus* egg extracts, suggesting a role for cyclin A1-Cdk2 in apoptosis. Hallmarks of apoptosis, such as internucleosomal DNA fragmentation, pyknotic and uniformly condensed nuclei, and loss of intercellular attachments, were evident in embryos exposed to γ -IR before the midblastula transition. Apoptotic cells accumulated in the blastocoel, suggesting that before the midblastula transition *Xenopus* embryos use apoptosis to eliminate cells containing damaged DNA. However, embryos treated with the same dose of γ -IR after the midblastula transition developed normally and exhibited no signs of apoptosis, no change in cyclin A1 level, and no increase in cyclin A1-Cdk2 activity. These results indicate that there is a change in the response to DNA damage at the midblastula transition in *Xenopus* embryos.

INTRODUCTION

Cell cycle progression is regulated by checkpoints that ensure genomic integrity and completion of early cell cycle events before late events begin (Hartwell and Weinert, 1989). Upon DNA damage, most somatic cells arrest cell cycle progression at G₁ or G₂ to prevent the inheritance of incomplete or damaged genetic information, protecting cells and their progeny from excessive mutations. Most embryos exposed to DNA-damaging agents, however, fail to arrest cell division and some embryonic cell cycles lack DNA-based checkpoints (Hartwell and Weinert, 1989; Murray and Kirschner, 1989b). Failure of embryonic systems to activate a cellular DNA damage response could lead to developmental abnormalities and cancer. Therefore, it is likely that embryos have evolved an alternate system to deal with DNA damage.

Upon DNA damage in somatic cells, an increase in the tumor suppressor protein p53 induces either cell cycle arrest or cell death (apoptosis), thereby protecting the genome from accumulating excess mutations (for review, see Lane, 1992; Ko and Prives, 1996). To halt the cell cycle, increased p53 induces transcription of p21^{WAF1/CIP1}, which binds to and inhibits cyclin-dependent kinases (cdks) required for cell cycle progression (El-Deiry *et al.*, 1993, 1994; Harper *et al.*, 1993). Elevated p53 also functions to trigger deletion of damaged cells by apoptosis (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993). p53 is thought to activate the transcription of "death genes" such as *bax* or repress the transcription of "survival genes" such as *bcl-2* to induce apoptosis (Miyashita *et al.*, 1994; Selvakumaran *et al.*, 1994; Miyashita and Reed, 1995). Whether DNA damage induces cell cycle arrest or cell death appears to be cell-type dependent (Kastan *et al.*, 1992; Clarke *et al.*, 1993; Lowe *et al.*, 1993).

In the developing *Xenopus* embryo, the first 12 cell divisions after fertilization are rapid and synchronous,

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oscillating between DNA synthesis and mitosis with no discernible G phases in cycles 2–12. Before the 12th division, there is no detectable transcription, and thus the entire developmental program is maternally controlled. After the 12th division, the cell cycle lengthens, transcription of zygotic genes begins, and cells become motile and divide asynchronously. This change in cellular events at the 12th division is called the midblastula transition or the MBT (Newport and Kirschner, 1982a,b). The timing of cell divisions before the MBT is regulated by the synthesis of mitotic cyclins (Murray and Kirschner, 1989a; Hartley *et al.*, 1996). At the MBT, cyclin levels change as the zygotic developmental program is initiated. Cyclin E1 disappears followed by an increase in B cyclins and the disappearance of cyclin A1 (Hartley *et al.*, 1996). The disappearance of cyclin A1 coincides with an increase in cyclin A2 (Howe *et al.*, 1995).

It has been suggested that before the MBT, during the period of rapid synchronous divisions, embryos detect and eliminate damaged cells rather than sacrifice speed and synchrony to repair damage (Hartwell and Weinert, 1989). This has not been investigated directly but would explain the lack of controls on cell cycle progression in some early embryonic systems. No transcription occurs during early *Xenopus* development, obviating a transcriptionally controlled DNA damage response before the MBT. Apoptosis, a specific form of cell suicide, can be induced in the absence of transcription or translation (Searle *et al.*, 1975; Martin *et al.*, 1990; Bansal *et al.*, 1991; Collins *et al.*, 1991; Sible *et al.*, 1997) and is a possible mechanism by which early embryos could remove cells containing damaged DNA.

Apoptosis is a pathway of cell death required for normal development and differentiation (Raff, 1992; Hengartner and Horvitz, 1994). For example, apoptosis is active in the degeneration of the tadpole tail during frog metamorphosis (Kerr *et al.*, 1974) and in the elimination of interdigital webs during mammalian limb development (Kerr *et al.*, 1987). Apoptosis can also be induced by a variety of stimuli, including anticancer drugs, hormone withdrawal or addition, mild hyperthermia, cytotoxic T lymphocytes, and ionizing radiation (for review, see Kerr and Winterford, 1994). Despite a variety of inducers, there is a common sequence of intracellular events accompanying the apoptotic death process. In particular, apoptotic cells display a characteristic form of chromatin condensation into distinct dense masses (Wyllie *et al.*, 1980). Endogenous endonucleases that cleave genomic DNA between nucleosomes are activated, resulting in a characteristic "DNA ladder" when resolved by agarose gel electrophoresis (Arends *et al.*, 1990). Apoptotic cells also lose their intercellular attachments and nu-

clear lamins undergo proteolysis (Lazebnik *et al.*, 1993; Oberhammer *et al.*, 1994).

Some of the morphological and biochemical events associated with apoptosis suggest that apoptosis is intimately linked to the cell cycle (Ucker, 1991; Sen and D'Incalci, 1992; Lazebnik *et al.*, 1993; Rubin *et al.*, 1993). An increase in cyclin-cdk activity is associated with apoptosis in some cell types (Freeman *et al.*, 1994; Gazitt and Erdos, 1994; Meikrantz *et al.*, 1994; Shi *et al.*, 1994; Shimizu *et al.*, 1995; Kranenburg *et al.*, 1996; Meikrantz and Schlegel, 1996) but not in others (Norbury *et al.*, 1994; Oberhammer *et al.*, 1994; Ongkeko *et al.*, 1995). It has been suggested that apoptosis results when cyclin-cdk activities are misregulated, triggering incompatible signals for cell division and cell cycle arrest (Yonish-Rouach *et al.*, 1993). Cyclin-cdks acting in late G₁ or early S-phase have been implicated in the induction of apoptosis. More specifically, cyclin A-cdk activity is stimulated by many apoptosis-inducing agents (Buchou *et al.*, 1993; Jacobson *et al.*, 1993; Jansen-Durr *et al.*, 1993; Hoang *et al.*, 1994; Meikrantz *et al.*, 1994) and cyclin A-cdk activation is required for apoptosis induction in some systems (Shi *et al.*, 1994).

Herein, we have investigated the effects of ionizing radiation in early *Xenopus* development. Our results reveal that before but not after the MBT, *Xenopus* embryos use apoptosis to remove damaged cells, and the apoptotic signaling pathway involves aberrant activation of cyclin A1-Cdk2.

MATERIALS AND METHODS

Preparation of Embryos

Eggs were fertilized in vitro, dejellied in 2% cysteine (pH 7.8), and incubated at room temperature in 0.1× MMR (10 mM NaCl, 0.2 mM KCl, 0.1 mM MgSO₄, 0.2 mM CaCl₂, and 0.5 mM HEPES, pH 7.8). Embryos were staged according to Nieuwkoop and Faber (1975). Microinjections were performed at the one-cell stage, between 30 and 70 min after fertilization. After microinjection, embryos were maintained in 0.1× MMR containing 5% Ficoll. To inhibit transcription, α -amanitin was injected into one-cell embryos to an internal concentration of 50 μ g/ml. γ -Irradiation (γ -IR) was performed by exposing embryos in 0.1× MMR to 20 Gy (2000 rad) from a ⁶⁰Co source.

Embryo Fixation and Sectioning

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 12 to 24 h and were dehydrated by sequential incubation in increasing concentrations of ethanol (50, 70, 95, and 100%) and cleared in xylenes. Embryos were embedded in paraffin and incubated at 56°C overnight. Serial sections were cut 7- μ m thick, mounted on glass slides with 1% Mayer's albumin, and placed on a 35°C slide warmer overnight. Embryo sections were cleared in xylenes and rehydrated in a decreasing series of ethanol. DNA was stained with 1 μ g/ml 4,6-diamidino-2-phenylindole (DAPI), washed in PBS, and mounted with Fluoromount (Southern Biotechnology Associates, Birmingham, AL).

DNA Isolation

Embryos were homogenized in DNA extraction buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA, 50 μ g/ml RNase A, 0.5% SDS) and incubated

at 37°C for 1 h. Proteinase K was added to a final concentration of 100 µg/ml with continued incubation at 50°C for 2 h. Sequential phenol and phenol:chloroform extractions were performed, and the DNA was ethanol precipitated. Total genomic DNA was electrophoresed on a 1% agarose gel and stained with ethidium bromide.

Antibodies and Immunoblotting

Embryos were collected at the indicated stages or times and frozen on dry ice. For analysis, embryos were homogenized in 10 volumes of extract buffer or EB [80 mM β-glycerophosphate, 20 mM HEPES, pH 7.5, 15 mM MgCl₂, 20 mM EGTA, 1 mM dithiothreitol, 1 mM Pefabloc (Boehringer Mannheim, Indianapolis, IN), 3 µg/ml leupeptin, 50 mM NaF, 1 mM sodium vanadate, and 1 µM microcystin] and centrifuged for 10 min to remove insoluble proteins. The supernatants were used for immunoblotting and immunoprecipitation.

Antisera raised against full-length *Xenopus* cyclins B1, B2 (sheep), and E1 (goat) were affinity purified as described (Rempel *et al.*, 1995). Affinity-purified antibodies to cyclin A1 (sheep) and Cdc2 and Cdk2 (both rabbit) were generated as described previously (Gautier *et al.*, 1990; Hartley *et al.*, 1996).

For immunoblots, embryo extracts were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose. Blots were blocked in 10% nonfat dry milk in PBS and incubated with primary antibody. Blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA) diluted 1:5000 in 10% nonfat dry milk and visualized by ECL (Amersham, Arlington Heights, IL).

Immunoprecipitations

Immunoprecipitation of cyclin-associated kinase activity was performed essentially as described previously (Hartley *et al.*, 1996). Embryos were homogenized in EB (1 embryo per 10 µl of EB) and centrifuged, and the supernatant was diluted 10-fold and precleared with 50% protein G-Sepharose beads in EB. Ten microliters were removed for Western blotting and the remaining sample was divided into equal aliquots and incubated with affinity-purified cyclin A1 antiserum or crude cyclin A2, B1, B2, E1, or Cdc2 antiserum for 1 h on ice. After incubation for 1 h at 4°C with 50% protein G-Sepharose beads, immunocomplexes were precipitated and washed as previously described (Hartley *et al.*, 1996). To measure cyclin-associated kinase activity, samples were incubated for 20 min at 25°C with kinase assay buffer (0.5 mg/ml histone H1, 20 mM HEPES, pH 7.5, 15 mM MgCl₂, 5 mM EGTA, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, and 200 µM [γ -³²P]ATP, (5 CPM/fmol). Reactions were stopped by the addition of 5× sample buffer. Samples were subjected to SDS-PAGE, the gels were stained and dried, and specific phosphorylation of histone H1 was measured by scintillation counting of the H1 band.

[³H]Uridine Incorporation Assay

Embryos were incubated in 0.8 mCi/ml [5,6-³H]uridine in Danilchik's medium (Peng, 1991) 30 min after fertilization. Embryos were removed from the medium at stage 7 (about 4 h after fertilization), washed, and placed in 0.1× MMR. Embryos were exposed to a ⁶⁰Co source at the indicated stages, collected, and frozen on dry ice. Total RNA was isolated using the Tri-Reagent protocol (Molecular Research Center, Cincinnati, OH). The incorporation of [³H]uridine into RNA was measured by scintillation counting of trichloroacetic acid-precipitable material.

Egg Extracts

Metaphase II-arrested cytotstatic factor (CSF) extracts were prepared from *Xenopus* eggs as described (Murray, 1991). Extracts were supplemented at time 0 with 400 µM CaCl₂ and demembrated sperm nuclei (800–1000 nuclei/µl). Recombinant proteins or buffer were

added to extracts 60 min after CaCl₂ addition. Two-microliter aliquots were removed at the indicated times and added to an equal volume of fixative containing 3.6% formaldehyde, 48% glycerol, and 1 µg/ml DAPI in 1× MMR.

Production and Purification of Recombinant Cyclin A1-Cdk2

Xenopus Cdk2 was expressed in baculovirus-infected Sf9 cells as a fusion protein with glutathione S-transferase (GST). The *Xenopus* cdk2 gene was cloned into the baculovirus expression vector pVL1392 (Invitrogen, San Diego, CA), modified to encode a GST tag (our unpublished results). The dominant-negative (DN) form of Cdk2 (Van den Heuvel and Harlow, 1993) was generated by polymerase chain reaction-based site-directed mutagenesis to change Asp-145 to Asn (our unpublished results). Sf9 cells were coinfectd with baculovirus expressing *Xenopus* cyclin A1 and wild-type (WT) Cdk2 or the DN Cdk2 mutant.

WT and DN cyclin A1-Cdk2 complexes were purified essentially as described (Izumi and Maller, 1993) with the following modifications. The Sf9 cell pellet (approximately 2 g) was resuspended in 3 ml of buffer B (50 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM Pefabloc, 0.01% Brij 35, 1 µM microcystin LR, 0.5 µg/ml adenosine 5'-[γ-thio]triphosphate, 1% Nonidet P-40, 10 µg/ml chymostatin, 10 µg/ml pepstatin, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). The cell suspension was incubated at 4°C for 15 min and sonicated (ten 10-s bursts), and the sonicate was centrifuged at 20,000 × g at 4°C for 10 min. The supernatant was incubated with 1.5 ml of glutathione-agarose beads at 4°C for 2 h with agitation. The beads were washed twice with buffer B containing 0.5 M NaCl, twice with buffer B, twice with EB, and once with EB lacking protease inhibitors. The GST-Cdk2/cyclin A1 complex was eluted at 4°C for 30 min in EB (without protease inhibitors) containing 50 mM glutathione. The activities of the WT and DN kinase complexes were measured as previously described with histone H1 as a substrate (Rempel *et al.*, 1995).

RESULTS

Ionizing Radiation Before the Midblastula Transition Induces Apoptosis

DNA damage leads to cell growth arrest or cell death to protect cells and their progeny from accumulating excessive mutations. To study the effects of ionizing radiation-induced DNA damage during early development, *Xenopus* embryos were exposed to γ-IR at various stages. *Xenopus* embryos treated with high doses (20 Gy, 2000 rad) of γ-IR prior to the MBT at stage 8 failed to undergo gastrulation by 6 h, and no neural plates were visible 8 h after treatment (Figure 1). The embryos appeared as unorganized "balls" of cells 6–8 h after treatment at stage 8. Approximately 50% of the treated embryos appeared abnormal at 6 h and 90–100% at 8 h. However, when treated with the same dose of γ-IR after the MBT (stage 9), embryos underwent normal gastrulation and neural plate formation. These embryos were virtually indistinguishable from controls at 6–8 h (Figure 1). The same results were observed in numerous experiments and in embryos treated with 40 Gy (4000 rads) and 50 Gy (5000 rads) of γ-IR (our unpublished results). Thus, there is an apparent change at the MBT in the cellular response to very high doses of γ-IR.

Figure 1. Exposure to γ -IR before but not after the MBT results in arrested abnormal embryonic development. Embryos treated with γ -IR received 20 Gy (2000 rad) from a ^{60}Co source. Control [stage (St) 8 and St 9] and 20-Gy-treated embryos (St 8 and St 9) were collected and photographed 4, 6, and 8 h after treatment.

Ionizing radiation activates cell growth arrest or apoptosis (Lennon *et al.*, 1991; Sen and D'Incalci, 1992), a program of cell death required for normal development and differentiation (Raff, 1992; Hengartner and Horvitz, 1994). Experiments were designed to evaluate whether *Xenopus* embryonic cells treated with γ -IR before the MBT became apoptotic. Interestingly, cellular events accompanying the apoptotic death process were observed only when embryos were treated with γ -IR prior to the MBT. During apoptosis, double-strand cleavage of genomic DNA occurs at the linker regions between nucleosomes, leading to the formation of DNA fragments that are multiples of 180–200 bp; these form a characteristic "DNA ladder" on agarose gel electrophoresis. Eight hours after treatment with γ -IR, internucleosomal DNA cleavage was seen in genomic DNA isolated from embryos treated at stages 5–8 but not in DNA isolated from embryos treated at stages 9 and 10 (Figure 2). A general feature

Figure 2. Internucleosomal DNA fragmentation occurs in pre-MBT embryos exposed to γ -IR. Embryos were untreated (–) or treated at the indicated stages with 20 Gy of γ -IR (+). Genomic DNA was isolated 8 h after exposure and electrophoresed on a 1% agarose gel. Arrows indicate internucleosomal fragments in multiples of 180–200 bp. MW markers indicate *Hind*III-digested λ DNA.

of apoptotic cells is fragmentation and condensation of their DNA into sharp uniformly dense masses that can be visualized by nuclear staining (Kerr *et al.*, 1987). In DAPI-stained sections of irradiated embryos collected 8 h after treatment, the nuclei had a pyknotic appearance indicative of apoptosis only when irradiated prior to stage 9 (Figure 3, B and D), whereas nuclei in embryos irradiated at stage 9 lacked sharp uniformly condensed masses of DNA (Figure 3F). Thus, these results indicate that apoptosis is induced by ionizing radiation administered before but not after the MBT in *Xenopus* embryos.

Further analysis by phase-contrast microscopy revealed cells in the blastocoel of embryos treated with γ -IR at stages 7 and 8 (Figure 4, B and C), but the blastocoel of embryos treated at stage 9 were devoid of cells (Figure 4D). The cells contained within the blastocoel of pre-MBT-treated embryos had fragmented nuclei indicative of apoptosis (Figure 3, B and D). This may reflect the tendency of apoptotic cells to lose intercellular attachments and separate from surrounding cells (Kerr and Winterford, 1994). Pre-MBT *Xenopus* embryos given lower doses of γ -IR (2–5 Gy) contained fewer apoptotic cells in the blastocoel and developed normally to the tadpole stage (our unpub-

Figure 3. γ -IR induces apoptosis in pre-MBT embryonic cells. DAPI staining of sections of control embryos collected 8 h after stage 7 (A), stage 8 (C), and stage 9 (E). DAPI staining of sections of γ -IR-treated embryos collected 8 h after treatment at stage 7 (B) and stage 8 (D) indicates apoptotic bodies (condensed or fragmented DNA, indicated by arrows) in the nuclei, but intact nuclei lacking apoptotic bodies in embryos treated at stage 9 (F). Bars, 10 μ m (A, C, and E, bar in A; B, D, and F, bar in B).

lished results), suggesting that the removal of some DNA-damaged cells can be tolerated and is not lethal.

The p53 tumor suppressor protein, known to accumulate after DNA damage by γ -IR (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992), is part of the apoptotic signaling pathway in some systems (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Miyashita *et al.*, 1994; Miyashita and Reed, 1995). However, Western blots probed with antibodies to *Xenopus* p53 revealed no detectable change in p53 in extracts from stages 6 to 10 embryos treated with 20 or 50 Gy of γ -IR (our unpublished results). These results suggest that the apoptotic program in *Xenopus* is not initiated by an increase in p53.

Ionizing Radiation Before the Midblastula Transition Does Not Block Zygotic Transcription

In early *Xenopus* development, after the 12th division (approximately 5 h after fertilization), several new cell activities define the major developmental transition known as the MBT. These changes include the initiation of zygotic transcription, the onset of cell motility, and asynchronous cell divisions (Newport and Kirschner, 1982b). Before the MBT, there is no observable transcription, and thus the entire embryonic developmental program is under maternal control.

Apoptosis in response to γ -IR may be inhibited by a regulatory mechanism that requires new transcription at the MBT. In some cell populations, inhibitors of transcription or translation induce apoptosis (Searle *et al.*, 1975; Martin *et al.*, 1990; Bansal *et al.*, 1991; Collins *et al.*, 1991). This suggests that the apoptotic program in these cells is normally inhibited by newly synthesized factors. When transcription or translation of these inhibitory factors is prevented, the apoptotic "machinery" is no longer held in check and programmed cell death ensues.

In *Xenopus*, blocking new transcription at the MBT by α -amanitin, a RNA polymerase inhibitor, induces apoptosis in the absence of γ -IR (Sible *et al.*, 1997). DNA isolated from embryos injected with α -amanitin undergoes internucleosomal DNA cleavage indicative of apoptosis 8–9 h after fertilization. Apoptosis induced by α -amanitin appears earlier (8–9 h after fertilization) than apoptosis induced by γ -IR (12–13 h after fertilization). This result suggested that prior to the MBT, γ -IR prevents the onset of zygotic transcription and induces a similar apoptosis signaling pathway observed in the presence of α -amanitin. To directly test this possibility, incorporation of [3 H]uridine into RNA was measured in embryos exposed to γ -IR before and after the MBT. There was no significant difference in the time of transcription initiation in control versus treated embryos (Figure 5). Therefore, exposure to high doses of γ -IR does not block the onset of zygotic transcription. This does not rule out the possibility that γ -IR affects a specific transcript(s) required for inhibition of apoptosis after the MBT. However, apoptosis induced by γ -IR is a response to DNA damage that appears to involve a triggering mechanism different from that induced by blocking zygotic transcription.

The Level of Cyclin A1 and Cyclin A1-Cdk2 Activity Increases in Apoptotic Embryos

Apoptosis is induced in many different cell types in response to a variety of stimuli, but these different cell types undergo similar intracellular events upon apoptosis. These similarities suggest that, although many signals appear cell-type specific, all signals converge to a final common pathway. It has been suggested that the pathway signaling apoptosis is linked to the cell cycle (for review, see Meikrantz and Schlegel, 1995). Inappropriate activation of some cyclin-cdks leads to premature chromatin condensation and other cellular events seen in apoptosis (Freeman *et al.*, 1994; Gazitt and Erdos, 1994; Meikrantz *et al.*, 1994; Shi *et al.*, 1994; Shimizu *et al.*, 1995; Kranenburg *et al.*, 1996; Meikrantz and Schlegel, 1996).

On the basis of these considerations, the levels and associated kinase activities of cyclins A1, E1, B1, and

Figure 4. Apoptotic cells accumulate in the blastocoel of embryos treated with γ -IR before the MBT. Phase-contrast photomicrographs of embryo sections showing the blastocoel from an untreated embryo (A) and embryos treated at stage 7 (B), stage 8 (C), and stage 9 (D). Bar, 50 μ m.

B2 were analyzed in apoptotic *Xenopus* embryos. To induce apoptosis, embryos were either treated with 20 Gy of γ -IR at stages 7–9 or injected with α -amanitin. Embryos treated with γ -IR were collected 8 h after treatment and embryos injected with α -amanitin were collected 9 h after fertilization. Immunoblotting of embryo extracts revealed that although the levels of cyclins E1, B1, and B2 did not change appreciably in control versus apoptotic embryos, cyclin A1 level increased dramatically (Figures 6A and 7A). Embryos exposed to γ -IR after the MBT (stage 9) did not undergo apoptotic cellular events (Figures 2–4) and did not exhibit an increased accumulation of cyclin A1 (Figure 6A). During normal development, the level of cyclin A1 decreases between stages 8 and 11, before the time embryos were evaluated for apoptosis. Cyclin A1 accumulated in apoptotic embryos and did not decrease, in contrast to what was observed in the controls and in embryos treated after the MBT (Figure 6). Cyclin A1 normally decreases as the synthesis of cyclin A2 increases (Howe *et al.*, 1995). The initiation of cyclin A2 synthesis in embryos injected with α -amanitin was not altered compared with control embryos (our unpublished results), suggesting that the degradation of maternal cyclin A1 is not required for the synthesis of maternal cyclin A2. In addition, the timing of the degradation of cyclin E1 is not affected in the presence of α -amanitin (Hartley *et al.*, 1997) or upon treatment with γ -IR (Figure 6A). It is possible that, like cyclin E1, the degradation of cyclin A1 is not affected by the inhibition of zygotic tran-

scription or exposure to γ -IR. In this case, the increase in the level of cyclin A1 after γ -IR would be due to increased synthesis and not decreased degradation.

Extracts of embryos were also analyzed for specific cyclin-associated kinase activity. Cyclins A1, E1, B1, and B2 were immunoprecipitated and assayed for associated histone H1 kinase activity. Cyclin A1-cdk

Figure 5. Ionizing radiation does not affect the onset of zygotic transcription. Embryos loaded with [3 H]uridine were untreated (□) or treated with 20 Gy of γ -IR at stage (St) 8 (Δ) or 9 (○). Embryos were collected at the indicated stages and the incorporation of [3 H]uridine into RNA was analyzed by trichloroacetic acid precipitation. Similar results were obtained in two separate experiments.

Figure 6. Level of cyclin A1 and its associated kinase activity increase in γ -IR-induced apoptosis. Embryos were exposed to 0 Gy or 20 Gy of γ -IR at the indicated stages and frozen 8 h after treatment. (A) Cyclin immunoblots reveal that only cyclin A1 level increases significantly in extracts of apoptotic embryos [stages (St) 7 and 8] but not in untreated embryos or embryos treated after the MBT (stage 9). (B) Samples were assayed for H1 kinase activity in cyclin immunoprecipitates. Results similar to those presented were observed in three separate experiments. Open bars, activity in control embryo extracts; solid bars, activity in extracts from embryos treated with 20 Gy.

activity was increased only in embryos that had undergone apoptosis in response to γ -IR prior to the MBT (Figure 6B) and α -amanitin (Figure 7B). The associated kinase activities of cyclins E1, B1, and B2 decreased in both apoptotic and normal embryos (Figures 6B and 7B), possibly due to slowing of the cell cycle in response to γ -IR and α -amanitin. These results

Figure 7. Level of cyclin A1 and its associated kinase activity increase during α -amanitin-induced apoptosis. Embryos were injected at the one-cell stage with buffer or 1 mg/ml α -amanitin and 9 h after fertilization the embryos were frozen for analysis. (A) Cyclin immunoblots reveal that only cyclin A1 increases significantly in α -amanitin-injected embryos (+ α) but not in embryos injected with buffer (- α). (B) Samples were assayed for H1 kinase activity in cyclin and Cdc2 immunoprecipitates. Results similar to those presented were observed in three separate experiments. Open bars, activity in buffer-injected embryo extracts; solid bars, activity in extracts from embryos injected with α -amanitin.

indicate that increased cyclin A1-cdk activity correlates with an apoptotic response in early *Xenopus* development.

Cyclin A1-Cdk2 Induces Apoptotic Nuclear Morphology in Egg Extracts

In *Xenopus* eggs and during early cleavages, cyclin A1 associates primarily with Cdc2, but low concentrations of cyclin A1-Cdk2 complexes have been detected at stage 8 (Minshull *et al.*, 1990; Rempel *et al.*, 1995; Strausfeld *et al.*, 1996). To determine the kinase partner(s) of cyclin A1 in apoptotic embryos, cyclin A1 was immunoprecipitated from control embryos and embryos treated with γ -IR. Western blot analysis was performed on these immunoprecipitates with antibodies against Cdk2 and Cdc2. An increased level of Cdk2

Figure 8. Cyclin A1 associates with Cdk2 in apoptotic embryos. Embryos were exposed to 0 Gy or 20 Gy of γ -IR at the indicated stages, and 8 h after treatment the embryos were frozen for analysis. Cyclin A1 and A2 immunoprecipitates (IP) blotted for Cdk2 reveal an increase in cyclin A1-Cdk2 complexes in γ -IR-treated apoptotic embryos [stages (St) 7 and St 8] but not in untreated embryos or embryos treated after the MBT (St 9 and St 10); the level of cyclin A2-Cdk2 complexes remain constant. Similar results were obtained in two separate experiments.

was detected in cyclin A1 immunoprecipitates from apoptotic embryos treated before the MBT (stages 7 and 8) but not in control embryos or those treated after the MBT (stages 9 and 10; Figure 8). Cdc2 was detectable in cyclin B immunoprecipitates, was undetectable in cyclin A1 and A2 immunoprecipitates (our unpublished results), and Cdc2-specific kinase activity did not increase in apoptotic embryos injected with α -amanitin (Figure 7B). These results indicate that cyclin A1 associates with Cdk2 during apoptosis.

Increased cyclin A1-Cdk2 activity correlates with apoptosis in *Xenopus* embryos (Figures 6B and 7B). To determine whether the observed increase in cyclin A1-Cdk2 activity is involved in the induction of apoptosis or merely a consequence of the apoptotic program, we designed experiments to directly test the effect of increased cyclin A1-Cdk2 in *Xenopus* egg extracts. Purified recombinant cyclin A1-WT Cdk2 and cyclin A1-kinase-dead Cdk2 (DN) complexes, generated in a baculovirus expression system, were added to 200 μ l of CSF-released egg extract. The activity of the WT cyclin A1-Cdk2 was approximately 3.5 pmol per min per μ l. Forty microliters of the active kinase complex were added during interphase, 60 min after CSF release with CaCl_2 . Nuclear morphology resembling that observed during apoptosis was apparent 30 min after the addition of WT (Figure 9). The addition of buffer or an equal amount of the kinase-dead complex with less than 0.2% of WT activity induced no apoptotic-like nuclei in the extract (Figure 9). Compared with buffer and kinase-dead controls, a 2- to 2.5-fold increase in the recoverable cyclin A1-cdk activity was observed after the addition of WT. Cyclin B-cdk and cyclin E-cdk activities were not affected by the addition of cyclin A1-Cdk2 (our unpublished results), suggesting that increased cyclin A1-Cdk2 activity directly induced the nuclear morphology evident during apoptosis. Although this does not indicate that

Figure 9. Recombinant cyclin A1-Cdk2 induces apoptosis-like nuclear morphology in egg extracts. Equal volumes of buffer, kinase-dead cyclin A1-Cdk2 (DN) or WT cyclin A1-Cdk2 were added to CSF-released egg extracts 60 min after CaCl_2 addition. Aliquots were fixed, stained with DAPI, and photographed at 60, 80, and 90 min after CaCl_2 addition. Nuclear morphology consistent with apoptosis is observed at 90 min in extracts containing recombinant WT cyclin A1-Cdk2. Results similar to those presented were observed in three separate experiments.

cyclin A1-Cdk2 is required for apoptosis, it does establish a potential link between increased cyclin A1-Cdk2 activity and the characteristic chromatin condensation observed in apoptosis.

DISCUSSION

The work described in this article establishes that during the rapid synchronous cell divisions before the MBT, *Xenopus* embryos use apoptosis for the removal of cells damaged by γ -IR and that apoptosis is associated with an increase in the level and activity of cyclin A1-Cdk2.

It is interesting to note that irregularly shaped nuclei, resulting from failed mitoses in *Drosophila* embryos, accumulate in the interior of the egg before cellularization (Minden *et al.*, 1989). The vacancy left by removal of these cells is filled by neighboring normal cells. Thus, *Drosophila* has the capacity to remove and replace defective cells in early divisions so that they do not affect normal development (Minden *et al.*, 1989). Our results suggest that a similar mechanism for removal of cells with damaged DNA exists at the MBT in *Xenopus*.

Because the embryonic response to γ -IR is altered at the MBT, this transition in *Xenopus* should prove use-

ful in studying the induction and inhibition of apoptosis during development. It has been reported that apoptosis in a *Xenopus* cell-free system is inhibited by human Bcl-2 (Newmeyer *et al.*, 1994), an oncoprotein that prevents or delays many forms of apoptosis (for review, see Vaux, 1993; Reed, 1994). Two *Xenopus bcl-2*-like genes have been cloned and the mRNAs have been detected in RNA from stage 12 to stage 50 tadpoles (Cruz-Reyes and Tata, 1995). Preliminary results also indicate that *Xenopus bcl-2* mRNA is not present in eggs or embryos before stage 12 (our unpublished results). Therefore, little or no *Xenopus* Bcl-2 is present in the embryo before the MBT. At the MBT, *bcl-2*-related genes may be transcribed to synthesize a level of Bcl-2 sufficient to inhibit apoptosis. In addition, apoptosis can be detected after the MBT in cells treated with the protein synthesis inhibitor cycloheximide (Sible *et al.*, 1997), further suggesting that an inhibitor of apoptosis is synthesized after the MBT.

It is also possible that DNA repair is more efficient after the MBT, resulting in a weaker signal to trigger the apoptotic response. Preliminary results suggest, however, that this is not the case. Double-strand DNA breaks are repaired with similar efficiency before and after the MBT (our unpublished results). Furthermore, the activity of DNA-dependent protein kinase, a kinase activated in response to double-strand breaks and involved in double-strand break repair (Finnie *et al.*, 1995), is similar in embryos treated with DNA-damaging agents before and after the MBT (our unpublished results). Before the MBT, the cell cycles are rapid and lack G phases. In the absence of G phases, there may be insufficient time for DNA repair. After the MBT, the cell cycles become more somatic-like with the establishment of G phases, possibly allowing more time for DNA repair in each cycle and fewer DNA lesions to induce apoptosis.

We have determined that the apoptotic program in *Xenopus* is not initiated by a detectable increase in p53. Most evidence suggests that p53 functions as a transcription factor to initiate a DNA damage response. A transcription-dependent mechanism for apoptosis would not exist in early *Xenopus* development since there is no transcription before the MBT. However, evidence exists suggesting that transcriptional activation by p53 is not the sole mechanism for induction of apoptosis (Caelles *et al.*, 1994). In addition, because of a high basal level in *Xenopus* embryonic cells (Tchang *et al.*, 1993), p53 may not be limiting for activation of a signaling cascade. The levels of p53 RNA and protein are high and constant throughout normal *Xenopus* oocyte maturation and early development. It is estimated that a single mature oocyte contains 40–50 ng of p53 protein, and this level remains invariant until the tadpole stage (Tchang *et al.*, 1993). Furthermore, it has been shown that diverse tumor-derived cell lines

lacking p53 function undergo apoptosis in response to DNA-damaging drugs (Levine *et al.*, 1991), suggesting that there may not be a general requirement for p53 in DNA damage-induced cell death. Further studies that eliminate or reduce the level of p53 in early embryos are required to determine whether p53 is involved in the apoptotic signaling pathway in *Xenopus*.

Apoptosis and cell cycle progression are linked in many cases because apoptotic cells often contain abnormal levels of cell cycle components (King and Cidlowski, 1995). This suggests that either apoptosis causes the misregulation of cyclin-cdks or apoptosis results when cyclin-cdk activities are misregulated. Activation of cyclin B-Cdk2 is correlated with apoptosis induced by DNA damage in promyelocytic leukemia cells (Shimizu *et al.*, 1995) and Cdk2 activation is required for apoptosis in lymphoma cells (Shi *et al.*, 1994). These results suggest that cyclin B-Cdk2 might be involved in some of the nuclear changes that occur during apoptosis.

Our results suggest that the apoptotic pathway is established early in *Xenopus* development and involves, at least in part, the activation of cyclin A1-Cdk2. In the cell cycle, cyclin A-cdk activity is associated with S- and M-phase entry in a variety of systems (Minshull *et al.*, 1990; Girard *et al.*, 1991; Walker and Maller, 1991; Pagano *et al.*, 1992; Knoblich and Lehner, 1993; Strausfeld *et al.*, 1996). Therefore, activation of apoptosis might involve aberrant entry into S-phase or M-phase through cyclin A-cdk activation. Apoptosis-inducing agents activate cyclin A-Cdk2 in S phase-arrested HeLa cells (Meikrantz *et al.*, 1994), and cyclin A induction alone promotes apoptosis in serum-starved fibroblasts (Hoang *et al.*, 1994). DN forms of Cdk2 block apoptosis in response to two general inducers of apoptosis and this block can be overcome by coexpression of cyclin A (Meikrantz and Schlegel, 1996). In addition, overexpression of cyclin A and Cdk2 bypasses the antiapoptosis activity of Bcl-2 (Meikrantz and Schlegel, 1996).

Phosphorylation of the p34 subunit of replication protein A, required for the initiation and maintenance of DNA replication (Fang and Newport, 1993; Waga and Stillman, 1994), is dependent on Cdk2 (Fang and Newport, 1993) and is stimulated by exposure of some cell types to ionizing radiation (Liu and Weaver, 1993). When administered before the MBT, ionizing radiation induces activation of cyclin A-Cdk2 by an as yet unknown mechanism that may cause premature initiation of DNA replication, leading to apoptosis. Alternatively, cyclin A might target activated Cdk2 to substrates involved in chromatin condensation or other morphological changes common to apoptosis and mitosis, a possibility supported by the initiation of chromatin condensation in egg extracts by cyclin A1-Cdk2 (Figure 9).

It has been suggested that embryos lack DNA-based checkpoints (Hartwell and Weinert, 1989; Murray and Kirschner, 1989b). We have determined that although DNA damage before the MBT does not induce checkpoint arrest in *Xenopus* embryos, a maternal checkpoint may exist at the MBT. This checkpoint assesses the extent of DNA damage and initiates apoptosis to prevent cells with irreparable damage from contributing to embryonic development.

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