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Requirement of CDC45 for Postimplantation Mouse Development

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CDC45 is required for the initiation of DNA replication in *Saccharomyces cerevisiae* and functions as a DNA polymerase α loading factor in *Xenopus*, but its role in mammalian DNA replication is unknown. To investigate the genetic and physiological functions of CDC45, we used a gene targeting strategy to generate mice lacking a functional *CDC45* gene. Homozygous mutant mice lacking a functional *CDC45* gene underwent uterine implantation and induced uterine decidualization but did not develop substantially thereafter. Detailed analysis of *CDC45* null embryos cultured in vitro revealed impaired proliferation of the inner cell mass. These findings make CDC45 the only putative replication factor experimentally proven to be essential for mammalian development. The *CDC45* gene localizes to human chromosome 22q11.2 in the DiGeorge syndrome critical region (DGCR). Almost 90% of individuals with congenital cardiac and craniofacial defects have a monoallelic deletion in the DGCR that includes *CDC45*. We report here that heterozygous mutant mice develop into adulthood without any apparent abnormalities, so that it is unlikely that hemizygosity of *CDC45* alone is responsible for the cardiac and craniofacial defects in the congenital syndromes.

CDC45 is an essential gene for the initiation of DNA replication in *Saccharomyces cerevisiae* (7, 17, 30). Yeast CDC45 interacts genetically with the origin recognition complex (ORC) and physically with the minichromosome maintenance (MCM) family members and the yeast replication origins (1, 2, 4, 5, 7, 30, 31). Recent studies indicate that CDC45 functions in late G_1 and associates with the prereplicative complex after activation of S-phase-promoting cdk (31). Yeast CDC45 plays an essential role during elongation (24) and is involved in recruiting replication protein A and DNA polymerase α to the DNA (26). It has also been shown that *Xenopus* CDC45 plays a pivotal role in the loading of DNA polymerase α onto chromatin, a process dependent on S-phase-specific cdk activity (16).

A human homolog of yeast *CDC45* has been isolated, and the encoded protein has been shown to associate with human ORC2 protein in cells in culture (22). The human CDC45 protein associates with chromatin in G_1 but progressively loses attachment to a nuclear tether as S phase proceeds, such that none of the protein is detected in the nuclear fraction in G_2/M. Based on this circumstantial biochemical evidence and the strong conservation of the replication apparatus from yeasts to mammals, the mammalian CDC45 protein is expected to be essential for DNA replication, although this has not been formally proven.

The human *CDC45* gene is located in the chromosome region 22q11.2, where interstitial deletions are associated with many developmental disorders, including DiGeorge syndrome (DGS), velocardiofacial syndrome, and conotruncal anomaly face syndrome (3). DGS can be characterized by growth retardation, psychiatric disorders, hypoproliferation of thymus and parathyroid, and outflow tract congenital heart defects, the latter being the most common cause of death (12). A unifying hypothesis is that all these defects might be explained by selective failure of development of the third and fourth branchial arches and pharyngeal pouches that give rise to the conotruncal region of the heart, the thymus, and the parathyroid glands (10, 25). Interestingly, CDC45 is widely expressed in the developing mouse embryo, including the brain, pharyngeal arches, thymus, and kidney, all of which are affected in DGS (23). We know little, however, about the effect of a decrease in *CDC45* gene dosage (as seen in hemizygosity) on the proliferation and differentiation of specific lineages, such as neural crest cells.

One patient with DGS has a heterozygous de novo 20-kb microdeletion that removes only exons 1 to 5 of *CDC45* and exons 1 to 3 of *UFD1* (encoding ubiquitin fusion degradation protein 1) (29). More recently, chromosome-engineering technology was used to model DGS, at least for cardiac anomalies, in mice (13). Hemizygous loss of a 1.2-Mb segment of mouse chromosome 16, syntenic to the human DGS critical region (DGCR), deletes 14 genes, including *CDC45* and *UFD1*, and produces mice with cardiovascular abnormalities of the same type as those seen in DGS. When the hearts of the heterozygous mouse embryos were examined just before birth, a quarter had defects in derivatives of the fourth-branchial-arch artery. Interestingly, mice with a 150- or 550-kb deletion of the proximal region of the DGCR that overlaps with the deleted segment described by Lindsay et al. (13) but does not remove *CDC45* show none of the structural abnormalities of DGS (9, 19). Therefore, the genes responsible for the structural defects in DGS map to the distal region of DGCR, where *CDC45* maps. Surprisingly, although loss of the entire DGCR produced cardiac anomalies, hemizygosity of just *UFD1* did not produce any phenotypic effect (13). Together with the mi-

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crodeletion reported by Yamagishi et al. (29), where hemizygosity of only *UFD1* and *CDC45* produced cardiac defects, these results raised the tantalizing possibility that haploinsufficiency of *CDC45* alone could contribute to the cardiovascular defects of DGS.

To test whether *CDC45* is essential for mammalian DNA replication and to test whether haploinsufficiency of *CDC45* alone produces any of the congenital anomalies seen in DGS, we generated mutant mice carrying a disrupted allele by gene targeting in embryonic stem (ES) cells. Analysis of the heterozygous mice shows no anomalies. Further analysis of the progeny from mating of *CDC45*$^{+/-}$ mice indicates that CDC45 is essential for early embryonic development, consistent with a role of the protein in mammalian DNA replication.

MATERIALS AND METHODS

Construction of the targeting vector. A mouse bacterial artificial chromosome clone (GenBank accession no. AC005816) derived from mouse strain 129/SvJ and containing the entire mouse *CDC45* gene was obtained from Bruce Roe (University of Oklahoma). To delete exons 1 and 2 of *CDC45*, the 2.4-kb (nucleotides [nt] 30513 to 32970) and 5.3-kb (nt 33431 to 38760) regions were cloned into the targeting vector pKO (Stratagene) with phosphoglycerate kinase I promoter driving the neomycin gene (PGK-*neo*) for positive selection and polyomavirus enhancer/herpes simplex virus thymidine kinase (MC1) promoter driving the thymidine kinase gene for negative selection (Fig. 1A). Both regions of homology were amplified by PCR, and the identities were confirmed by sequencing.

Gene targeting in ES cells. ES cells (line J1) were maintained in culture on γ-irradiated primary *neo*-resistant mouse embryo fibroblast (MEF) feeder cells. The culture medium was supplemented with leukemia inhibitory factor (1,500 U/ml; Gibco-BRL). The *Not*I-linearized targeting vector (25 μg) was electroporated into 10^7 ES cells. Targeted clones were selected for 7 to 10 days in the presence of G418 (200 μg/ml) and 1-(2-deoxy-2-fluoro-D-arabinofuranosyl)-5-ioduracil (200 nM) and expanded onto 24-well plates. To screen by Southern blot analysis, candidate clones were grown to confluence on 12-well gelatin-coated plates in the absence of a MEF feeder layer. Individual targeted clones, confirmed by Southern blot analysis, were further expanded for microinjection.

Generation of chimeras. Chimeric animals were generated by injection of three independent targeted ES cells (clones 22, 48, and 98) into 3.5-day-postcoitus (dpc) C57BL/6 blastocysts by standard procedures (6). After microinjection, the blastocysts were reimplanted into pseudopregnant females. Six- to eight-week-old male progeny with a high percent chimerism (>50%, based on agouti coat color) were bred with C57BL/6 females to produce heterozygous mice capable of transmitting the targeted allele through the germ line. Heterozygous mice were mated with each other to generate homozygous mice.

Genotyping of ES cells, embryos, and animals. ES cells, embryos, and 2-week-old mice were genotyped by Southern blot or PCR analysis. Genomic DNA was isolated from embryos and tail clipping by digestion overnight at 55°C in lysis buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate [SDS], 100 μg of proteinase K/ml) followed by isopropanol precipitation. Cultured blastocyst outgrowth was frozen in 10 μl of water, heated to 94°C, treated with 1 μl of 2-mg/ml proteinase K for 1 h at 55°C, heated again to 94°C to inactivate the proteinase K, then subjected to amplification using 5 μl of the solubilized cells per reaction. For Southern blot analysis, genomic DNA was digested with *Eco*NI and resolved on 0.8% agarose gels. The gels were blotted to Nytran membranes (Schleicher & Schuell) by capillary transfer in 10× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membranes were prehybridized for 2 h at 42°C in a buffer consisting of 6× SSC, 10× Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), and 50 μg of denatured salmon sperm DNA/ml. Hybridization was overnight at 65°C in a buffer consisting of 6× SSC, 10% dextran sulfate, 1% SDS, and 50 μg of denatured salmon sperm DNA/ml and containing the ^{32}P-labeled DNA probe. The probe, a 1.1-kb DNA fragment (nt 38844 to 39960) that maps upstream of the *CDC45* gene (probe A in Fig. 1A), was radiolabeled by random priming using [^{32}P]dCTP. The blots were washed twice for 15 min at 65°C in 3× SSC–0.2% SDS and once for 15 min at 65°C in 0.2× SSC–0.2% SDS before autoradiography.

PCR genotyping of genomic DNA from cultured embryos, yolk sacs, and tail clippings was performed using nested primer sets to detect wild-type and mutant

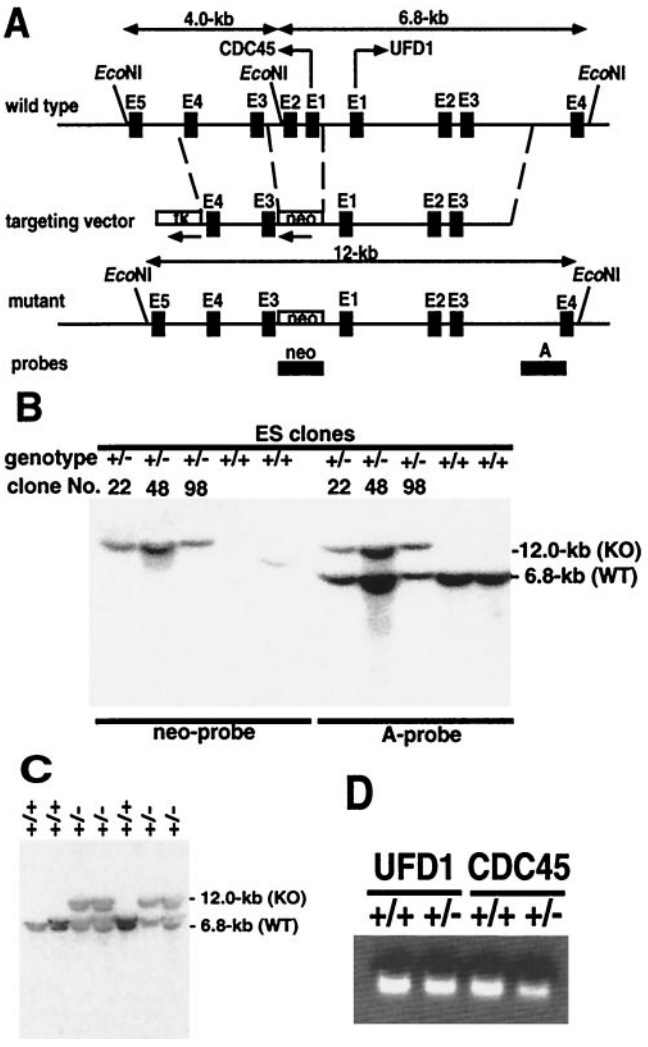

FIG. 1. Generation of *CDC45* mutant mice by gene targeting. (A) The gene structures of the *CDC45* and *UFD1* loci, the targeting vector, and the targeted allele are shown schematically. Diagnostic restriction fragments indicating the presence of a wild-type (6.8-kb) or targeted (12.0-kb) allele are diagrammed above the wild-type and targeted loci, respectively. The solid boxes and straight lines represent exon and intron sequences, respectively. *Eco*NI sites are indicated. A 460-bp region of the *CDC45* gene, encompassing exons 1 and 2, was replaced with a *neo* gene. The thymidine kinase (tk) gene was inserted in the 3′ end of the targeting construct. Arrows at 5′ ends or beneath the respective genes indicate the relative orientations of *CDC45*, *UFD1*, *neo*, and tk gene transcripts. (B) Southern blot analysis of the targeted *CDC45* allele. Genomic DNA isolated from G418-resistant ES cells, digested with *Eco*NI, was hybridized with the neo probe (*neo* gene coding sequence) or probe A (sequence derived from the outside of the targeting vector). The neo probe detects a 12-kb (KO) fragment from a targeted locus. The non-12-kb fragments detected in *CDC45*$^{+/+}$ ES cells indicate random integration of the *neo* gene elsewhere in the genome. Probe A detects a 12-kb (KO) fragment from the targeted *CDC45* locus and a 6.8-kb (WT) band from the wild-type *CDC45* locus. (C) Genomic DNA isolated from progeny (derived from ES clone 98) of *CDC45*$^{+/-}$ matings was genotyped with probe A as described for panel B. No homozygous mutant mice were recovered. (D) Levels of *CDC45* and *UFD1* transcripts were measured by RT-PCR analysis of RNA from 13.5-dpc embryos. The *CDC45* transcript level in *CDC45*$^{+/-}$ mice is roughly half that in *CDC45*$^{+/+}$ mice, whereas the *UFD1* transcript level is the same in both types of mice.

alleles (see Fig. 3A). 5′-ACACTACGTACTTGTCTCACTGTTTGCACT-3′ and 5′-TTGGGTTCCTCACCTCCTGC-3′ were used as forward *CDC45* primers (45F1 and 45F2); 5′-TATTGGCTGCTGGCGTGGACCAATCAGAAG-3′ and 5′-TCCGTGTCTCAGCGCCAGTT-3′ were used as reverse *CDC45* primers (45R1 and 45R2). 5′-GCCAATATGGGATCGGCCAT-3′ and 5′-GAACA AGATGGATTGCACGC-3′ were forward *neo* primers (Neo F1 and Neo F2), and 5′-AGCGGCGATACCGTAAAGCA-3′ and 5′-ACAACGTCGAGCACA GCTGC-3′ were reverse *neo* primers (Neo R1 and Neo R2). The *neo* primer set (Neo F2 and Neo R2) amplifies a 245-bp fragment from the correctly targeted locus and no fragment from the wild-type *CDC45* locus. The wild-type internal primer set (45F2 and 45R2) amplifies a 430-bp DNA fragment from the wild-type *CDC45* allele and no product from a Neo-targeted *CDC45* allele. PCR products were resolved on 1.0% agarose gels and visualized by UV fluorescence. PCR conditions were 95°C for 3 min, followed by 30 (or 35) cycles of 94°C for 30 s, 58°C for 30 s, and then 72°C for 30 s. After the final cycle, we used a 5-min extension period at 72°C.

Histological analysis. For histological examination, samples were fixed in phosphate-buffered saline-buffered 4% formaldehyde, processed, and embedded in paraffin. Sagittal sections (10 μm thick) were cut and stained with hematoxylin and eosin. Embryos (5.5 to 6.5 dpc) obtained from timed matings were dissected from uteri. Serial sections through the whole decidua were mounted onto polyionic slides. Two investigators scored the presence of embryos (or deciduae) in the uterus.

MEF generation and culture. *CDC45*$^{+/+}$ and *CDC45*$^{+/-}$ embryos (13.5 dpc) were dissected and trypsinized, and MEF were transferred to Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum as described elsewhere (21). γ-Irradiation of the MEF was performed using a ^{137}Cs γ-irradiator (2.12 Gy/s; Gammacell 1000; Atomic Energy of Canada Limited Industrial Products, Mississiagua, Ontario, Canada). Subsequently, 10^4 cells were plated in triplicate onto six-well plates with 2 ml of medium/well. Viable cells were quantitated by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche) at 5 days after irradiation.

RT-PCR. Total RNA (10 μg) was prepared from 13.5-dpc embryos and reverse transcribed using oligo(dT) and Superscript II reverse transcriptase (RT; Gibco-BRL). cDNA obtained from 0.5 μg of total RNA was amplified by PCR using the following primer pairs: 5′-CTGAAGCAAGTCAAGCAG-3′ (exon 12) and 5′-CACAAGAGCGTCCAGGAA-3′ (exon 18) as *CDC45* primers and 5′-GTGG CGACCTACTCTAAG-3′ (exon 5) and 5′-CCAGGACAAGCTTGATAG-3′ (exon 12) as *UFD1* primers. PCR was at 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and then 72°C for 30 s. After the final cycle, we used a 5-min extension period at 72°C. The *CDC45* and *UFD1* primer sets amplify DNA fragments of 723 and 708 bp, respectively.

In vitro culture of preimplantation embryos. *CDC45*$^{+/-}$ males and females were intercrossed, and 3.5-dpc embryos were flushed out from the uteri of the plugged females (21). Blastocysts were individually cultured in eight-well chamber slides (Nalge Nunc) in ES medium without leukemia inhibitory factor, in 5% CO_2 at 37°C. Photographs of the cultured embryos were taken at 5 or 8 days. After 5 to 8 days in culture, the genotype was determined by nested PCR as described above.

RESULTS

Gene targeting strategy. Our strategy was to delete exon 1 (including the initiation codon) and exon 2 from the *CDC45* gene (Fig. 1A). An internal methionine first occurs in exon 8; thus, at least the first 7 out of 19 exons will be nontranslatable in the mutant. Even if a protein is produced from an internal methionine it will have 199 N-terminal amino acids deleted out of a total of 566 amino acids. These N-terminal 199 residues include acidic patches and nuclear localization signals that are conserved in yeast and *Xenopus*, so that it is unlikely that such a deletion-containing form of CDC45 would be functional (7, 16). Approximately 170 G418-resistant ES cell clones were screened by Southern blot analysis; six of these were positive for the targeted allele as determined by the presence of a diagnostic 12.0-kb *Eco*NI fragment (Fig. 1B). Three of the targeted ES cell clones (clones 22, 48, and 98) were microinjected into blastocysts to generate chimeric strains of mice that transmitted the disrupted allele through the germ line. We

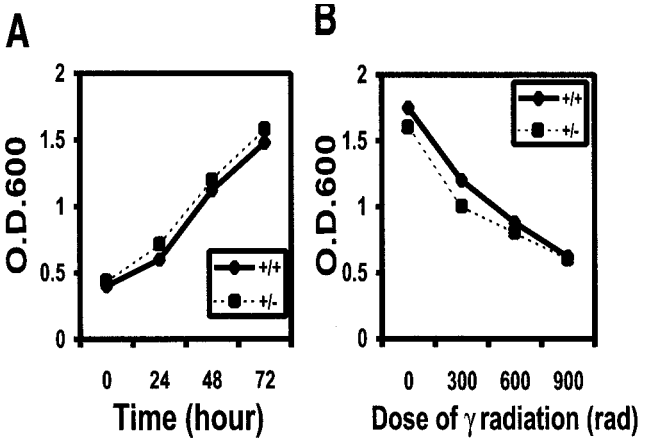

FIG. 2. In vitro culture of MEF derived from *CDC45*$^{+/+}$ and *CDC45*$^{+/-}$ embryos. Cell proliferation (A) and the effect of ^{137}Cs γ-irradiation on the number of viable cells (B) were measured by MTT assay, and assays were performed in triplicate. Cell proliferation rates in low serum concentration (0.5%) also showed no difference between *CDC45*$^{+/+}$ and *CDC45*$^{+/-}$ MEF (data not shown). O.D.600, optical density at 600 nm.

generated two independent lines from chimeras derived from clones 22 and 98 from a C57BL/6 background. Heterozygous mice generated from each of the independently derived chimeras were identified by Southern blotting and/or PCR analysis of genomic DNA isolated from tail samples of the offspring (Fig. 1C; see also Fig. 3A).

Phenotype of *CDC45*$^{+/-}$ mice. No gross anatomical abnormalities have been detected in *CDC45* heterozygous mice up to 6 months of age. The mice grow to normal size, are fertile, and do not display any obvious behavioral deficiencies. About 25% of E18.5 embryos hemizygous for a deletion encompassing 22 known genes including *CDC45* exhibited various velocardiofacial syndrome- and DGS-associated cardiovascular abnormalities (13). We therefore recovered and examined 18.5-dpc embryos from a cross of *CDC45*$^{+/-}$ mice. Thirteen *CDC45*$^{+/-}$ embryos as well as seven wild-type embryos were examined and found to have no abnormal development of the major vessels of the heart (data not shown). RT-PCR analysis of 13.5-dpc embryos revealed that the expression level of *CDC45* transcripts in *CDC45*$^{+/-}$ mice was roughly half that in wild-type mice, whereas levels of *UFD1* were the same in *CDC45*$^{+/-}$ and wild-type mice (Fig. 1D).

To investigate whether hemizygosity of *CDC45* has any phenotypic effect at the cellular level, we prepared MEF from *CDC45*$^{+/+}$ and *CDC45*$^{+/-}$ embryos and measured proliferation rates. Hemizygosity of *CDC45* did not have any effect on cell doubling time (Fig. 2A). Since *CDC45* is expected to play a role in DNA repair (because of its requirement in the loading of DNA polymerase α), *CDC45*$^{+/+}$ and *CDC45*$^{+/-}$ MEF were γ-irradiated, and the cells surviving radiation was measured. No difference was seen in the radiation sensitivity of *CDC45*$^{+/+}$ and *CDC45*$^{+/-}$ MEF (Fig. 2B).

Embryonic lethality of the *CDC45*$^{-/-}$ mutation. Of 61 live births resulting from crosses between *CDC45*$^{+/-}$ mice, 39 were heterozygous (*CDC45*$^{+/-}$) and 22 were wild-type (*CDC45*$^{+/+}$), indicating that disruption of *CDC45* results in embryonic le-

TABLE 1. Progeny from *CDC45* heterozygotes[a]

Age (dpc)	Genotype			No. (%) resorbed	Total
	+/+	+/−	−/−		
Neonate	22	39	0	NA	61
18.5	7	13	0	8 (29)	28
13.5	6	10	0	9 (36)	25
7.5	7	19	0	8 (24)	34
6.5	ND	ND	ND	7 (27)	26[b]
5.5	ND	ND	ND	6 (25)	24[b]
3.5	6	9	3	NA	22[c]

[a] NA, not available ND, not determined.
[b] All decidua were histologically checked.
[c] Four failed to hatch and produced no PCR products upon genotyping.

thality (Table 1). The observed ratio of heterozygote to wild-type births is consistent with a predicted ratio of 2:1 and suggests that the *CDC45*$^{-/-}$ mutation is embryonically lethal.

To define the stage at which the *CDC45*$^{-/-}$ animals die, postimplantation embryos (7.5 to 18.5 dpc) from heterozygous matings were surgically explanted from the uterine tissue of pregnant females and genotyped by PCR analysis. At 7.5 dpc, a total of 34 implanted embryos, including 7 resorptions (27%), were found (Table 1). Attempts to genotype the tissue from resorbed embryos were unsuccessful. However, analysis of 62 intact embryos (42 *CDC45*$^{+/-}$ and 20 *CDC45*$^{+/+}$) between 7.5 and 18.5 dpc confirmed the absence of the *CDC45*$^{-/-}$ genotype (Table 1), suggesting that the *CDC45*$^{-/-}$ animals die before 7.5 dpc.

To gain more insight into the mutant phenotype, we analyzed fixed paraffin-embedded deciduae from matings of *CDC45*$^{+/-}$ mice. At 5.5 and 6.5 dpc, we noted two distinct phenotypes. Of a total of 50 deciduae, 74% contained healthy animals (Table 1), characterized by a well-developed embryonic structure (data not shown). However, 24% of the decidua contained no embryos, a number consistent with the possibility that they were derived from the missing *CDC45*$^{-/-}$ embryos (Table 1). In contrast, out of 19 5.5- and 6.5-dpc deciduas examined from *CDC45*$^{+/+}$ × *CDC45*$^{+/-}$ matings, only 2 were abnormal. These results suggest that *CDC45*$^{-/-}$ embryos induced the decidual reaction upon implantation but the embryo proper was unable to develop beyond the point of implantation. Even embryos that die or are degraded shortly after attachment stimulate decidualization, a process whereby stromal cells expand, elaborate extracellular matrix, and form tight junctions (28). Since a normal mouse blastocyst attaches to the uterus between 4.5 and 5.0 dpc (20), it is likely that the *CDC45*$^{-/-}$ mice die between 4.5 and 5.5 dpc.

In vitro growth of blastocysts. We assessed in vitro growth of blastocysts from heterozygote intercrosses. When 22 blastocysts were individually cultured for 5 to 8 days, 4 did not emerge from the zona pellucida (genotype unknown). Three embryos with trophoblast outgrowths but no proliferation of the inner cell mass (see Fig. 4B and D) were subsequently identified as *CDC45*$^{-/-}$ (Fig. 3B). The remaining 15 embryos showed trophoblast outgrowths and proliferation of their inner cell mass (Fig. 4A and C); these embryos were found by nested PCR analysis to be either wild type or heterozygous at the *CDC45* locus (Fig. 3B). These results strongly suggest that the growth of *CDC45*$^{-/-}$ embryos is affected at the peri-implantation stage.

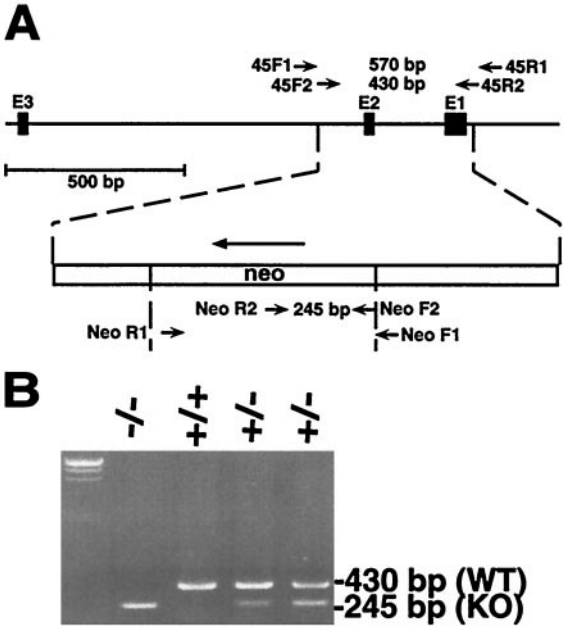

FIG. 3. PCR analysis of DNAs from embryos resulting from *CDC45*$^{+/-}$ crosses. (A) PCR strategy for amplification of wild-type and disrupted *CDC45* alleles. DNA samples derived from cultured embryos were subjected to PCR amplification using a combination of two sets of nested primers (see Materials and Methods for sequences). Nested PCR of the wild-type *CDC45* allele by forward and reverse 45 primers produces a 430-bp DNA fragment, whereas amplification of the disrupted *CDC45* allele by forward and reverse Neo primers produces a 245-bp DNA fragment. The arrow indicates the direction of transcription of PGK-*neo* plus poly(A). (B) Genotype analysis of 5- to 8-day-cultured blastocysts by PCR. Wild-type and targeted *CDC45* alleles are amplified as 430-bp (WT) and 245-bp (KO) fragments. λ/*Hin*dIII fragments are on the left.

DISCUSSION

To the best of our knowledge, this is the first study that genetically demonstrates the requirement of a putative DNA replication factor for normal mammalian development. Because of the difficulty of performing genetic analyses in mammals, most of our knowledge of DNA replication factors in mammals is dependent on biochemical studies in vitro or genetic studies in yeasts and *Drosophila*. Important roles of *CDC45* in the initiation of DNA replication in lower organisms such as yeast (7, 17, 30) as well as *Xenopus* (16) have been reported. One reasonable explanation for the early lethality of *CDC45*$^{-/-}$ embryos is that DNA replication initiation is impaired after maternal CDC45 in the embryo is lost. Consistent with this possibility, CDC45 is expressed in multiple tissues from 9.5 to 14.5 days of mouse embryonic development (23). Although evolutionary conservation of core life processes like DNA replication leads us to expect that mammalian homologs of replication factors like CDC45 are essential for viability, this is the first experimental demonstration of this fact. The results reported here eliminate the possibility that mammals have

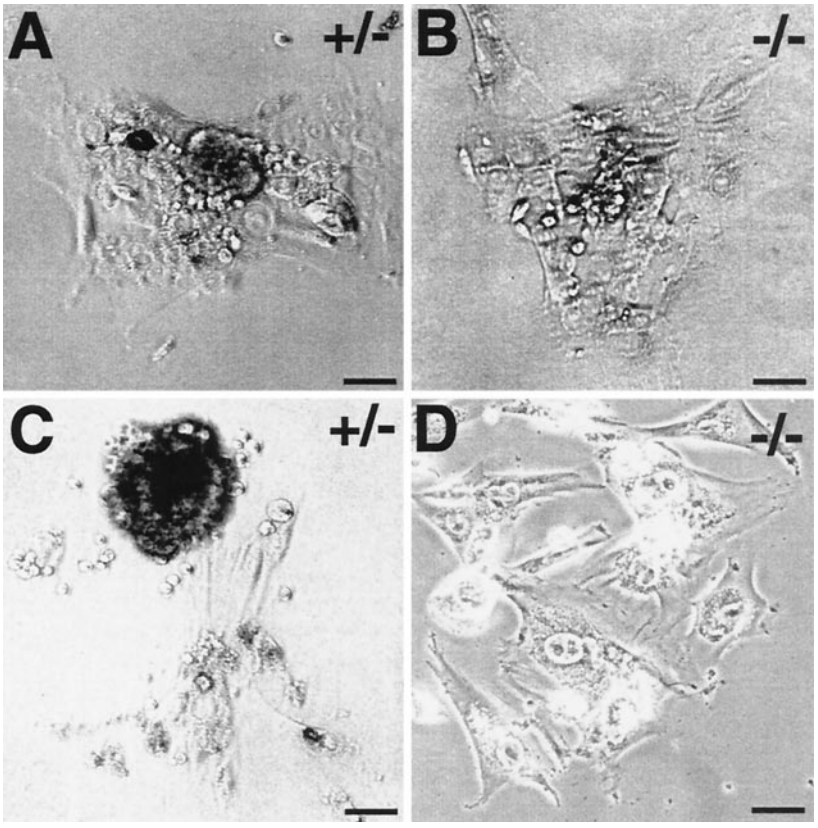

FIG. 4. . Morphology of cultured blastocyst outgrowths from crosses of *CDC45*$^{+/-}$ mice. Cultured heterozygous (A and C) and homozygous (B and D) embryos were photographed using phase microscopy after 5 (A and B) and 8 (C and D) days in culture. The tightly packed inner cell mass is found in heterozygous but not in homozygous embryos. Bars, 100 μm.

evolved pathways that bypass the requirement of CDC45 in DNA replication.

Maternal stockpiles of replication factors in eggs have been shown to support many rounds of cell division without zygotic transcription. For example, null mutations in *ORC2* or *ORC3* in *Drosophila* allow development of the fly to late larval stages, implying that the maternal supply of these proteins can sustain at least 20 cell divisions without any contribution from the embryo (11, 18). *Xenopus* eggs have a vast excess of ORC proteins, such that more than 99% of the supply has to be immunodepleted before one sees a defect in replication initiation in vitro (27). In light of these observations, it is somewhat surprising that the *CDC45*$^{-/-}$ mice exhibit such an early embryonic lethality. By 5.5 dpc, there are about 128 to 256 cells in the embryo, so our results suggest that maternal supplies of CDC45 in mammalian eggs can support only about seven to eight cell divisions before lethality. Either the maternal stockpile of CDC45 and other replication factors is low in mouse eggs (relative to that in *Xenopus* and *Drosophila* eggs), or CDC45 is unstable in early embryonic cell divisions or the early lethality is an indirect effect of CDC45 deficiency. Since CDC45 is involved during the elongation stage of DNA replication, a decrease in the amount of the protein might lead to defects in replication fork movement well before factors become limiting for replication initiation. Stalled replication forks may lead to DNA damage, so that the early embryonic lethality could be a result of cell cycle arrest due to the activation of cell cycle checkpoints. Examining the phenotypes of *CDC45*$^{-/-}$ mice that are concurrently defective in p53 and other checkpoint-activating genes will be used to test this hypothesis.

Mice that lack one *CDC45* allele develop normally, with no obvious defects in cardiovascular development. We conclude that the loss of one copy of *CDC45* alone is not responsible for the congenital defects seen in patients with DGS or in mouse models of the disease. While this paper was under review, three other papers reported that hemizygosity of *TBX1*, a gene that maps to the distal region of DGCR, was sufficient to produce conotruncal defects in cardiac development in a certain fraction of the mice (8, 14, 15). Homozygous deletion of *TBX1* produced more profound defects in cardiac development than seen in the hemizygous mice. Furthermore, *TBX1*$^{-/-}$ but not *TBX1*$^{+/-}$ mice had additional features of DGS, such as hypoplasia of the thymus and parathyroid and cleft palate, that could be detected in late-stage embryos (8). The fact that a homozygous deletion of *TBX1* is necessary in mice to phenocopy all the defects of DGS (which is seen in humans with hemizygosity of DGCR) might suggest that additional genes in the DGCR contribute to the full spectrum of congenital defects seen in humans.

A hemizygous deletion that removes both *TBX1* and *CDC45* still does not produce the full spectrum of defects in mice, ruling out *CDC45* as a gene that synergizes with *TBX1* to produce the congenital defects in mice. We cannot, however,

rule out species-specific differences that might allow hemizygosity of the *CDC45* and *TBX1* to cooperate to produce the full spectrum of defects of DGS in humans but not in mice. It will be necessary to identify DGS patients with mutations only in the *TBX1* gene and without any defect in the structure or expression of any other gene in the DGCR (including *CDC45*) before concluding that *TBX1* is solely responsible for all the congenital defects in humans.

The $CDC45^{+/-}$ mice appear to have half the amount of CDC45 mRNA that is found in the $CDC45^{+/+}$ controls, indicating that both copies of the gene are transcribed in normal development. Although expression of half the normal amount of CDC45 mRNA did not impair cell proliferation or development, this demonstrable decrease in CDC45 transcript levels might leave $CDC45^{+/-}$ cells vulnerable to a similar decrease in levels of another transcript involved in cell proliferation or development. Such a synergistic impairment of cell proliferation in the hemizygous state may be tissue specific if the second gene (like *TBX1*) has tissue-specific functions. Alternatively, hemizygosity of *CDC45* might impair proliferation in specific tissues, as seen with hypomorphic mutations in another gene involved in general cell proliferation, *Drosophila ORC3* (18). Future experiments will be directed at generating organisms which are doubly heterozygous for *CDC45*, a gene involved in cell proliferation, and other genes implicated in normal development or cell proliferation. The results will test whether subtle lesions in different genes, which by themselves have subtle phenotypic effects, interact to produce more profound changes in development or cell proliferation.

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