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Targeted Disruption of the Mouse Topoisomerase I Gene by Camptothecin Selection

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Topoisomerase I has ubiquitous roles in important cellular functions such as replication, transcription, and recombination. In order to further characterize this enzyme in vivo, we have used gene targeting to inactivate the mouse *Top-1* gene. A selection protocol using the topoisomerase I inhibitor camptothecin facilitated isolation of embryonic stem cell clones containing an inactivated allele; isolation of correctly targeted clones was enhanced 75-fold over that achieved by normal selection procedures. The disrupted *Top-1* allele is embryonic lethal when homozygous, and development of such embryos fails between the 4- and 16-cell stages. Both sperm and oocytes containing the inactive allele maintain viability through the fertilization point, and thus gene expression of topoisomerase I is not required for gamete viability. These studies demonstrate that topoisomerase I is essential for cell growth and division in vivo. The *Top-1* gene was also shown to be linked to the agouti locus.

Eukaryotic topoisomerase I has a key role in many fundamental biological processes. The enzyme alters DNA topology by catalyzing transient single-stranded breakage of the phosphodiester backbone; this event allows passage of the intact strand through the break site, and subsequent religation of the nicked strand reforms the intact helix. Both positive and negative supercoils can be relaxed by eukaryotic topoisomerase I, and the linking number of the DNA can be changed by increments as small as one. This simple type of topological manipulation is known to be a crucial process for basic DNA and RNA metabolism in mammalian cells (40, 41).

A variety of studies, both in vitro and in vivo, indicate the importance of topoisomerase I in cellular function. The enzyme is necessary for efficient simian virus 40 replication in vitro (12, 42, 43), and *Saccharomyces cerevisiae TOP1* mutants have delayed onsets of DNA short-chain elongation during early S phase (15). Topoisomerase I is known to interact with regions of actively transcribed chromatin (8, 34, 35) and has also been found to be an essential component of some in vitro transcriptional systems (18). In addition, inactivation of *TOP1* affects the rate of transcription in *S. cerevisiae* (5).

Experiments with yeast *TOP1* mutants showed that topoisomerase I was dispensable for cell growth (38, 39). This is not the case with *Drosophila melanogaster*, in which topoisomerase I is required for development past the blastocyst stage (21). The gene may thus be essential in more-complex eukaryotes.

DNA topoisomerase I is considered an attractive target for cancer chemotherapy. Levels of the enzyme are found to be elevated in several types of leukemia, lymphoma, and colon carcinoma cells (9, 27), and a variety of promising antineoplastic agents that inhibit topoisomerase I activity are currently under examination (3, 9). Several such agents are derived from the cytotoxic plant alkaloid camptothecin (CPT), including topotecan, 9-amino-CPT, and CPT-11.

CPT seems to act by stabilizing the topoisomerase I-DNA covalent intermediate, significantly slowing the reclosure step of the nicking-closing reaction (11, 26). Cellular cytotoxicity of CPT is associated with persistent double-stranded breaks in replicating cellular DNA that are postulated to be highly lethal (30). Resulting DNA damage can lead to cell cycle arrest and/or cell death by apoptosis (7). Stepwise exposure of several cell types to increasing levels of CPT has resulted in the isolation of drug-resistant cell lines. These lines have reduced topoisomerase I levels (13, 33), mutations in the coding region of the gene, or both (19, 36). Point mutations resulting in CPT resistance and reduced enzymatic activity have been used to detect structure-function relationships between various regions of the protein and drug resistance. The lack of mutations completely eliminating topoisomerase I activity suggests an important role for the enzyme in cellular viability.

Although many studies link topoisomerase I to important cellular functions, the precise role of the enzyme in these processes remains obscure. In order to investigate whether the gene is essential for mammalian development, we have used gene targeting to develop mice in which *Top-1* has been interrupted. To accomplish this, the topoisomerase I inhibitor CPT was used to develop a procedure for selection of homologous recombinants in which one copy of the gene has been disrupted.

MATERIALS AND METHODS

Cloning the topoisomerase I gene and construction of the targeting vector. Genomic DNA was isolated from E14TG2a (10) mouse embryonic stem (ES) cells and partially digested with *Mbo*I. Fragments of approximately 15 kb were isolated and used to make a library in lambda phage Charon 35 (22). Primers corresponding to highly conserved regions of exon 15 of the human topoisomerase I gene (4, 20) were used for PCR amplification of the mouse gene (1), thus generating a 150-bp probe for screening use. The primers used were from the 5′ region of exon 15 (5′-GCTCTGAGAGCAGGCAATGAAAA-3′) and the 3′ region of the exon 15 coding sequence (5′-ATGGAGTCCTTCCCGAGGAAG TCAAA-3′); the resulting probe corresponds to amino acid residues 486 to 535 (4). Clones containing portions of the topoisomerase I gene were isolated, and two fragments (4.5-kb *Bgl*II and 6.0-kb *Bam*HI fragments) containing exon 15 were subcloned (Fig. 1A). A 3.8-kb *Bgl*II-*Pst*I fragment and a 1.4-kb *Bgl*II-*Hin*dIII fragment were then used to create the targeting vector, pTGT-2 (Fig. 1A and B).

Cell culture, electroporation, and selection. BK4 cells (a subclone of E14TG2a, provided by B. Koller, University of North Carolina at Chapel Hill) were cultured on murine embryonic fibroblasts in Dulbecco modified Eagle medium-high glucose (DMEM-H) (Life Technologies)–100 μM β-mercaptoethanol–2 mM L-glutamine. The cells were trypsinized and resuspended in 0.04 ml of DMEM-H. Electroporation was carried out in the presence of 2 to 5 nM

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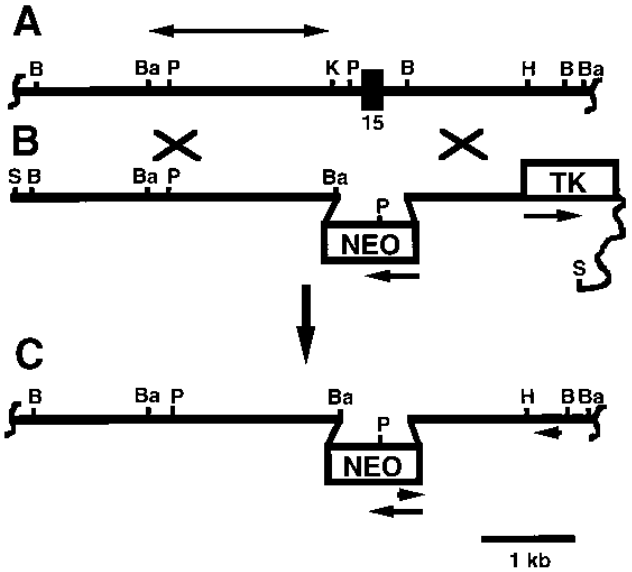

FIG. 1. Disruption of the mouse *Top-1* gene. (A) Part of the wild-type *Top-1* gene. Exon 15 (black box) and the *Bam*HI-*Kpn*I fragment used as a probe for Southern analysis (double arrow) are indicated. B, Ba, H, K, P, and S, *Bgl*II, *Bam*HI, *Hin*dIII, *Kpn*I, *Pst*I, and *Sal*I, respectively. (B) The targeting construct. Neomycin resistance (NEO) and thymidine kinase (TK) genes, oriented as indicated by the arrows, and plasmid sequence (wavy line) (not drawn to scale) are shown. The most 5′ *Sal*I site in the construct is from the lambda phage vector. (C) The targeted *Top-1* locus, with *Neo* replacing exon 15. PCR primers used to identify targeted cell lines are indicated (arrowheads).

pTGT-2 plasmid which had been linearized with *Sac*II; a 1-s discharge from a 200-μF capacitor charged to 300 V was used. Selection with G418 and ganciclovir was carried out as described elsewhere (23). Half of each clone was picked with a micropipette and placed in a 24-well tissue culture plate overnight. The next day, the colony was trypsinized and expanded for use or frozen in 50% serum–40% DMEM-H–10% dimethyl sulfoxide (DMSO) at −80°C. The other half of the clone was utilized for genotypic PCR analysis.

In a separate experiment, cell colonies surviving G418-ganciclovir selection were dispersed with trypsin, replated, and allowed to grow for 3 days. The cells were then exposed to DMEM-H containing 250 nM, 500 nM, or 1 μM CPT (made from a 1 mM stock in DMSO) to directly select for cells containing reduced topoisomerase I activity. The selection was maintained for 7 days, at which time CPT-resistant clones were chosen from each plate. Cells from each individual plate were regarded as clonal, since the clonality of cells within an individual plate could not be assessed. Clones were picked with a micropipette and placed in a 24-well tissue culture plate. Colonies were subsequently expanded for use or frozen in 50% serum–40% DMEM-H–10% DMSO at −80°C.

Targeted cells heterozygous for the inactivated *Top-1* allele were further selected in higher concentrations of G418 by the method of Mortenson et al. (25) in an attempt to select clones homozygous for the targeted allele.

Genotypic analysis by PCR and Southern blotting. Potential recombinant clones were initially screened by recording the presence of a PCR product diagnostic for a correct homologous recombination event (14). The two primers used were derived from the *neo* gene (5′-ACGCGTCACCTTAATATGCG-3′) and from an intron region that was not included in the targeting construct (5′-ATGTTGTTGTCTTGGGTTCTG-3′) (Fig. 1C). DNAs from clones that had undergone homologous recombination (as judged by the amplification of the correct PCR fragment) were then used for Southern blot analysis to confirm that *Top-1* had been disrupted.

The genotypes of the mice were determined by using DNA isolated from their tails. Genomic DNA totalling 8 to 10 μg was digested with *Pst*I or *Bam*HI overnight and applied to a 0.8% agarose gel. After separation by agarose gel electrophoresis, the DNA was transferred to Hybond nylon membranes. The membranes were probed with a 2.0-kb *Bam*HI-*Kpn*I fragment from the *Top-1* genomic clone (Fig. 1A) labeled with ^{32}P. The membranes were washed and exposed to Kodak XAR film overnight with an intensifying screen.

Analysis of CPT resistance in heterozygous ES cells. Wild-type and heterozygous mutant ES cells were grown without feeder cells, and nuclear extracts were prepared from these cells as described by Tanizawa and Pommier (37). The nuclear extracts containing topoisomerase I were then assayed for CPT sensitivity as previously described (24).

Microinjection and derivation of a heterozygous mouse line. Cells from one of the targeted clones were microinjected into C57BL/6J (B6) host blastocysts and implanted in pseudopregnant female recipients to generate chimeric mice. A male mouse that transmitted the 129 germ line to his progeny at 100% frequency was identified. This male was bred to B6 females, and approximately 50% of his F_1 pups were heterozygous for one wild-type and one disrupted allele. The heterozygous F_1 progeny were then interbred to obtain F_2 mice. These mice were used for further studies.

Quantitation of topoisomerase I levels. Enzyme quantitation was done by Western blot (immunoblot) analysis. Equivalent weights of liver tissue were isolated from wild-type and heterozygous F_2 mice, and salt-soluble nuclear protein fractions were obtained as described by Tanizawa and Pommier (37). A 50-μg amount of total salt-soluble nuclear protein from wild-type and heterozygous animals was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The protein was then transferred to nitrocellulose by using a Bio-Rad electroblotter and transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, and 20% methanol) at 4°C and 100 mA.

After protein transfer, the nitrocellulose membrane was incubated in blocking solution (3% nonfat dried milk in Tris-buffered saline [TBS], 50 mM Tris-HCl [pH 8.0], 150 mM NaCl) for 2 h with gentle agitation. All incubations were done at room temperature. The membrane was then incubated with an anti-topoisomerase I antibody (anti-Scl-70 antigen antibody, purchased from Immunovision Inc.) diluted 1:5,000 in blocking solution. The blot was washed in 200 ml of TBS three times before incubation with a secondary reagent (goat anti-human polyvalent immunoglobulin–alkaline phosphatase conjugate or –horseradish peroxidase conjugate, purchased from Sigma Immunochemicals, St. Louis, Mo.) at a 1:10,000 dilution in TBS for 1 h. The membrane was then washed three times with TBS. Protein was detected by incubation with either a luminescent or a chromogenic substrate (31). Densitometry was used to determine the relative amounts of protein from wild-type and heterozygous cells.

Collection of embryos. F_2 females heterozygous for the topoisomerase I-deficient allele were superovulated with 5 U of pregnant mare serum gonadotropin and given 5 U of human chorionic gonadotropin within 45 to 48 h. They were then mated with heterozygous F_1 males. Pregnant females at 3 to 4 days of gestation (vaginal plug counted as day 0) were euthanized by cervical dislocation. The oviducts were removed and flushed with modified M2 medium (Sigma Chemicals, St. Louis, Mo.). One- to 64-cell embryos were collected and placed in drops of M2 medium covered with paraffin oil.

Microscope slide preparation. Detergent-cleaned glass microscope slides were coated twice with a 2-mg/ml poly-L-lysine hydrobromide solution (molecular weight, >300,000; Sigma Chemicals) dissolved in distilled water (32).

Embryo preparation. The zona pellucida was removed with acidic Tyrodes solution (Sigma Chemicals). The embryos were allowed to equilibrate in M2 medium for 30 min and were washed with phosphate-buffered saline (pH 7.5) without calcium or magnesium (PBS). The tissue culture plate was coated with 1% Bacto-agar to prevent adhesion of the embryos (32).

Embryo fixation and permeabilization. A 7-μl drop of PBS was placed on the coated microscope slide. Embryos were dispensed into the drop. After a 5-min incubation at 37°C in a humidified chamber, 1 μl of 3.5% formaldehyde–0.1% Triton X-100 solution was added and the slide was placed at room temperature for 5 min. A 1-μl volume of 2.5% glutaraldehyde was then added. It was found to be important to keep the slides in a humidified chamber to prevent evaporation of solutions. After 5 min, the slides were submersed in PBS.

Immunofluorescence staining of embryos. Affixed embryos were immersed in blocking solution (15% bovine serum albumin, 0.1% Triton X-100, and 10% horse serum in PBS) for 1 h at room temperature, at which time they were incubated with an anti-topoisomerase I antibody (anti-Scl-70 antigen antibody; Immunovision Inc.) at a 1:100 dilution in blocking solution. The embryos were washed three times in 50 ml of blocking solution before incubation with a secondary reagent (goat anti-human polyvalent immunoglobulin–alkaline phosphatase conjugate; Sigma Immunochemicals). The three washes were repeated, and the embryos were then examined by immunofluorescence microscopy.

RESULTS

Enrichment for recombinant *Top-1* clones by using CPT in a selection protocol and attempts to isolate homozygous mutant ES cells. After electroporation of the targeting construct into BK4 cells and selection using G418 and ganciclovir, 192 resultant colonies were screened by PCR for clones having undergone homologous recombination events. Cell lines having undergone the planned targeting event generate a 1.8-kb PCR product when the two primers illustrated in Fig. 1C are used. Two colonies were detected, yielding a frequency of 1/96.

In a separate set of experiments, a selection protocol based on the topoisomerase I-specific cellular toxin CPT was used to attempt selection of homozygous recombinants. In these experiments, cells electroporated with the targeting construct were first selected with G418 and ganciclovir; approximately 100 resistant colonies per plate were found. Cells from nine

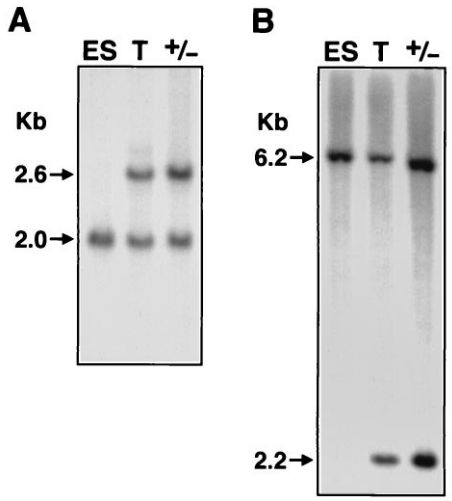

FIG. 2. (A) Autoradiogram from a Southern blot of mouse genomic DNA digested with *Pst*I showing the expected fragments for the wild-type (2.0 kb) and targeted (2.6 kb) alleles as indicated by the arrows. (B) Autoradiogram from a Southern blot of mouse genomic DNA digested with *Bam*HI showing the expected fragments for the wild-type (6.2 kb) and targeted (2.2 kb) alleles indicated by the arrows. Lanes ES, T, and +/−, genomic DNA from the parental ES cell line, the targeted ES cell line, and a heterozygous *Top-1* mutant mouse, respectively. The radioactive probe used for detection was a *Kpn*I fragment from the genomic clone of *Top-1*.

plates were then dispersed with trypsin and allowed to grow for 3 days in DMEM. At this time, the plates, which were approximately 70% confluent, were selected with various concentrations of CPT (250 nM, 500 nM, and 1 μM) in an attempt to select colonies completely lacking topoisomerase I activity. Only the 250 and 500 nM concentrations yielded colonies; all cells were killed by the 1 μM concentration. One colony from each of nine plates was then screened by PCR, and none of the nine independently isolated colonies were found to be homozygous for the inactivated allele. However, seven of the nine colonies were heterozygous for the inactive allele. Thus, although the protocol failed to select homozygous mutant clones, the isolation of *Top-1* heterozygous cell lines was significantly enhanced by selection in the presence of 250 or 500 nM CPT. Thus, a 75-fold enrichment of correctly targeted homologous recombinants was obtained by using G418, ganciclovir, and subsequent CPT selection (7 of 9), compared with G418 and ganciclovir selection alone (2 of 192).

We also attempted to isolate cells homozygous for the targeted allele according to the protocol of Mortenson et al. (25), which uses a G418 concentration higher than the original selection concentrations to select mutants homozygous for the *neo*-containing targeted allele. Even though we were successful with this protocol in isolating homozygous mutants for both *Ptgs-1* and *Ptgs-2* (23a), thus demonstrating the efficacy of the method in our hands, all attempts to isolate homozygous *Top-1* mutants were unsuccessful.

Confirmation of gene targeting and germ line transmission from chimeras to F_1 progeny. Genomic DNAs from the PCR-positive cell lines were digested with *Pst*I or *Bam*HI for Southern blot analysis. Correctly targeted cell lines generate a 2.6-kb *Pst*I fragment corresponding to the interrupted allele and a 2.0-kb *Pst*I fragment corresponding to the wild-type allele, or a 2.2-kb *Bam*HI fragment corresponding to the interrupted allele and a 6.2-kb *Bam*HI fragment corresponding to the wild-type allele (Fig. 1A and B). One of the targeted cell lines (Fig. 2) was injected into B6 blastocysts. This cell line gave rise to male chimeric animals which, when mated to B6 females, subsequently passed the targeted allele to their F_1 129/B6 offspring. F_1 heterozygotes were interbred, and Southern blot analysis of genomic DNAs from their F_2 progeny showed the 2.6- and/or 2.0-kb *Pst*I fragments expected for the wild-type or heterozygous genotypes (Fig. 2A). The expected 6.2- and/or 2.2-kb *Bam*HI fragments expected for wild-type and heterozygous genotypes were also observed (Fig. 2B).

Selection in CPT does not affect subsequent development of ES cells. A cell line that had been selected in 500 nM CPT was subsequently used for injection into blastocysts. The resultant chimeras displayed no overt pathology and were able to pass the 129 lineage on to a subsequent generation. This demonstrates that ES cells selected in CPT can retain their totipotent ability to recapitulate all necessary tissue types.

Heterozygotes have cellular levels of topoisomerase I lower than that of the wild type. Quantitation of topoisomerase I levels in F_2 animals was carried out by Western blotting. Six wild-type and six heterozygous animals were analyzed. An average of 45% reduction in protein levels was observed in the heterozygotes as measured by densitometry. A representative Western blot showing topoisomerase I levels for two individual wild-type animals (lanes +/+) and two individual heterozygous animals (lanes +/−) is shown in Fig. 3.

The remaining allele in the heterozygous mutant cell line remains CPT sensitive. In order to determine whether the wild-type allele in the heterozygous mutant cell line had also undergone a mutational event (resulting in CPT resistance), nuclear extracts from both wild-type and heterozygous mutant ES cells were assayed for CPT sensitivity (data not shown). Equivalent amounts of nuclear extracts from heterozygous and wild-type ES cells were assayed for strand breakage in the absence and presence of CPT. Both extracts demonstrated increased strand breakage in the presence of CPT, indicating that the other allele in the ES cell line heterozygous for the disrupted allele had not been mutated to a CPT-resistant form. Titration of the nuclear extract demonstrates that there is less strand breakage activity in the mutant than in the wild type. This would be expected, since only one copy of the wild-type allele is present in heterozygous cells. Western blotting confirms a reduced level of topoisomerase I in heterozygous cells (Fig. 3).

Genotypic analysis of F_2 progeny from heterozygous F_1 matings. The genotypes of progeny from F_1 heterozygous females mated to heterozygous F_1 males were analyzed after Southern

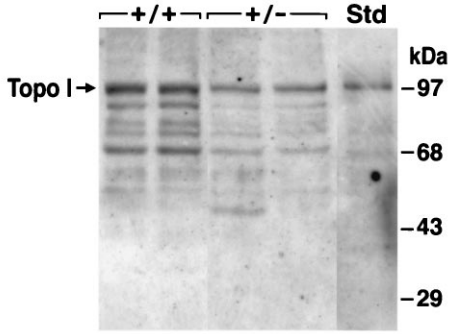

FIG. 3. Western blot of salt-soluble nuclear protein from wild-type and heterozygous mutant *Top-1* liver cells. Lanes: +/+ and +/−, nuclear protein fractions (50 μg per lane) from two individual wild-type and two individual heterozygous mutant mice, respectively; Std, purified HeLa topoisomerase I standard. Positions of molecular mass markers are indicated on the right. The position of the full-size topoisomerase I protein (Topo I) is indicated on the left.

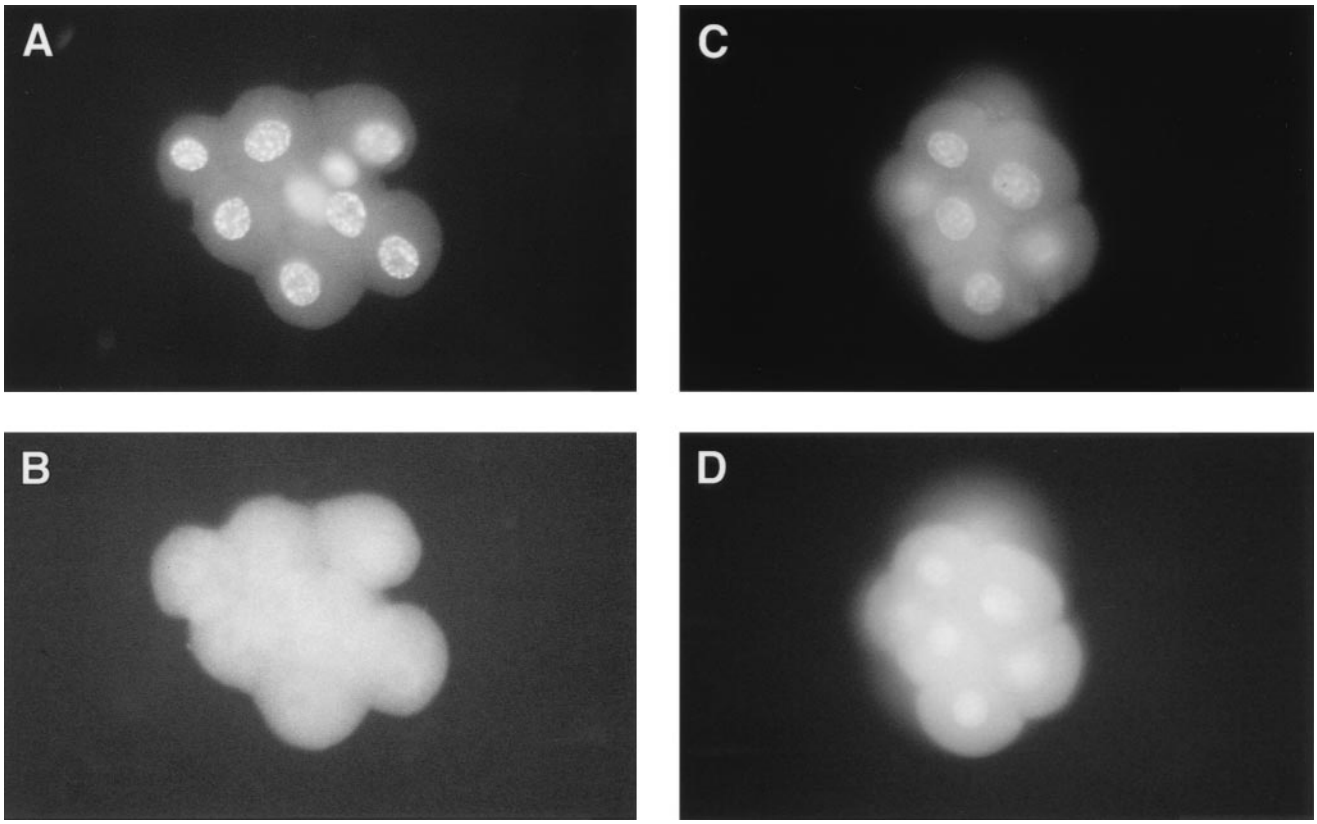

FIG. 4. Cytological analysis of wild-type (C and D) and topoisomerase-deficient mutant (A and B) embryos. Embryos were fixed with formaldehyde and stained with bisbenzimide (A and C) and anti-Scl-70, an antitopoisomerase antibody (B and D).

blotting. No homozygotes were found among the 125 F_2 progeny genotyped. Thus, the *Top-1* inactivated allele is lethal when homozygous. However, the genotypic ratios of the progeny lead to some interesting corollaries. The ratio of wild-type to heterozygous progeny at weaning (3 weeks after birth) was 41/84, which does not differ from the expected ratio of 1:2 ($P > 0.5$). This shows that both sperm and oocytes containing the inactive allele are derived from a productive gametogenesis and maintain their viability through fertilization. Thus, gene expression of topoisomerase I is not required for sperm or oocyte viability until after fertilization occurs. We find that a haploid complement of the enzyme is not lethal or obviously deleterious for the animal.

Topoisomerase I is linked to the agouti locus. Mouse strain 129 has a recessive wild-type allele (*a*) at the agouti locus. Strain B6 is homozygous for the dominant mutant allele (*A*). Inspection of the coat colors of the heterozygotes allows the linkage of *Top-1* to the agouti locus (*A*) to be determined. Of 68 mice heterozygous for the targeted *Top-1* mutation, 4 had solid black coats and 64 were agouti; of 33 wild-type mice, 2 were agouti and 33 were black. Both ratios, 4 of 68 and 2 of 33, indicate a 6% recombination frequency, i.e., a map distance of approximately 13 centimorgans between the agouti locus (*A*) and *Top-1*. This is in agreement with current data which demonstrate that both agouti and *Top-1* reside on mouse chromosome 2 (16, 44).

Determination of lethality in early-stage embryos. Embryos were isolated from superovulated females that had been bred in either Het × Het or WT × WT crosses (where Het is heterozygote and WT is wild type). In order to determine at what stage homozygous embryos become inviable, we used immunofluorescence to detect topoisomerase I in the nuclei of embryos at various stages of development. The embryos were fixed and treated with human anti-topoisomerase I antibody, and detection was accomplished by using a goat anti-human antibody conjugated with fluorescein. Embryos were also stained with bisbenzimide for visualization of DNA. All 25 Het × Het embryos at the >32-cell stage were found to contain topoisomerase I in all nuclei, as were all 30 Het × Het embryos at the 16-cell stage. Seven of 52 embryos from the eight-cell stage and 6 of 21 four-cell-stage Het × Het embryos were found to lack topoisomerase I (Fig. 4C), even though all the nuclei were readily visible with bisbenzimide staining (Fig. 4A). However, all 28 two-cell-stage Het × Het embryos contained topoisomerase I. The nuclei of all WT × WT embryos at all stages were visible with bisbenzimide staining (Fig. 4B), and they also contained topoisomerase I in all nuclei (Fig. 4D). These data show that homozygous mutants develop to the 4- or 8-cell stage but all fail to reach the 16-cell stage.

DISCUSSION

With all gene targeting procedures currently in use, some type of selection is necessary to allow the isolation of recombinants. Here, we have used a selection protocol for the isolation of a topoisomerase I knockout allele that depends on a dominant selection against cells containing wild-type levels of topoisomerase I. The selection protocol uses a protein-specific toxin as the selective agent. CPT is such an agent for topoisomerase I (2, 11, 26, 30), and its use greatly simplified our isolation of cells heterozygous for inactivation of the *Top-1* allele. The effectiveness of the approach is exemplified by the follow-

ing: only 2 of 192 G418- and ganciclovir-selected clones (1%) were found to have undergone the desired recombination, whereas 7 of 9 of the clones selected first with G418-ganciclovir and then with CPT (77%) gave the desired homologous recombinant. This represents a 75-fold increase in selection efficiency.

Toxins specific to other enzymes might be used to select targeted clones in conjunction with the more traditional selection methods using G418 and ganciclovir. This eliminates much of the more laborious genotypic screening usually needed to isolate targeted clones. Our success using CPT as a selection agent validates this type of approach and may thus have a broad utility in developing selection procedures for specific gene-targeting events. Indeed, by using an enzyme-specific toxin selection protocol, it may be possible to isolate ES cell clones homozygous for the targeted allele without further steps.

We have demonstrated that the untargeted topoisomerase I allele isolated by our CPT selection protocol is sensitive to CPT in vitro (data not shown). In addition, heterozygous cells contain a reduced level of topoisomerase I (Fig. 3). This suggests that a lower level of topoisomerase I protects cells from the drug, a contention supported by data from cell selection experiments using CPT (13, 33). These data from our experiments do not, however, exclude the possibility that the nontargeted allele harbors a subtle mutation which renders it partially resistant to CPT. In addition (for G418-, ganciclovir-, and CPT-selected cells), we cannot rule out the presence of other mutations elsewhere in the genome affecting CPT resistance. Note, however, that the wild-type allele present in the heterozygous F_1 and F_2 animals comes from the B6 lineage of the female that was mated to the chimera and is thus a wild-type B6 *Top-1* gene.

There is evidence that CPT causes differentiation in various cell lines (17), although the reason for this is unknown at present. However, in our experiments, recombinant ES cells selected in 500 nM CPT retained their totipotent ability to develop into a whole organism; thus, they show no evidence of having differentiated after treatment.

In *Escherichia coli*, inactive mutants of the topoisomerase I gene are isolated only in the presence of compensatory mutations in the topoisomerase II gene or other genes (6, 28, 29). Topoisomerase I is not essential in the eukaryotes *S. cerevisiae* and *Schizosaccharomyces pombe* (38, 39) but is required for development past the blastocyst stage in *D. melanogaster* (21). In addition, cellular selection in vitro with CPT results in mutations which reduce cellular topoisomerase I levels (13, 33) or render the enzyme resistant to the drug (19, 36). The gene may thus be essential in more-complex eukaryotes.

The importance of topoisomerase I to cellular function in mammals has been demonstrated in the present study by the deleterious effect of its absence on the developing mouse embryo. Thus, failure of *Top-1* $-/-$ embryos to continue development at the 4- to 16-cell stage is strongly suggestive that topoisomerase I is essential for cell division and that enzyme expression is required very early after fertilization. Since we can detect the presence of the enzyme in all two-cell Het $\times$ Het embryos, it is likely that a maternal complement of topoisomerase I or *Top-1* message is present in oocytes; this allows development to the four- to eight-cell stage in homozygous mutant embryos. However, because no new topoisomerase I message can be synthesized in *Top-1* $-/-$ cells, this maternal complement of enzyme eventually becomes limiting, and further cell division ceases. In any case, it is clear that topoisomerase I is essential for development in the mouse.

Our failure to isolate a homozygous ES cell line by using either of two powerful selection procedures supports the proposition that *Top-1* is essential for cell growth in tissue culture. Thus, while both an increased level of G418 (25) and a high level of CPT (1 μM) were used to attempt selection of homozygous mutant ES cells, neither yielded surviving colonies. Although this does not directly demonstrate that cells require topoisomerase I for growth, the inability to isolate cells carrying homozygous targeted alleles strongly suggests that *Top-1* is necessary for cellular division.

Breeding data also demonstrate the essential nature of the topoisomerase I gene, since no animals homozygous for the mutation have been found among the 125 progeny examined. However, because the numbers of heterozygotes and wild-type animals obtained in the F_1 by F_1 matings do not differ from the expected 2:1 ratio, the data also demonstrate that topoisomerase I is not essential for gamete viability. If both the sperm and the oocytes were inviable in the mutant haploid state, only wild-type pups would be born. If either the sperm or the oocytes were inviable, then the ratio of heterozygotes to wild type would be 1:1. Breeding data also indicate that the topoisomerase I gene is linked to the agouti gene (*A*) at a distance of approximately 13 centimorgans.

Our demonstration that topoisomerase I is a crucial enzyme for cell growth and embryo development suggests that cell lines derived from the heterozygous animals will be useful for mutagenesis studies to investigate the mechanisms of topoisomerase I action. Since such cell lines contain only a single copy of the wild-type *Top-1* allele, the isolation of mutants is simplified and hence a variety of complementation studies can be attempted. Temperature-sensitive mutant cells would provide a valuable tool for discerning how the enzyme acts in replication, transcription, and recombination.

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