

# Mitochondrial NAD-Dependent Methylene-tetrahydrofolate Dehydrogenase-Methenyltetrahydrofolate Cyclohydrolase Is Essential for Embryonic Development

E. Di Pietro,<sup>1</sup> J. Sirois,<sup>2</sup> M. L. Tremblay,<sup>2</sup> and R. E. MacKenzie<sup>1\*</sup>

Department of Biochemistry<sup>1</sup> and McGill Cancer Center,<sup>2</sup> McGill University, Montreal, Quebec, Canada H3G 1Y6

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**Folate-dependent enzymes are compartmentalized between the cytoplasm and mitochondria of eukaryotes. The role of mitochondrial folate-dependent metabolism and the extent of its contribution to cytoplasmic processes are areas of active investigation. NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase (NMDMC) catalyzes the interconversion of 5,10-methylenetetrahydrofolate and 10-formyltetrahydrofolate in mitochondria of mammalian cells, but its metabolic role is not yet clear. Its expression in embryonic tissues but not in most adult tissues as well as its stringent transcriptional regulation led us to postulate that it may play a role in embryonic development. To investigate the metabolic role of NMDMC, we used a knockout approach to delete the *nmdmc* gene in mice. Heterozygous mice appear healthy, but homozygous NMDMC knockout mice die in utero. At embryonic day 12.5 (E12.5), homozygous null embryos exhibit no obvious developmental defects but are smaller and pale and die soon thereafter. Mutant fetal livers contain fewer nucleated cells and lack the characteristic redness of wild-type or heterozygous livers. The frequencies of CFU-erythroid (CFU-E) and burst-forming unit-erythroid (BFU-E) from fetal livers of E12.5 null mutants were not reduced compared with those of wild-type or heterozygous embryos. It has been assumed that initiation of protein synthesis in mitochondria requires a formylated methionyl-tRNA<sup>met</sup>. One role postulated for NMDMC is to provide 10-formyltetrahydrofolate as a formyl group donor for the synthesis of this formylmethionyl-tRNA<sup>met</sup>. To determine if the loss of NMDMC impairs protein synthesis and thus could be a cause of embryonic lethality, mitochondrial translation products were examined in cells in culture. Mitochondrial protein synthesis was unaffected in NMDMC-null mutant cell lines compared with the wild type. These results show that NMDMC is not required to support initiation of protein synthesis in mitochondria in isolated cells but instead demonstrate an essential role for mitochondrial folate metabolism during embryonic development.**

Folic acid is essential for the synthesis of purines and thymidylate and is also required for the remethylation of homocysteine to methionine to support the many cellular methylation reactions that use [S]adenosylmethionine. Because of its numerous roles, it is not surprising that the status of folic acid has been linked with a number of conditions that include megaloblastic anemia, cardiovascular disease, and birth defects, as well as with several types of cancer (for a review, see reference 13). The metabolically active form, tetrahydrofolate (THF), functions to transfer one-carbon units that exist in different oxidation states (14). These one-carbon folates are interconverted by enzymes that are present in cytosolic and mitochondrial compartments in mammalian cells (Fig. 1). Recently, there has been a considerable interest in understanding the metabolic advantages for this compartmentalization, supported by studies in mammalian cell lines. A mutant of a Chinese hamster ovary (CHO) cell line, GlyA, that lacks mitochondrial serine hydroxymethyltransferase was observed to be a glycine auxotroph even though it retains cytoplasmic serine hydroxymethyltransferase (2). Studies on another CHO

mutant cell line, AUXB1, that lacks all folylpoly- $\gamma$ -glutamate synthetase activity have shown that mitochondrial folylpoly- $\gamma$ -glutamate synthetase activity is also required to support the cellular requirement for glycine (5, 11, 12). Studies on these two cell lines demonstrate that the mammalian mitochondrial folate pathways contribute to overall cellular metabolism.

Trifunctional methylene-THF dehydrogenase-methenyl-THF cyclohydrolase-formyl-THF synthetase (DCS) consists of an amino-terminal 33-kDa domain containing the dehydrogenase and cyclohydrolase activities linked to a 67-kDa synthetase domain. In yeast, the cytoplasm and mitochondria each contain a trifunctional NADP-dependent DCS enzyme, encoded by different nuclear genes (26). Appling's group has demonstrated that yeast mitochondria produce formate (and ATP) via synthetase activity (18, 30). In this model, one-carbon flow through the mitochondrial DCS is in the direction of methylene- to formyl-THF to free formate that is released from the mitochondria and can be reconverted to methylene-THF by the cytosolic DCS (requiring ATP) in the opposite direction.

Mammalian cells (Fig. 1) contain a trifunctional NADP-dependent DCS in the cytoplasm (14). It has been found to be ubiquitously expressed in tissues and cell lines (15, 27, 29) and functions as a housekeeping gene (20). However, no DCS has been purified from mammalian mitochondria, and the homolog of the mitochondrial DCS in mammals is a bifunctional NAD-dependent methylene-THF dehydrogenase-methenyl-THF

\* Corresponding author. Mailing address: Department of Biochemistry, McGill University, McIntyre Medical Sciences Building, 3655 Promenade Sir William Osler, Montreal, Quebec, Canada H3G 1Y6. Phone: (514) 398-3523. Fax: (514) 398-7384. E-mail: robert.mackenzie@mcgill.ca.

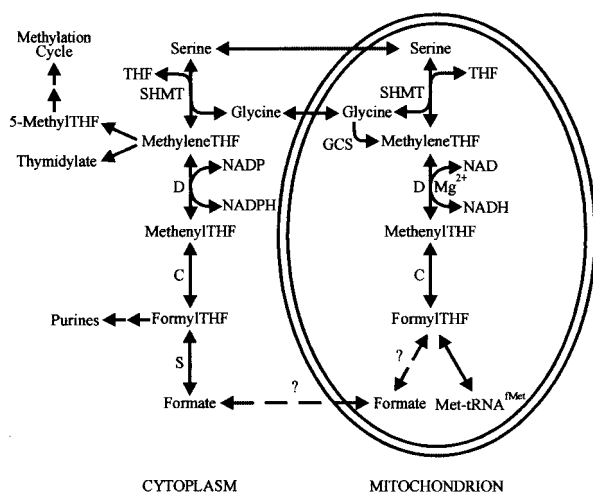


FIG. 1. Compartmentalization of folate-dependent activities in mammalian cells. Abbreviations are SHMT, serine hydroxymethyltransferase; D, methylene-THF dehydrogenase; C, methenyl-THF cyclohydrolase; S, 10-formyl-THF synthetase; and GCS, glycine cleavage system. Adapted from Yang and MacKenzie (31).

cyclohydrolase (NMDMC) that lacks a synthetase domain (1, 16, 17, 31). It has been detected in many transformed and established cell lines, as well as in embryonic but not in differentiated tissues (15, 27), and its expression is much more highly regulated than that of the cytoplasmic DCS enzyme (21). These observations lead us to postulate that NMDMC has an important role in early development and might relate to folate-responsive birth defects. Its location in mitochondria suggests a possible role in mitochondrial biogenesis. However, it is not known whether this bifunctional NMDMC is required to support mitochondrial protein synthesis by providing 10-formyl-THF to formylate the initiator methionyl-tRNA, or if it plays some other role such as a variation of the formate cycle as in yeast. Either of these functions might be particularly important during embryonic development. We have used a gene inactivation approach in mice to gain some initial insight into its metabolic function.

#### MATERIALS AND METHODS

**Generation of gene-targeted cell lines.** The NMDMC gene was cloned from a 129/SvJ mouse genomic library, in the bacteriophage lambda DASH, using the full-length murine cDNA as a probe. A 1.6-kb *EcoRI* fragment containing exon 2 and part of exon 3 and a 3.5-kb *HindIII* fragment containing part of exon 8 were inserted into pMC1neopA (Stratagene). The herpes simplex virus thymidine kinase gene was also subcloned into the vector for negative selection. The resulting targeting vector, pMC1neo $\Delta$ 3-8/TK, replaces part of exon 3, exons 4 to 7, and part of exon 8 with a neomycin resistance gene (Fig. 2A). The DNA construct was prepared by the QIAGEN midprep protocol and linearized with *HindIII*.

Embryonic stem (ES) cells were grown on mitotically inactivated feeder cells. Dulbecco's modified Eagle's medium was supplemented with leukemia inhibitory factor at 1,000 U/ml (ESGRO; Gibco BRL). The 129/Sv ES cell line R1 ( $1 \times 10^7$  to  $2 \times 10^7$  cells) was electroporated with 50  $\mu$ g of linearized construct DNA using a gene pulser (240 V, 500  $\mu$ F; Bio-Rad, Hercules, Calif.). After electroporation, cells were plated at a density of  $2 \times 10^6$  cells/10-cm plate on neomycin-resistant feeder cells. G418 (300  $\mu$ g/ml) and ganciclovir (2  $\mu$ M) selection was applied 24 h after electroporation. Doubly resistant clones were transferred to 96-well plates 10 to 12 days after electroporation and were expanded for freezing and DNA extraction as previously described (24). Colonies doubly resistant to

G418 and ganciclovir were screened for homologous recombination by Southern blot analysis.

**Generation of chimeric and heterozygous mice.** Targeted ES cells were injected into 3.5-day-old BALB/c blastocysts and were transferred into CD-1 foster mothers, and animals classified as chimeras by coat color were mated with BALB/c mice. Germ line transmission was achieved with two independently targeted cell lines. For timed pregnancies, mice were mated overnight and female mice scored for vaginal plaques the next morning were taken to represent embryonic day 0.5 (E0.5).

**Isolation of homozygous mutant NMDMC cells.** NMDMC<sup>-/-</sup> ES cells were generated in two different ways. NMDMC<sup>+/-</sup> ES clones were cultured at increasing concentrations of G418 (up to 2 mg/ml), and highly resistant clones were genotyped by Southern blot analysis to identify <sup>-/-</sup> cells. In a second approach, transformed mouse embryonic fibroblast cell lines were derived from primary embryonic fibroblast cultures isolated from mouse embryos. Briefly, embryos were isolated at E9.5 from the mating of <sup>+/-</sup> mice. Embryos were trypsinized for 10 min at 37°C, and the cells were cultured. Cells were transformed by infection with large T antigen for 2 h at 37°C in serum-free medium.

**DNA probe preparation.** A 161-bp fragment (E2/161) to be used as a probe was prepared by PCR amplification of exon 2 using primers: left primer 5'-GAAGCCGTGTTCATT TCTGG-3' and right primer 5'-GCCCTGGTTTGTGAGAAC-3'. A 504-bp fragment containing part of exon 8 (E8/504) and the *neo* cassette from pMC1neopA were prepared by restriction enzyme digestion of plasmid DNA followed by agarose gel electrophoresis and QIAquick (QIAGEN) purification of the excised DNA fragments. The purified fragments were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham) using the multiprime DNA-labeling kit (Amersham). Removal of the unincorporated nucleotides was achieved by Sephadex G-25 (Pharmacia Biotech) spin chromatography.

**Southern blot analysis.** DNA from individual ES cell clones grown in 96-well plates (24) and 5 to 10  $\mu$ g of genomic DNA isolated from mouse tails or from yolk sac DNA (6) were digested to completion with *Bam*HI (25 to 50 U). Digested DNA was electrophoresed through a 0.8% agarose gel (20 by 25 cm) at 25 V overnight and was then vacuum transferred onto Hybond N+ (Amersham) at 4 mm Hg for 1.5 h. The membranes were pretreated with hybridization solution (35% deionized formamide, 5 $\times$  SSPE [1 $\times$  SSPE is 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1 mM EDTA {pH 7.7}], 5 $\times$  Denhardt's solution, 0.5% sodium dodecyl sulfate [SDS], and 100  $\mu$ g of salmon sperm DNA/ml) for at least 2 h at 42°C and were hybridized in the presence of  $\alpha$ -<sup>32</sup>P-labeled probe (E2/161, E8/504, or *neo*) overnight. Membranes were soaked in 1 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS to 65°C (from 42°C), 1 $\times$  SSC and 0.1% SDS at 65°C for 30 min, and 0.1 $\times$  SSC and 0.1% SDS at 65°C for 30 min and were exposed to BIOMAX MS film (Kodak) with a BIOMAX intensifying screen at -80°C.

**PCR.** Genotypes of embryos that were harvested at  $\leq$ E10.5 were determined by PCR. DNA was isolated from the yolk sacs of embryos using the DNeasy protocol for animal tissues (QIAGEN). Yolk sac DNA (100 to 200 ng) was used with the following PCR primers: P1 5'-GAAAGTGCTAAGACCAGAGGAC-3' and P2 5'-TAACCCAAGCTCTGGATAGC-3' to amplify a 916-bp wild-type fragment or P3 5'-ATCTCTGTACATCCATCTGC-3' to amplify a 1,306-bp targeted fragment. PCR conditions were as follows: 200 nM concentrations of 5' and 3' primers, 250  $\mu$ M concentrations of deoxynucleoside triphosphates, 1 $\times$  QIAGEN *Taq* buffer, 2 mM MgCl<sub>2</sub>, and 2.5 U of QIAGEN *Taq* polymerase in a final volume of 50  $\mu$ l with 1 cycle at 94°C for 3 min; 30 cycles at 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

**Enzyme assays.** Cells were harvested from three or four T175 tissue culture flasks to obtain 200 to 500  $\mu$ l of packed cells. Cells harvested for enzyme assays were removed by a rubber policeman 24 h after the last feeding. Cell pellets were resuspended in 3 volumes of 0.1 M potassium phosphate, pH 7.3, 35 mM 2-mercaptoethanol, 1 mM benzimidazole, and 1 mM phenylmethylsulfonyl fluoride and were sonicated twice for 10 s. Glycerol (0.25 volumes) was added to the extract and was centrifuged for 10 min in a microcentrifuge at 4°C. The supernatant was used for determination of enzyme activity as previously described (15). Assays were performed in triplicate.

**Cell staining with Mitotracker mitochondrion-selective probes.** Lyophilized Mitotracker Green FM (Molecular Probes) was dissolved in dimethyl sulfoxide to a final concentration of 1 mM. Cells were grown on tissue culture chamber slides (Nunc Lab-Tek). Once the cells had reached the desired confluence, fresh medium containing 500 nM Mitotracker Green FM was added and cells were incubated at 37°C for 45 min. The probe-containing medium was replaced with fresh medium. The cells were observed using a fluorescence microscope.

**Analysis of mitochondrial translation products.** Exponentially growing embryonic fibroblast cells in six-well plates were washed twice with phosphate-buffered saline (PBS) and were then preincubated in methionine- and cysteine-

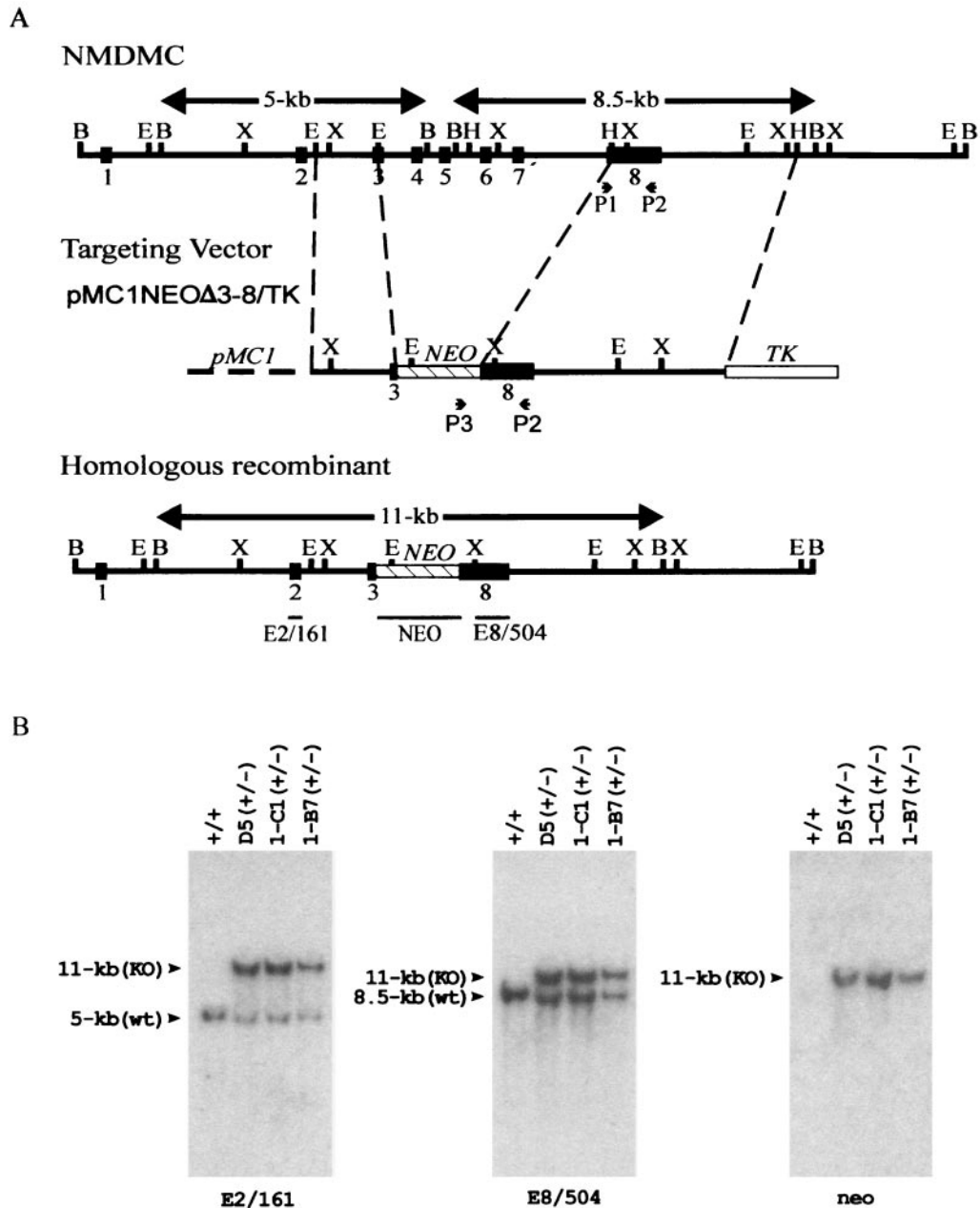


FIG. 2. Disruption of NMDMC in ES cells. (A) Gene-targeting strategy. A partial restriction map of the *nmdmc* gene is displayed (top). Exons are numbered and indicated by shaded boxes, and thin lines represent introns. The location of probes for hybridization (E2/161, E8/504, and *neo*) and primers for PCR (P1, P2, and P3) are indicated. The targeting vector pMC1neoΔ3-8/TK is shown, and the predicted targeted allele is indicated. The vector contains a 5' arm, including exon 2 and part of exon 3, and a 3' arm including part of exon 8. The E2/161 probe is specific for *Bam*HI fragments containing exon 2 and detects a 5-kb fragment from the wild-type allele and an 11-kb fragment from the targeted allele generated by *Bam*HI digestion. The E8/504 probe is specific for *Bam*HI fragments containing exon 8 and hybridizes to an 8.5-kb fragment from the wild-type allele and an 11-kb fragment from the targeted allele. The *neo* probe hybridizes to the neomycin cassette. Abbreviations used: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; X, *Xba*I; NEO, neomycin resistance gene; and TK, herpes simplex virus thymidine kinase gene. (B) Southern blot analysis of DNA isolated from ES cell clones. *Bam*HI-digested ES cell DNA was hybridized with E2/161, E8/504, or *neo* probes. The size (in kilobases) of the NMDMC-specific *Bam*HI fragments is indicated on the left. KO, knockout; wt, wild type.

free medium with the cytoplasmic translation inhibitor, emetine (100  $\mu$ g/ml), for 5 min at 37°C. A parallel control experiment was performed that also included chloramphenicol (100  $\mu$ g/ml), an inhibitor of mitochondrial protein synthesis. Cells were labeled for 1 h at 37°C with [<sup>35</sup>S]methionine (200  $\mu$ Ci of Trans<sup>35</sup>S label [ICN] per ml) in methionine- and cysteine-free medium. The radiolabel was diluted with unlabeled methionine (30  $\mu$ g/ml) and cysteine (48  $\mu$ g/ml), and the cells were incubated for 10 min at 37°C and washed three times with PBS.

Trypsinized cells were lysed, and identical amounts of <sup>35</sup>S-labeled total protein were loaded on a 12 to 20% gradient gel (9) containing 1% glycerol to prevent the gel from cracking. Gels were stained, fixed, treated with En<sup>3</sup>Hance (Dupont), dried, and exposed to X-Omat AR film (Kodak) for 2 days. Mitochondrial translation products were assigned as described by Chomyn et al. (4).

**Hematopoietic progenitor cell colony-forming assays.** Individual fetal livers from E12.5 embryos were dissected, disaggregated into single-cell suspensions in

TABLE 1. Breeding data from heterozygous matings

Cell line	Background	No. of litters	No. of pups	No. of female pups				No. of male pups			
				+/+	+/-	-/-	Total	+/+	+/-	-/-	Total
D5	BALB/c	137	637	120	210	0	330	105	202	0	307
1-B7	BALB/c	19	160	25	61	0	86	26	48	0	74
D5	129/Sv (inbred)	10	37	8	10	0	18	5	14	0	19

PBS, pH 7.2, and passed through a cell strainer (70- $\mu$ m pore size; Falcon). An aliquot of cells was diluted in 3% acetic acid with methylene blue (StemCell Technologies Inc., Vancouver, Canada) to lyse nonnucleated mature erythrocytes, and the remaining cells were counted. Fetal liver cells for burst-forming unit-erythroid (BFU-E) assays ( $2 \times 10^4$  cells) or for CFU-erythroid (CFU-E) assays ( $10^4$  cells) were plated in duplicate in methylcellulose medium containing erythropoietin only for CFU-E assays (MethoCult M3334; StemCell Technologies Inc.) or containing erythropoietin, stem cell factor, interleukin 3 (IL-3), and IL-6 for BFU-E assays (MethoCult M3434 GF; StemCell Technologies Inc.). Colonies were counted by placing each 35-mm-diameter tissue culture plate within a gridded 60-mm-diameter tissue culture plate. Benzidine-positive CFU-E colonies were counted after 3 days. Benzidine-positive, benzidine-negative, and mixed colonies were counted after 7 to 10 days (BFU-E assays). For benzidine staining, 5% diaminobenzidine tetrahydrochloride in 0.1 M phosphate buffer, pH 7, was mixed with 10 ml of PBS, pH 7.2, and 10  $\mu$ l of 30% hydrogen peroxide. One milliliter of the cold mixture was carefully layered on top of the methylcellulose culture and incubated at 37°C for 10 to 15 min, and colonies were counted.

## RESULTS

**Targeted disruption of the NMDMC gene.** To disrupt the NMDMC gene, a targeting vector designated pMC1neo $\Delta$ 3-8/TK was constructed that was designed to replace part of exon 3, exons 4 to 7, and part of exon 8 with the neomycin resistance gene from pMC1neoP. A herpes simplex virus thymidine kinase gene was also included in the vector for negative selection (Fig. 2A). The construct was electroporated into the 129/Sv ES cell line R1, and targeted colonies were identified by genomic Southern blot analysis using three different probes (Fig. 2A). Out of approximately 1,100 neomycin-resistant ES cell clones subjected to Southern blot analysis, seven clones (D5, 1-C1, 2-B11, 1-B7, 4-A8, 5-H8, and 6-G10) had properly targeted alleles. As shown in Fig. 2B, the targeted clones were identified by using a 161-bp probe containing exon 2 (E2/161), lying outside the targeted region, that identifies a shift in a 5-kb wild-type *Bam*HI fragment to an 11-kb targeted fragment. These positive clones were confirmed with a 504-bp probe containing part of exon 8 (E8/504), which hybridizes to an 8.5-kb wild-type *Bam*HI fragment and an 11-kb targeted fragment. The probe to the *neo* cassette detected a single band in all these cell lines, hybridizing with the same 11-kb fragment from the targeted allele, indicating that the neomycin gene was present only at the NMDMC locus. ES clones derived from three independent cell lines, D5, 1-C1, and 1-B7, were used for blastocyst injection.

**Lethality of NMDMC deletion.** ES cells were injected into 3.5-day-old BALB/c blastocysts and were then implanted into the uteri of CD-1 pseudopregnant females. A total of 12 female and 13 male chimeras were obtained and were crossed with BALB/c mice to generate heterozygotes for the knockout allele. Three female chimeras derived from the D5 cell line and three males from the 1-B7 cell line gave germ line transmission, while the female chimera obtained with cell line 1-C1 was not germ line competent. Mice heterozygous for the disrupted

gene appear phenotypically normal. Out of 637 mice obtained from intercrosses of heterozygous mice generated from the D5 cell line and 160 obtained from the 1-B7 cell line, none was homozygous for the NMDMC gene disruption as determined by Southern blot analysis of tail DNA (Table 1). Heterozygous intercrosses of mice from the D5 cell line in a 129/Sv inbred background also did not give rise to any NMDMC homozygous mice (Table 1), and heterozygous offspring appeared phenotypically normal. In all cases, sex ratios for offspring from heterozygous matings gave the expected equal numbers of males and females. These results indicate that the defect is lethal in utero at the homozygous level.

Embryos at various stages of development were then analyzed to detect any developmental defects and to estimate the embryonic age at which death occurs. Genotypes of embryos that were  $\leq$  E10.5 were determined by PCR (Fig. 3A). Genotypes of embryos that were  $\geq$  E11.5 were determined by Southern blot analysis (Fig. 3B). Homozygous embryos were identified at various days of gestation (Table 2). At E12.5, the live homozygous embryos for the NMDMC gene disruption are distinguishable from wild-type or heterozygous littermates. There are no obvious gross developmental abnormalities, but the homozygous null mutants are smaller and paler than the normal littermates (Fig. 4). In particular, the mutant fetal livers are smaller and do not have the typical redness of a normal liver. Beyond E13.5, most null mutant embryos were dead, and by E15.5, all recovered *nmdmc*<sup>-/-</sup> embryos were dead and appeared to be at different stages of resorption. Preliminary analysis of histological sections of E9.5-to-E14.5 *nmdmc*<sup>-/-</sup> embryos showed no apparent abnormalities.

To determine whether the paleness of the embryos was a result of a defect in the formation of progenitor cells for hematopoiesis, we performed colony-forming assays. Single-cell suspensions of fetal liver cells from E12.5 embryos of each genotype were plated in methylcellulose culture supplemented with a variety of growth factors supporting the development of hematopoietic colonies. At E12.5, the total number of nucleated cells in livers of null mutant embryos was greatly reduced compared to that in fetal livers of wild-type or heterozygous littermates (Fig. 5A). However, the frequency of CFU-E (Fig. 5B) and BFU-E (Fig. 5C) was similar between genotypes. The frequency of benzidine-negative and mixed colonies from mutant fetal livers was also unchanged (data not shown).

**Characterization of +/+, +/-, and -/- cell lines.** The NMDMC enzyme is localized in mitochondria where it has been postulated that it is involved in providing the 10-formyl-THF necessary for formylation of the initiator methionyl-tRNA<sup>fmet</sup>. Impaired mitochondrial function might explain the slower development and be responsible for the death of embryos. To test this possibility, we generated transformed mouse

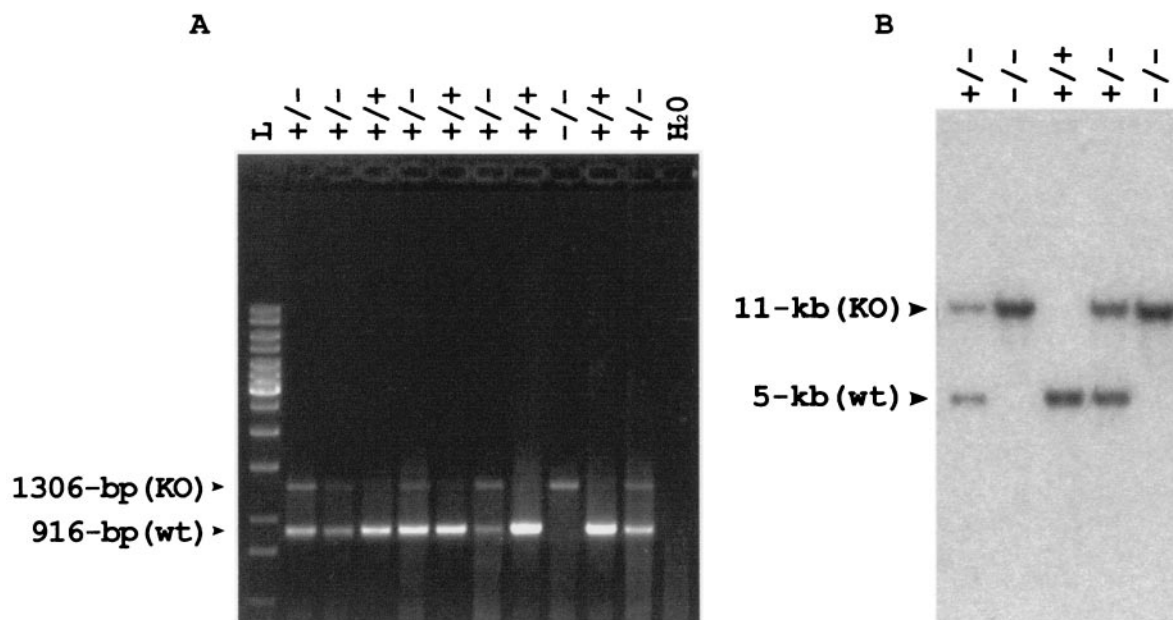


FIG. 3. Analysis of DNA isolated from embryos of  $+/-$  intercrosses. (A) PCR of yolk sac DNA from 9.5-day-old embryos with PCR primers P1 and either P2 or P3 amplified a 916-bp wild-type fragment or 1,306-bp targeted fragment. L, 100-bp DNA Ladder Plus (MBI Fermentas). (B) Southern blot analysis of yolk sac DNA digested with *Bam*HI and hybridized with the flanking probe E2/161. The 5-kb band indicates transmission of the wild-type (wt) allele, and the 11-kb band indicates transmission of the targeted (KO) allele.

embryonic fibroblasts from E9.5 embryos isolated from heterozygous intercrosses. NMDMC-null ES cell lines were also generated by culturing heterozygous ES cells in increasing concentrations of G418. Genotypes of the cell lines were determined by Southern blot analysis (Fig. 6).

Cellular extracts from ES cells and transformed mouse embryonic fibroblast cells were assayed for NAD-dependent methylene-THF dehydrogenase activity as well as cytosolic NADP-dependent activity. As shown in Table 3, both NMDMC-null mutant cell lines had undetectable levels of NAD-dependent enzyme activity, confirming that both alleles had been disrupted in these cell lines. As expected, all cell lines had NADP-dependent dehydrogenase activity.

As a first measure of mitochondrial function,  $+/+$ ,  $+/-$ , and  $-/-$  cell lines were stained with Mitotracker Green FM to visualize mitochondria. As shown in Fig. 7, there are no obvious differences in the mitochondrial network of the three cell lines.

TABLE 2. Genotypic analysis of embryos from heterozygous intercrosses

Day	No. of litters	No. of embryos	No. of embryos per genotype		
			$+/+$	$+/-$	$-/-$
9.5	19	122	42	48	32
10.5	12	65	22	30	13
11.5	2	16	9	6	1
12.5	14	104	27	56	21
13.5	8	46	14	22	10
14.5	5	44	12	22	10
15.5	12	69	19	34	16
18.5	5	31	5	24	2
19.5	1	8	6	2	0

To examine if deletion of NMDMC has any effect on mitochondrial protein synthesis, the translation of mitochondrially encoded genes was examined by pulse labeling exponentially growing cells with [ $^{35}$ S]methionine in the presence of the cytoplasmic translation inhibitor emetine, so that only mitochondrial translation products were labeled. There is no difference in the labeling and pattern of mitochondrial peptides among the three cell lines (Fig. 8). An identical experiment was conducted in the presence of chloramphenicol, in addition to that of emetine, to inhibit translation in both the cytoplasm and mitochondria to control for background labeling (Fig. 8).

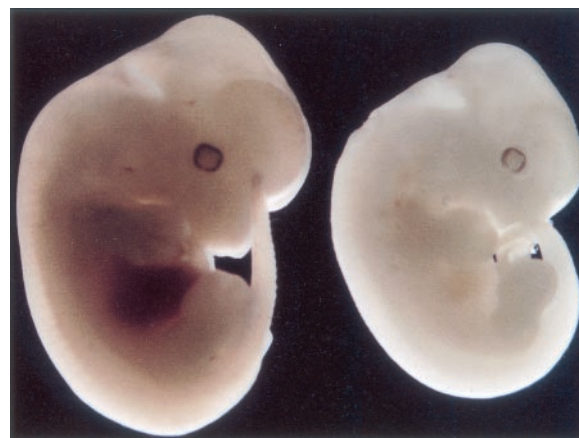


FIG. 4. Phenotypic comparison of E12.5 littermates from a mating between mice heterozygous for NMDMC. A mutant embryo is shown on the right, and a wild-type embryo is shown on the left. The mutant embryo appears to be developmentally normal in comparison with its littermate except for its paleness and smaller size.

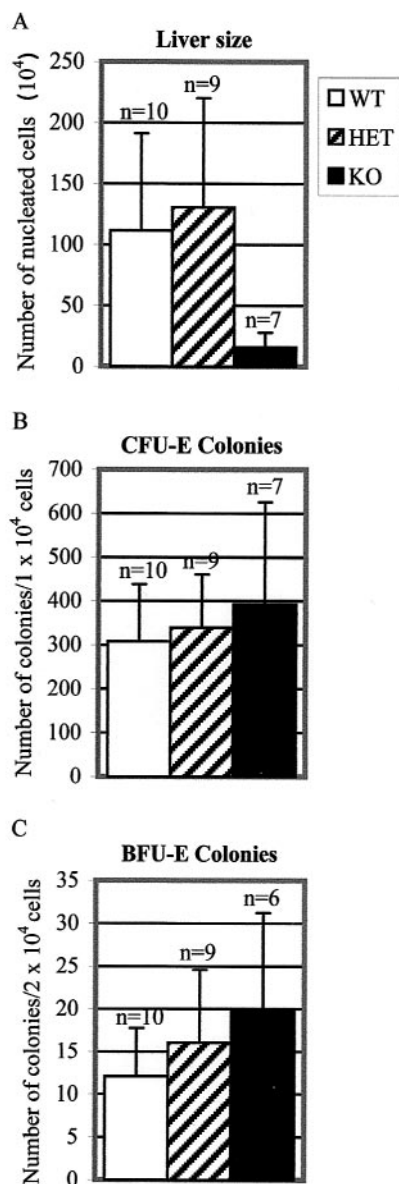


FIG. 5. Hematopoietic progenitor cell colony assays with livers from E12.5 wild-type, heterozygous, and *nmdmc*<sup>-/-</sup> embryos. (A) The average number of nucleated cells in wild-type (WT), heterozygous (HET), and *nmdmc*<sup>-/-</sup> (KO) fetal livers. (B) Numbers of CFU-E detected after 3 days of postplating in methylcellulose medium supplemented with erythropoietin. (C) Numbers of BFU-E detected after 7 to 10 days of postplating in methylcellulose medium supplemented with erythropoietin, stem cell factor, IL-3, and IL-6. The numbers at the top of each error bar indicate the number of embryos analyzed. CFU-E and BFU-E numbers are expressed per  $1 \times 10^4$  and  $2 \times 10^4$  nucleated liver cells, respectively. The height of each bar represents the average, and the error bars represent 1 standard deviation.

DISCUSSION

Folate-dependent metabolism requires that a suitable balance be maintained between the pools of one-carbon folates that provide methyl groups for methionine and thymidylate synthesis and those that donate formyl groups for purine syn-

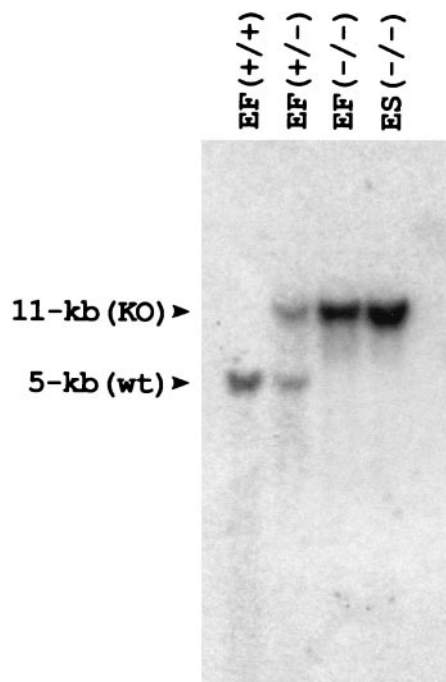


FIG. 6. Southern blot analysis of DNA isolated from mouse embryonic fibroblast cell lines and ES cell lines. *Bam*HI-digested DNA isolated from mouse embryonic fibroblast (EF) cell lines and ES cell lines was hybridized with the flanking probe E2/161. The 5-kb fragment indicates the wild-type (wt) allele, and the 11-kb band indicates the targeted (KO) allele.

thesis. Methylene-THF dehydrogenase-methenyl-THF cyclohydrolase interconverts the methylene- and formyl-THF intermediates that contribute to these functions. The existence of two nucleus-encoded DCS enzymes in yeast, one in the cytoplasm and the other in the mitochondria (26), has been explained as a mechanism to produce formate in mitochondria for use as a one-carbon source in the cytoplasm (18, 30). In this model, flux of metabolites through the DCS in the mitochondria is opposite to that in the cytoplasm, which not only explains the occurrence of both enzymes but indicates that mitochondrially generated formate can be an important intracellular one-carbon source for purine synthesis in the cytoplasm (8, 23).

On the other hand, the function of one-carbon metabolism in mitochondria of mammalian cells is not as clear. The mito-

TABLE 3. Specific activities of NAD- and NADP-dependent methylene-THF dehydrogenases

Genotype of cell line	Sp act for:			
	ES cells		Transformed mouse embryonic fibroblast cells	
	NADP <sup>a</sup>	NAD	NADP	NAD
+/+	7.1	1.5	2.2	2.9
+/-	8.2	0.5	1.5	0.5
-/-	6.3	ND <sup>b</sup>	2.2	ND

<sup>a</sup> Specific activities are given as nanomoles minute<sup>-1</sup> milligram<sup>-1</sup>.  
<sup>b</sup> ND, not detected.

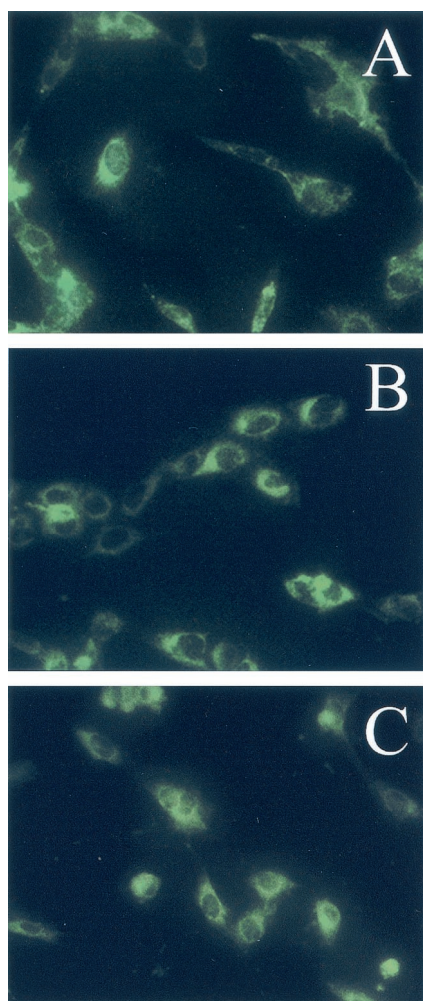


FIG. 7. Staining of mitochondria from mouse embryonic fibroblast cells with Mitotracker Green FM. Mouse embryonic fibroblast cells were incubated with 500 nM Mitotracker Green FM at 37°C for 45 min. (A) wild type; (B) heterozygous; and (C) homozygous null mutant cells.

chondrial enzyme that interconverts methylene-THF and 10-formyl-THF is only bifunctional (dehydrogenase-cyclohydrolyase) and is NAD dependent. It was originally reported in extracts of Ehrlich ascites tumor cells (25) and was later found to be expressed in immortal cell lines and fetal tissues but not in adult differentiated tissues (15). The human trifunctional DCS protein has been mapped to chromosome 14q24 and an intronless pseudogene to the X chromosome at Xp11 (7), suggesting that there is only one DCS gene in higher eukaryotes.

The function of NMDMC is not known, but its expression in fetal tissues suggests a role during embryonic development. In an attempt to determine its metabolic role, we generated NMDMC mutant mice and obtained both female and male transmitting chimeras. Heterozygous mice, derived from two independently targeted cell lines, are apparently healthy and reproduce normally. However, no homozygous mutant mice have been found from heterozygous matings of mice generated from either cell line in both mixed and inbred backgrounds,

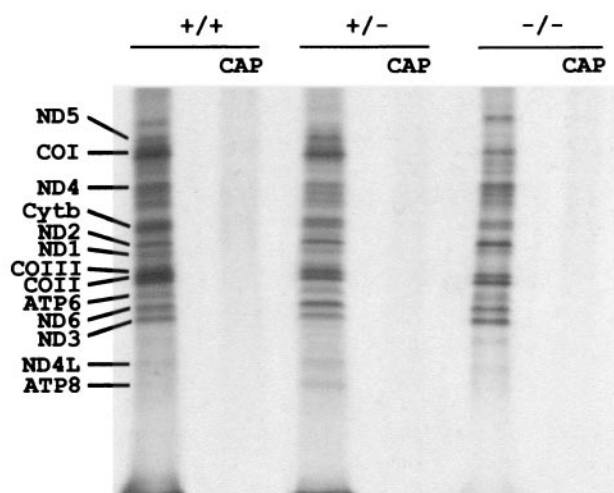


FIG. 8. Analysis of mitochondrial translation products. Exponentially growing cells were labeled with [<sup>35</sup>S]methionine in the presence of emetine for 60 min, and labeled polypeptides were separated on a 12 to 20% polyacrylamide linear gradient gel. An equal amount of protein from whole-cell extracts was loaded in each lane. Translation products are assigned according to Chomyn et al. (4) and are abbreviated as follows: ND1 to ND6, subunits of NADH dehydrogenase complex; COI to COIII, subunits of cytochrome oxidase complex; Cytb, cytochrome *b*; and ATP6 and ATP8, subunits of ATPase complex. CAP indicates an experiment conducted in the presence of chloramphenicol in addition to emetine.

indicating that the complete absence of NMDMC results in prenatal lethality. Examination of embryos at different stages of gestation revealed that, at E12.5, null mutant embryos are smaller than wild-type or heterozygous littermates and are pale. At later times, null mutant embryos were found at different stages of resorption. Preliminary examination of sections of embryos at various stages of development up to E14.5 did not identify any apparent malformations (data not shown). In particular, neural tube development does not appear to be altered. Although livers from E12.5 *nmdmc*<sup>-/-</sup> embryos were much smaller than those from normal littermates, there were no apparent differences in the frequencies of hematopoietic progenitors obtained from each genotype. The normal numbers of progenitor cells suggests that the mutant fetal liver does not provide the proper environment for the establishment of definitive erythropoiesis. This *nmdmc* gene does not code for a transcription factor, receptor, signaling molecule, or growth factor and has no obvious connection with erythropoiesis. The failure must be due to some type of metabolic defect relating to a product of folate-mediated pathways. Why this defect does not affect the apparent development of other tissues at this stage is not clear.

Recently, reports of the knockout of genes expressing proteins in the folate pathways have underscored their importance. Direct impairment of intracellular folate pools was obtained by knockouts of folate transporters that caused expected, very serious consequences. Loss of folate binding protein (Folbp1 but not Folbp2) that mediates folate internalization by endocytosis results in severe morphogenetic abnormalities, including failure of neural tube closure and death by E10 (22). Null mutants of the reduced folate carrier (RFC1)

die before E9.5 due to failure of hematopoietic organs (32). Disruption of the metabolic methylation cycle has been shown to have very serious consequences in two knockouts. The knockout of methionine synthase, in addition to impairing methionine synthesis, also causes depletion of the intracellular pools of methylene- and formyl-THF in favor of methyl-THF as a result of the methyl trap (28). Homozygous knockout mice for methionine synthase do not survive far beyond implantation (28). Methylene-THF reductase-null mutant mice are viable but show developmental defects with reduced survival at 5 weeks of age (3). This knockout does not have a general effect on the folate pool of intermediates but specifically reduces the synthesis of methyl-THF, interfering with the normal recycling of homocysteine. We would expect the loss of NMDMC to be metabolically more similar to that of methylene-THF reductase than to that of the other proteins just described.

Because its metabolic role is not obvious, we examined cells in culture for some initial insight into the importance of NMDMC in mitochondrial function. The staining of mitochondria with Mitotracker Green FM indicates that the mitochondria in the null cells are structurally intact. There is very convincing evidence that initiation of protein synthesis in *Saccharomyces cerevisiae* mitochondria can occur without formylation of the initiator methionyl-tRNA (10). We have also shown mitochondrial translation products to be identical in wild-type, heterozygous, and NMDMC-null mutant cell lines, which also demonstrates that the mitochondria are functional. We have not ruled out the possibility that 10-formyl-THF could be provided by other sources or that formylation of the initiator methionyl-tRNA might be more critical in cells carrying out particularly rapid mitochondrial biogenesis.

Because the NMDMC gene is highly expressed in embryos, it is possible that it serves a specialized role to adapt the embryo to a particular metabolic demand during a certain stage(s) of development. One possibility is that it participates in an analogous metabolic cycle generating formate as described for yeast. NMDMC is a good candidate to participate in such a role, since the NAD specificity shifts the equilibrium between methylene and 10-formyl-THF in mitochondria very significantly towards 10-formyl-THF (19). It could be argued that the demands of embryonic protein synthesis on serine and glycine, major sources of one-carbon units, are so great that the NAD- (rather than NADP-) dependent dehydrogenase activity is required to promote a more thermodynamically favorable pathway to balance the pools of 10-formyl-THF during development. This would be beneficial, regardless of whether the 10-formyl-THF is required to support rapid mitochondrial protein synthesis or to ensure that there is 10-formyl-THF as a source of formate. The latter is an attractive notion based on the results in yeast, but since there is no synthetase, an alternative mechanism is required to generate formate from 10-formyl-THF in mammalian mitochondria.

Our results demonstrate a critical role for mitochondrial folate metabolism during development, since complete loss of NMDMC activities results in embryonic lethality due to a metabolic block which impairs the establishment of erythropoiesis in the fetal liver. NMDMC does not appear essential to support protein synthesis in mitochondria, and its actual metabolic role during development remains an intriguing question.

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