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The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice

(redox regulation/DNA repair/A/P endonuclease/gene targeting/oxidative stress)

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ABSTRACT **The DNA-binding activity of AP-1 proteins is modulated, *in vitro*, by a posttranslational mechanism involving reduction oxidation. This mode of regulation has been proposed to control both the transcriptional activity and the oncogenic potential of Fos and Jun. Previous studies revealed that reduction of oxidized Fos and Jun by a cellular protein, Ref-1, stimulates sequence-specific AP-1 DNA-binding activity. Ref-1, a bifunctional protein, is also capable of initiating the repair of apurinic/apyrymidinic sites in damaged DNA. The relationship between the redox and DNA repair activities of Ref-1 is intriguing; both activities have been suggested to play an important role in the cellular response to oxidative stress. To investigate the physiological function of Ref-1, we used a gene targeting strategy to generate mice lacking a functional *ref-1* gene. We report here that heterozygous mutant mice develop into adulthood without any apparent abnormalities. In contrast, homozygous mutant mice, lacking a functional *ref-1* gene, die during embryonic development. Detailed analysis indicates that death occurs following blastocyst formation, shortly after the time of implantation. Degeneration of the mutant embryos is clearly evident at embryonic day 5.5. These findings demonstrate that Ref-1 is essential for early embryonic development.**

An emerging concept in cell biology proposes that the processes of transcription and DNA repair are intimately coupled. It has become apparent from recent studies that the cellular machinery required for basic transcription and DNA repair uses common components (1–4). For example, a subset of proteins known to catalyze nucleotide excision repair also comprise part of the TFIIH transcription initiation complex (1, 5). Mutations in these proteins can lead to inherited genetic disorders in humans such as Cockayne syndrome, xerodera pigmentosa, trichothiodystrophy, and cancer (1, 4, 6, 7). In addition, damage-induced alterations in the levels and function of several transcription factors, growth factors, and cell cycle proteins are known to occur (1, 8–10). However, the molecular mechanisms responsible for recognition of DNA damage and initiation of repair processes are not well understood.

Recently, two distinct lines of research converged on the identification of a dual function protein involved in base excision repair and transcriptional regulation (11–15). This novel protein, termed Ref-1 (also designated APE, HAP1, and APEX based on its A/P endonuclease activity), was first shown to stimulate the DNA-binding activity of AP-1 proteins (e.g., Fos and Jun) by a redox-dependent mechanism (13). A similar activity has recently been assigned to a Ref-1 homologue identified in plants (16). Ref-1 is the predominant AP-1 redox activity in HeLa cells and its stimulatory effect on Fos and Jun is mediated through reduction of a conserved cysteine residue located in the DNA-binding domain of each protein (13, 14, 17, 18). Oxidation or alkylation of the critical cysteine residue has an inhibitory effect on AP-1 DNA-binding activity (17, 19). In contrast, substitution of the cysteine with a residue that cannot be oxidized (i.e., serine) enhances the transforming capacity of Fos in cell culture (20). Cross-linking studies indicate that the cysteine residue is required for a direct interaction between Ref-1 and Jun *in vitro* (19). Furthermore, Ref-1 is also able to stimulate the DNA-binding activity of other classes of redox-regulated transcription factors including p53 (14, 21, 22). The effect on p53 is of particular interest since p53 is thought to orchestrate a crosstalk between transcription and DNA repair signaling pathways in response to genomic insult (23, 24). p53 plays a role in the cellular response to DNA damage by regulating cell cycle arrest and controlling the expression of genes induced by DNA damage (23–28). Thus, Ref-1 may represent a novel component of the signal transduction processes that regulate eukaryotic gene expression in response to DNA damage and cellular stress.

The redox and DNA repair activities of Ref-1 are encoded by distinct regions of the protein that can function independently of one another (19). With regard to its function as a class II A/P endonuclease, Ref-1 catalyzes the repair of oxidative lesions (primarily abasic sites) in DNA (29, 30). A minor 3′ phophodiesterase activity has also been assigned to this protein (31). Given the high rate of spontaneous base hydrolysis and oxidative damage that occurs on a continual basis in normal cells (20), the failure to repair A/P sites would be expected to increase the frequency of mutation (32, 33). DNA repair deficiencies can be manifested in several ways. For example, in the murine system, the disruption of critical mismatch repair genes predisposes the animals to neoplasia and results in a variety of chromosomal abnormalities (34, 35).

Expression of Ref-1 is induced in response to hypoxia in cultured carcinoma cells (36) and during regeneration of porcine epithelium after injury (37). Although no information is available regarding the expression of *ref-1* during early pre- and postimplantation development, studies have shown that the temporal and spatial patterns of *ref-1* expression change dramatically from the period of midgestation through adulthood in the mouse brain (38). At embryonic day (E) 13.5, expression in the brain is high. These levels diminish during development. In the adult mouse brain, *ref-1* is expressed at a low ubiquitous level with areas of elevated expression in the hippocampus, olfactory bulb, and cerebellar Purkinje cells (38). In the rat, high levels of *ref-1* expression have also been

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Abbreviations: E, embryonic day; ES cells, embryonic stem cells.

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observed in the suprachiasmatic, supraoptic, and paraventricular nuclei of the hypothalamus (39). The sites of elevated *ref-1* expression in the brain correlate with regions that express Fos and/or Jun in response to specific stimuli (40, 41).

To elucidate the function of Ref-1 *in vivo*, we generated mutant mice carrying a disrupted allele by gene targeting in embryonic stem (ES) cells. Analysis of the progeny from heterozygous matings of *ref-1*-deficient mice indicate that Ref-1 is essential for early embryonic development.

MATERIALS AND METHODS

Construction of the Targeting Vector. A 13.4-kb *Sal*I DNA fragment encompassing the *ref-1* gene and flanking sequences was isolated from a genomic library derived from mouse strain 129/SvJ (Stratagene). A 9.7-kb *Bgl*II–*Bst*I 1107 genomic DNA subfragment was used to generate the targeting vector. The 3.6-kb *Kpn*I fragment containing sequences encoding exons 1–4 of *ref-1* was deleted from the *Bgl*II–*Bst*I 1107 fragment and replaced with a neomycin (NEO) cassette driven by the phosphoglycerate kinase (PGK) gene promoter. The thymidine kinase (TK) gene cassette was inserted into the *Bgl*II site 5′ to the *ref-1* gene.

Culture, Selection, and Transfection of ES Cells. ES cells (line W9.5) (a gift from Collin Stewart, National Cancer Institute, Frederick) were derived from mouse strain 129/SvJ and maintained in culture on γ-irradiated primary mouse embryo fibroblast (PMEF) feeder cells carrying a neomycin gene. The culture medium was supplemented with leukemia inhibitory factor (1500 units/ml; GIBCO/BRL). The *Sal*I-linearized targeting vector (25 μg) was electroporated into 3×10^7 ES cells. Targeted clones were selected for 7–10 days in the presence of G418 (400 μg/ml) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ioduracil (FIAU) (200 nM) and expanded onto duplicate 96-well plates. To screen by Southern blot analysis, candidate clones were grown to confluence on 96-well gelatin-coated plates, in the absence of a PMEF feeder layer (42). Individual targeted clones, confirmed by Southern blot analysis, were further expanded for microinjection.

Generation of Chimeras and Breeding. Chimeric animals were generated by injection of targeted-ES cells into E3.5 C57BL/6J blastocysts (43). After microinjection, the blastocysts were reimplanted into pseudopregnant females (2.5 days post-coitum). Six- to eight-week-old male progeny with a high percent chimerism (>70%, based on agouti coat color) were bred with C57BL/6J females to produce heterozygous mice capable of transmitting the targeted allele through the germ line. Heterozygous mice were mated together to generate homozygous mice. Blastocysts (E3.5) from the heterozygous matings were isolated from superovulated females and cultured on feeder-free gelatin-coated 96-well plates for up to 2 weeks before analysis.

Genotyping of ES Cells, Embryos, and Animals. ES cells, embryos, and 10-day-old mice were genotyped by Southern blot (14, 42) or PCR analysis. Genomic DNA was isolated from cultured cells or blastocysts and tail clippings by digestion overnight at 55°C in lysis buffer (10 mM Tris·HCl, pH 7.5/100 mM NaCl/1 mM EDTA/0.5% SDS/100 μg/ml proteinase K/50 μg/ml RNase A) followed by phenol-chloroform extraction and ethanol precipitation. For Southern blot analysis, genomic DNA (1–10 μg) was digested with *Bgl*II and *Sac*I and resolved on 0.8% agarose gels. The gels were blotted to Hybond membranes (Amersham) by capillary transfer in 20× standard saline citrate (SSC). The membranes were prehybridized for 2 hr at 42°C in a buffer consisting of 6× SSC, 5× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 0.1% SDS, 50% formamide, and 100 μg/ml denatured salmon sperm DNA, followed by an overnight hybridization in the same buffer containing the ^{32}P-labeled DNA probe. The probe, a 1.1-kb *Bst*I 1107–*Bgl*II DNA fragment that maps 3′ to the *ref*-1 gene (see Fig. 1), was radiolabeled by random-priming (Amer-

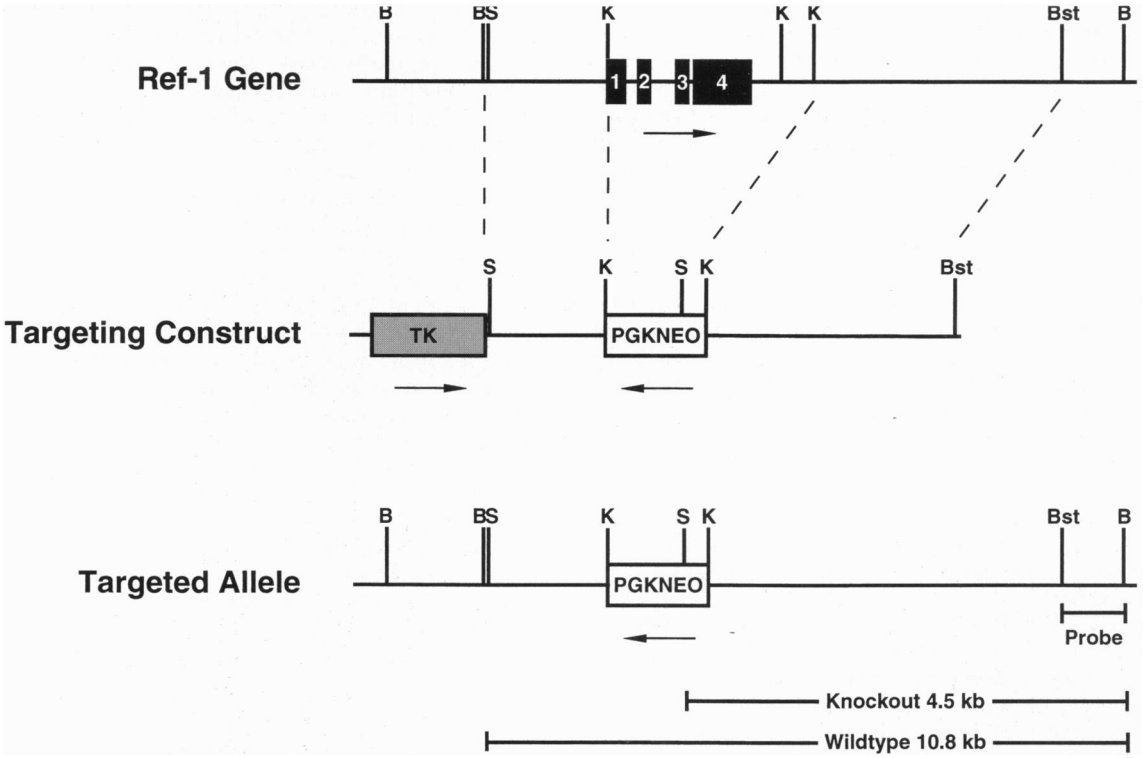

FIG. 1. Generation of Ref-1 mutant mice by gene targeting. The gene structure of the *ref-1* locus, the targeting vector, and the targeted allele are shown schematically. The 3′ probe (1.1 kb) and diagnostic restriction fragments indicating the presence of a wild-type (10.8 kb) or targeted (4.5 kb) allele are diagrammed below the targeted allele. The solid boxes and straight lines represent exon and intron sequences, respectively. Restriction enzyme sites are indicated (B, *Bgl*II; S, *Sac*I; K, *Kpn*I; Bst, *Bst*I 1107). A 3.6-kb region of the *ref-1* gene, encompassing exons 1–4, was deleted by *Kpn*I digestion and replaced with a neomycin-gene cassette (PGKNEO). The thymidine kinase gene (TK) was inserted at the 5′ end of the targeting construct. The relative orientation of the *ref*-1, NEO, and TK sequences is indicated by the arrows beneath the respective gene.

sham) using [^{32}P]dCTP. The blots were rinsed three times for 10 min at room temperature in 2× SSC and washed for 1 hr at 55°C in 0.2× SSC before autoradiography.

PCR genotyping of genomic DNA (1–50 ng) from embryos and tail clippings was performed using two primer sets: (*i*) NEO (NeoG, 5′-GAA CTG CAG GAC GAG GCA GCG-3′; NeoJ, 5′-AGC TCT TCA GCA ATA TCA CGG-3′) and (*ii*) APEX (APEX2, 5′-AAA TCA GGG AGC ACA TAC AA-3′; APEX4, 5′-GAC TCA CCC ATC AGT ACT GG-3′). The NEO and APEX primer sets amplify a 520-bp and 719-bp DNA fragment from the neomycin and *ref-1* genes that are diagnostic for the targeted and wild-type alleles, respectively. PCR products were resolved on 1.0% agarose gels or 4.0% nondenaturing polyacrylamide gels and visualized by UV fluorescence.

Histological Analysis. Histological analysis was performed on E5.5 embryos generated from heterozygous matings (plug date = E0). Pregnant females were killed by cervical dislocation, their uteri were exposed, dissected, and immersed into 4% paraformaldehyde. After a fixation of 24–48 hr, uteri were dehydrated through a series of graded alcohols, placed through several changes of xylene and embedded in paraffin (Paraplast-plus, Fisher Scientific). Serial (5 μm) sections through the uterus were mounted onto polyionic slides (Superfrost-plus, Fisher Scientific). After allowing the slides to dry, the sections were deparaffinized, rehydrated and stained with hematoxylin and eosin. The sections were then dehydrated and topped with a coverslip. The presence and classification (normal, abnormal, or empty) of embryos in the uterus was scored by two independent investigators. In all cases, the number of normal and abnormal embryos was identical.

RESULTS AND DISCUSSION

Genomic *ref-1* DNA clones, isolated from an isogenic strain of mice (129/Sv), were used to construct a gene targeting vector in which *ref-1* sequences encompassing most of exon 1 and all of exons 2–4 were deleted (44) (Fig. 1). This vector was designed to remove all of the *ref-1* protein coding sequences. The targeting vector was introduced into W9.5 embryonic stem cells by electroporation, and cell clones, resistant to G418 and FIAU, were isolated and propagated. Approximately 1000 resistant clones were screened by Southern blot analysis (42); five of these were positive for the targeted allele as determined by the presence of a diagnostic 4.5-kb *Sac*I–*Bgl*II fragment. The expected structure of the targeted allele was confirmed by restriction enzyme mapping. Two of the targeted ES cell clones were microinjected into blastocysts to generate chimeric strains of mice (R1 and R2) that transmitted the disrupted allele through the germ line. Heterozygous mice generated from each of the independently derived chimeras were identified by Southern blot analysis of genomic DNA isolated from tail samples of the offspring (Fig. 2).

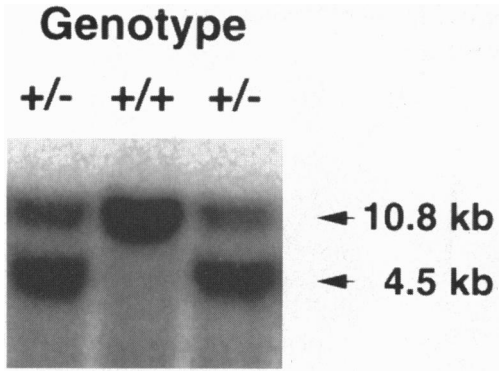

FIG. 2. Southern blot analysis of the targeted *ref-1* allele. Genomic DNA isolated from transgenic mice was digested with *Bgl*II and *Sac*I and resolved by electrophoresis on a 0.8% agarose gel. The gel was transferred to a nylon membrane and the blot was probed with a 1.1-kb fragment derived from the 3′ end of the *ref-1* gene. The size and position of the diagnostic bands indicative of the targeted (4.5 kb) and wild-type (10.8 kb) allele are shown.

Table 1. Genotype distribution of newborn progeny from heterozygote *ref-1* matings

Line	Total no. of progeny	Genotype +/−	Genotype +/+	Genotype −/−
R1	221	137	84	0
R2	243	154	89	0
R1 + R2	464	291	173	0

The genotype of the progeny was determined by Southern blot and/or PCR analysis as described.

No gross anatomical abnormalities have been detected in Ref-1 heterozygote mice up to 9 months of age. The mice grow to normal size, they are fertile, and they do not display any obvious behavioral deficiencies. However, crosses between heterozygous mice yielded smaller litter sizes and failed to produce offspring that were homozygous for the targeted allele. Of 464 live births examined to date, 291 were heterozygous (+/−) and 173 were homozygous wild type (+/+), indicating that disruption of *ref-1* results in early embryonic lethality (Table 1). The observed ratio of heterozygote to wild-type births (1.7:1) is consistent with the predicted ratio of 2:1.

To narrow the time at which the *ref-1* −/− animals die, postimplantation embryos (E6.5–E15.5) from heterozygous matings were surgically explanted from the uterine tissue of pregnant females and genotyped by Southern blot and/or PCR analysis. At E6.5, an average of six to seven implanted embryos (including one to two resorbtions) were found per female. Attempts to genotype the tissue from resorbing embryos were unsuccessful. However, analysis of 130 intact embryos (89 *ref-1* +/− and 41 *ref-1* +/+) between stages E6.5–E15.5 confirmed the absence of the *ref-1* −/− genotype.

To determine whether Ref-1 −/− embryos could develop into normal blastocysts (E3.5), embryos from heterozygous matings were flushed from the uteral lumen of pregnant females

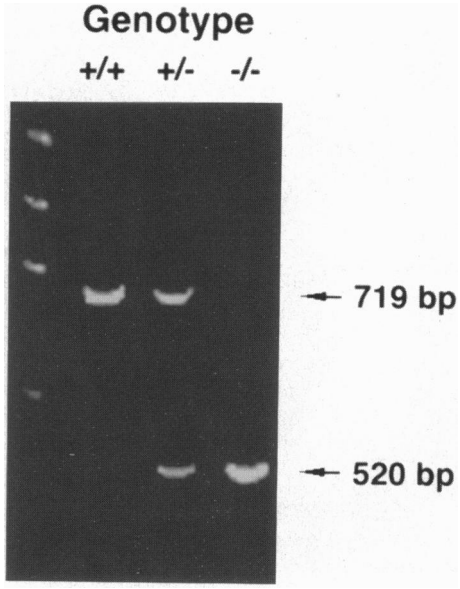

FIG. 3. Identification of Ref-1 −/− blastocysts. E3.5 embryos from matings of heterozygous (+/−) mice were cultured on gelatin coated plates for a period of up to 2 weeks. The genotype of individual blastocysts was determined by PCR analysis of genomic DNA. The size and position of the diagnostic bands indicative of the targeted (520 bp) and wild-type (719 bp) allele are shown.

and cultured for up to 14 days *in vitro*. The presence of Ref-1 −/− embryos was confirmed at this stage by PCR analysis (Fig. 3). A small percentage of the flushed embryos, identified later as Ref-1 −/− embryos, were isolated at the morula stage, and they continued to develop normally to the blastocyst stage while in culture. The Ref-1 −/− blastocysts were able to hatch from the zona pellucida and form trophoblast outgrowths when cultured on gelatin-coated plates. The growth and morphology of the inner cell mass appeared normal, regardless of the genotype. After several days in culture, outgrowths of the inner cell masses from the Ref-1 −/− embryos also gave rise to a normal pattern of visceral endoderm and parietal cell layers. The detection of Ref-1 −/− blastocysts implied that embryonic death was occurring between implantation and E6.5.

To gain more insight into the mutant phenotype, we analyzed fixed paraffin-embedded uterine tissue from heterozygous matings. At E5.5 (plug date = E0), we noted two distinct phenotypes. Approximately 71% of a total of 64 deciduae examined contained healthy animals, characterized by a well-developed embryonic endoderm and ectoderm (Fig. 4 *A* and *B*). However, approximately 23% of the deciduae contained embryos that appeared severely necrotic, characterized by disorganized patches of pyknotic cells (Fig. 4 *C* and *D*). The implantation site of these mutant embryos was discernible by the deciduized maternal tissue. The remaining 6% of the deciduae examined were empty. The percentage of degenerating embryos was significantly higher than would be expected from wild-type matings (1.5–2.6% in litter sizes greater than four) (45), suggesting that the death of these embryos is the direct consequence of the absence of a functional *ref-1* gene. Furthermore, these results demonstrate that Ref-1 −/− mice are unable to develop beyond the point of implantation.

At present, we do not know whether the phenotype of early embryonic death observed in the Ref-1 −/− mice results from loss of the redox or the DNA repair activity of Ref-1, since sequences encoding both these activities have been disrupted by the targeting vector (19). However, redox regulation of gene expression and DNA repair are not mutually exclusive processes and they are likely to be coordinately controlled throughout development. Thus, the function of Ref-1 during early embryonic

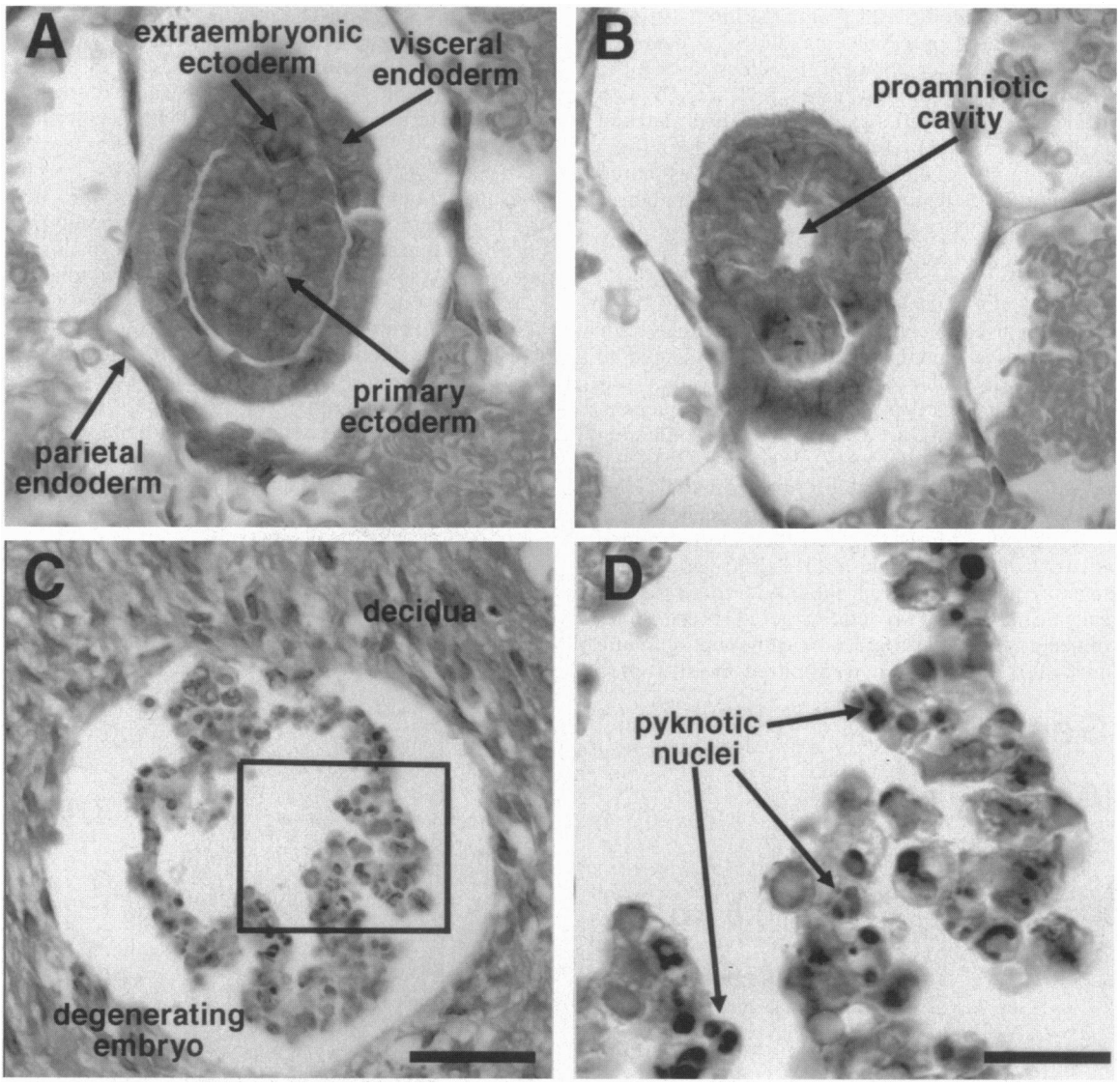

FIG. 4. (*A* and *B*) Photomicrographs of a *ref-1* (+/+ or +/−) E5.5 embryo demonstrating the normal cytoarchitecture of an egg cylinder subsequent to implantation. Note the clear organization of both the embryonic (primary ectoderm and visceral endoderm) and extraembryonic (extraembryonic ectoderm and parietal endoderm) layers. (*C*) Photomicrograph of a presumptive *ref-1* (−/−) E5.5 embryo demonstrating a loss of normal stratification, an expansion of the proamniotic cavity and a preponderance of pyknotic cells. (*D*) An enlargement of the inset shown in *C*. (*D*) Numerous fragmented and pyknotic cells (arrows) are visible throughout the decomposed primary ectoderm and visceral endoderm. (*A–C*, bar = 50 mm; *D*, bar = 133 mm.)

development may be twofold: (*i*) to modulate cellular antioxidant responses via regulation of transcription factor activity (i.e., Fos-Jun) and (*ii*) to ensure adequate repair of abasic lesions introduced into DNA (i.e., A/P sites). Exposure of whole embryos or embryonic tissue to oxidants has been shown to have deleterious effects in many instances. Recent studies indicate that the redox status of preimplantation embryos is altered dramatically during development (46, 47). Free-radical-mediated oxidative damage of embryonic DNA correlates with embryotoxicity (48). As with any proliferative state, active embryonic growth is accompanied by an oxidative burst. Uncontrolled accumulation of reactive oxidants may lead to irreversible damage of the nucleic acid, protein, and lipid components of the cell, resulting ultimately in cell death (49). Indeed, human and rodent cell lines expressing an antisense *ref-1* transcript display increased sensitivity to oxidizing agents (50, 51). Furthermore, studies in yeast indicate that A/P endonuclease-deficient strains are subject to a high rate of spontaneous mutation (32, 33). In higher eukaryotes disruption of specific DNA repair genes leads to several phenotypic abnormalities. Mice defective in the mismatch repair gene, PMS2, are prone to sarcomas and lymphomas and they exhibit abnormal chromosome synapsis during meiosis (35). Msh2-deficient mice show a similar predisposition to cancer as well as abnormal chromosomal recombination (34). Defective mismatch repair has also been implicated in the development of hereditary nonpolyposis colorectal cancer in humans (51, 52).

This study emphasizes the functional importance of Ref-1 *in vivo* by demonstrating that Ref-1 is necessary for early embryonic development of the mouse. Mice that lack one *ref-1* allele develop normally, whereas mice lacking both alleles die *in utero* following implantation. The period of embryonic death observed with the Ref $-/-$ mice is earlier than that observed with the Jun $-/-$ mice that die at midgestation (E12.5) (52). Mice carrying a disrupted c-*fos* gene survive to birth but develop bone disease due to a defect in the osteoclast lineage (53, 54). The phenotype of early embryonic death observed in the Ref-1 $-/-$ mice may be a consequence of defective DNA repair as well as inappropriate regulation of a large class of transcription factors whose DNA-binding activity is dependent on Ref-1 (14).

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