Complexity of CNC Transcription Factors As Revealed by Gene Targeting of the *Nrf3* Locus

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Cap'n'collar (CNC) family basic leucine zipper transcription factors play crucial roles in the regulation of mammalian gene expression and development. To determine the in vivo function of the CNC protein Nrf3 (NF-E2-related factor 3), we generated mice deficient in this transcription factor. We performed targeted disruption of two *Nrf3* exons coding for CNC homology, basic DNA-binding, and leucine zipper dimerization domains. *Nrf3* null mice developed normally and revealed no obvious phenotypic differences compared to wild-type animals. *Nrf3*$^{-/-}$ mice were fertile, and gross anatomy as well as behavior appeared normal. The mice showed normal age progression and did not show any apparent additional phenotype during their life span. We observed no differences in various blood parameters and chemistry values. We infected wild-type and *Nrf3*$^{-/-}$ mice with acute lymphocytic choriomeningitis virus and found no differences in these animals with respect to their number of virus-specific CD8 and CD4 T cells as well as their B-lymphocyte response. To determine whether the mild phenotype of *Nrf3* null animals is due to functional redundancy, we generated mice deficient in multiple CNC factors. Contrary to our expectations, an absence of Nrf3 does not seem to cause additional lethality in compound *Nrf3*$^{-/-}$/*Nrf2*$^{-/-}$ and *Nrf3*$^{-/-}$/*p45*$^{-/-}$ mice. We hypothesize that the role of Nrf3 in vivo may become apparent only after appropriate challenge to the mice.

Basic leucine zipper factors of the cap'n'collar (CNC) family regulate gene expression, tissue differentiation, and development in a variety of organisms. A unique region, the CNC domain, located amino terminal to the basic DNA-binding region, defines this group of proteins (4, 7, 43). The functional role of the CNC domain remains unknown (3). The CNC family includes the *Drosophila* CNC, *Caenorhabditis elegans* Skn-1, and vertebrate p45 NF-E2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2 proteins (4, 7, 9, 11, 12, 30, 32, 37, 43, 44, 48, 52). The roles of some of the mammalian CNC factors have been extensively analyzed. NF-E2 is a heterodimer composed of a p45 protein, a CNC family member, and a small Maf protein (4, 5, 48). The expression of the p45 NF-E2 subunit is restricted to hematopoietic progenitor, erythroid, megakaryocytic, and mast cells (4). Homozygous *p45* null mice suffer from a mild anemia and a profound decrease in platelets as a result of a block in the biogenesis of platelets (56, 57). This leads to the death of most animals from hemorrhage. Homozygous *Nrf1* null mice die in utero. One laboratory reported that the absence of Nrf1 in a mixed 129/C57BL/6 strain results in a non-cell autonomous defect in definitive erythropoiesis which leads to death at mid- to late gestation (14). Others reported the death at em-

bryonic day 7.5 due to abnormal mesoderm formation in an outbred Black Swiss background (24). When those investigators backcrossed the mutation in the C57BL/6J inbred mouse strain, homozygous mutant mouse embryos had no mesoderm block but did have a severe fetal anemia due to a defect in definitive hematopoiesis, which resolved at embryonic day 18.5 (39). Nrf1 appears to be critical for the redox balance during development (18). Targeted disruption of the *Nrf2* locus showed that this protein is dispensable for mouse development (16). Compound *p45*$^{-/-}$/*Nrf2*$^{-/-}$ mice do not exhibit defects beyond those seen with the loss of p45 alone, suggesting that Nrf2 is not the compensating factor in the mild erythrocyte phenotype of *p45* null mice (34, 38). A combined absence of Nrf1 and Nrf2 results in early embryonic lethality and severe oxidative stress (36). In the adult organism, Nrf2 appears to participate in the response to oxidative stress (29, 35, 61). Several reports showed that Nrf2 plays an important role in the expression of detoxification and antioxidant enzymes (2, 13, 15, 25, 29, 54, 60, 62).

The CNC factors have been shown to form heterodimers with the small Maf factors, members of the Maf (proto-)oncogene family of bZIP transcription factors (6, 46). The founding member of the Maf family, the v-Maf oncogene, was isolated as the transforming agent of an avian retrovirus (50). Related Maf proteins can be divided into two subgroups: the small Mafs, including MafF, MafG and MafK factors, and large Mafs, including c-Maf, MafA, MafB (Kreisler), L-Maf, and Nrl proteins (6, 46). Small Mafs are widely expressed, but levels
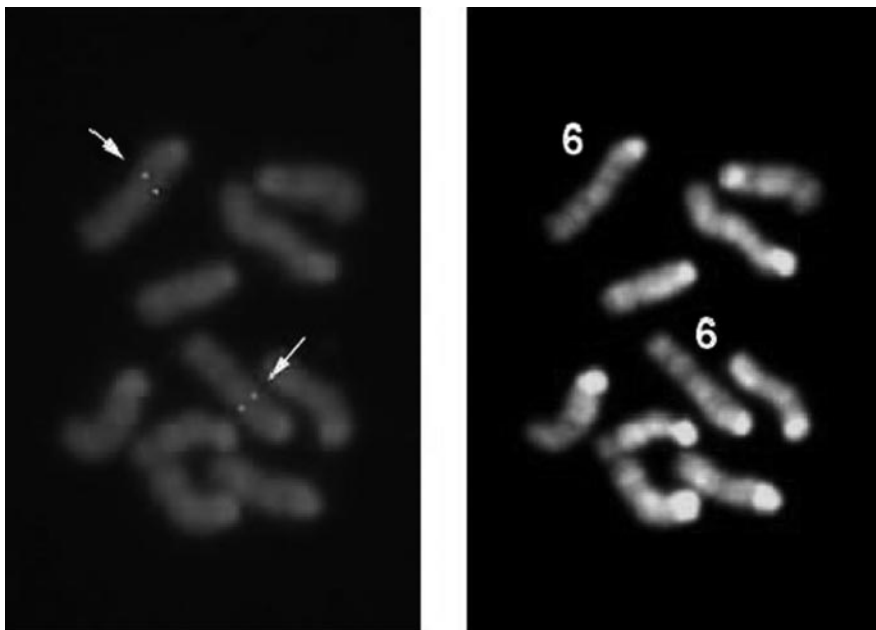
FIG. 1. Example of FISH mapping results for Nrf3. The left panel shows the FISH signals on the mouse chromosome, and the right panel shows the same mitotic figure stained with DAPI to identify mouse chromosome 6.

vary considerably (6). Small Maf homodimers that function as transcriptional repressors have been reported (22, 28, 49). In contrast, when small Mafs associate with the CNC factor p45 NF-E2 or Nrf2, the heterodimer serves as an activator of gene transcription (6, 25, 33, 62).

To better understand the possible in vivo functions of the recently identified Nrf3 protein, we isolated a mouse genomic clone comprising three exons coding for Nrf3. We performed genetic mapping and fluorescence in situ hybridization (FISH) analysis localizing the *Nrf3* gene to mouse chromosome 6. Gene targeting of the mouse *Nrf3* locus yielded healthy and fertile mice. Compound Nrf3/Nrf2- and Nrf3/p45-deficient animals were also viable. We speculate that a physiological role for Nrf3 may be uncovered by exposing the mice to challenging conditions.

MATERIALS AND METHODS

Isolation of a genomic clone. We used a human *NRF3* cDNA probe generated by random priming of a 950-bp BglII fragment, corresponding to amino acids 171 to 487 of human NRF3 (V. Blank, unpublished data) to screen a mouse 129SvJ genomic library (Stratagene) at reduced stringency. We isolated a 15-kb mouse genomic clone containing three exons of the mouse *Nrf3* gene that were identified by DNA sequencing.

FISH analysis. (i) Chromosomal slide preparation. Lymphocytes were isolated from mouse spleen and cultured at 37°C in RPMI 1640 medium supplemented with 15% fetal calf serum, 3 μg of concanavalin A/ml, 10 μg of lipopolysaccharide/ml, and 50 μM β-mercaptoethanol. After 44 h, the cultured lymphocytes were treated with 0.18 mg of bromodeoxyuridine/ml for an additional 14 h. The synchronized cells were washed and recultured at 37°C and treated with bromodeoxyuridine for 4 h in minimum essential medium alpha with thymidine (2.5 μg/ml). Chromosome slides were prepared by a conventional method used for human chromosome preparation (hypotonic treatment, fixation, and air drying).

(ii) Probe labeling and in situ hybridization. The DNA probe was biotinylated with dATP by using a Gibco BRL BioNick labeling kit (15°C, 1 h) (26). The procedure for FISH detection was performed as described previously (26, 27). Briefly, slides were baked at 55°C for 1 h. After RNase A treatment, the slides

were denatured in 70% formamide in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 2 min at 70°C followed by dehydration with ethanol. Probes were denatured at 75°C for 5 min in a hybridization mixture consisting of 50% formamide and 10% dextran sulfate and prehybridized for 15 min at 37°C. Probes were loaded on the denatured slides. After overnight hybridization, slides were washed, detected, and amplified by using a published method (26); FISH signals and the DAPI (4′,6′-diamidino-2-phenylindole) banding pattern were recorded separately. Images were captured and combined with a charge-coupled device camera, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI-banded chromosomes (27).

(iii) Analysis. Under the conditions used, the detection efficiency was 72% for this probe (among 100 checked mitotic figures, 72 showed hybridization signals on one pair of chromosomes). Since DAPI banding was performed to identify the specific chromosome, the assignment between signals from the probe and mouse chromosome 6 was obtained. The detailed position was further determined based on the summary from 10 photos.

Generation of mice lacking Nrf3. We used the isolated *Nrf3* genomic clone to generate 7.5- and 3.2-kb fragments to use as 5′ and 3′ segments flanking two exons of the *Nrf3* gene. These were cloned into the pTKLNCL targeting vector (a kind gift from Richard Mortensen). The resulting plasmid was linearized at a NotI site, chaperone oligonucleotide linkers were ligated onto the sticky ends to form hairpins (47), and the modified plasmid was electroporated into mouse TC1 embryonic stem (ES) cells (a kind gift from Phil Leder) (21). Subsequently, we grew the cells in the presence of G418 and ganciclovir and selected drug-resistant clones. Using Southern blot analysis, we identified clones that had undergone homologous recombination. All clones with a normal 40XY karyotype were injected into C57BL/6J blastocysts to generate chimeric offspring. High-level (up to 90%) chimeric mice were bred to C57BL/6J and 129S6 females to obtain heterozygous offspring. *Nrf3* null mice were obtained by further breeding of the *Nrf3*+/− animals. Mice were kept in sterile microisolators at all times.

RNA preparation and Northern blot hybridization analyses. Mouse tissues were isolated and immediately frozen in liquid nitrogen and stored at −80°C until RNA isolation. Total RNA was isolated by using the Trizol reagent according to the manufacturer's instructions (Invitrogen). The RNA concentration was determined by measuring the absorbance at a wavelength of 260 nm. For Northern blotting, 10 μg of each RNA sample was then separated by formaldehyde-agarose gel electrophoresis, transferred to a nylon membrane (Amersham), and subjected to hybridization as previously described, with minor modifications (20). The *Nrf3* probe was generated by random priming of a 572-bp SacII/PstI fragment (corresponding to amino acids 131 to 321 of mouse Nrf3) by using
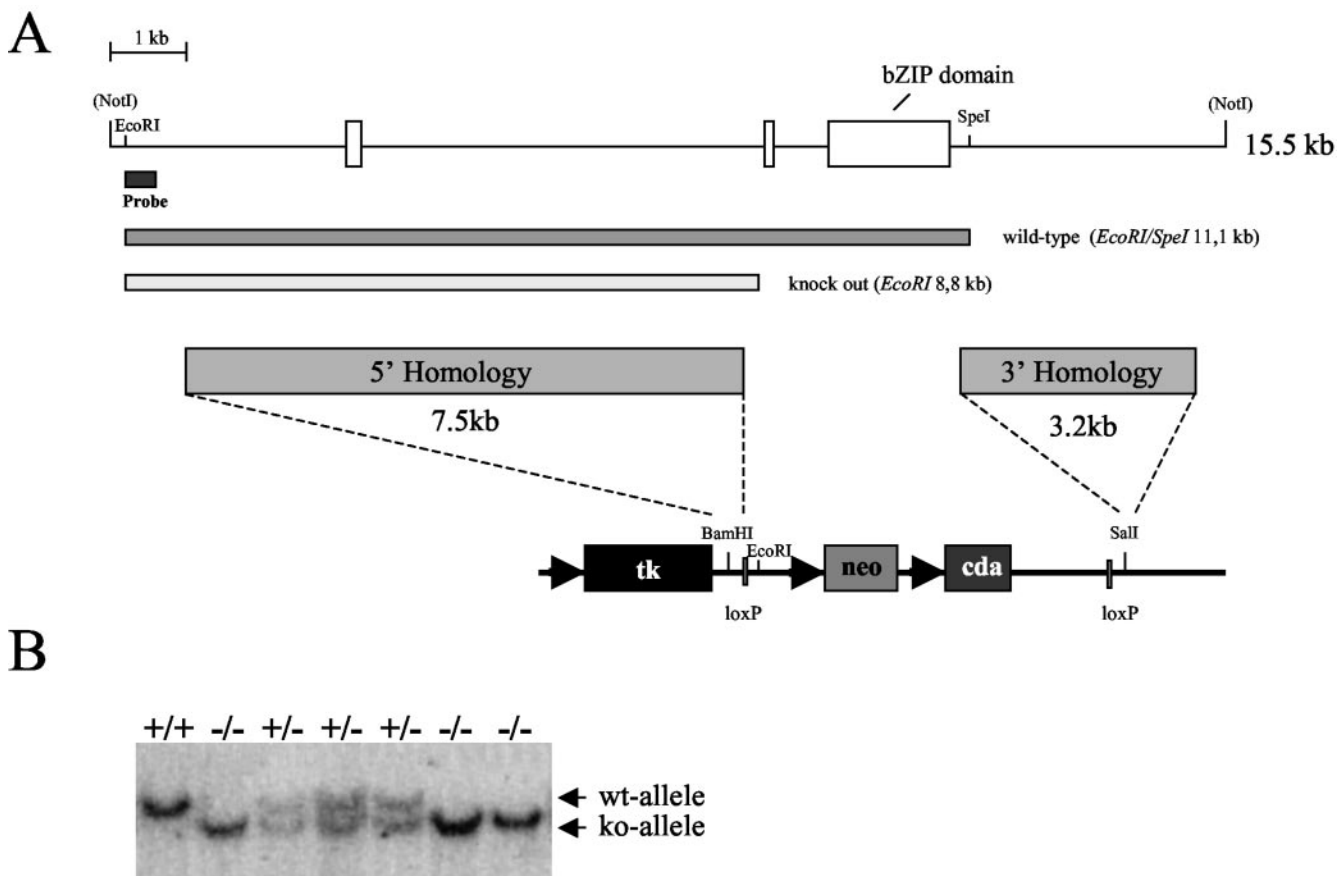
FIG. 2. Generation of Nrf3-deficient mice. (A) Replacement of two exons of the *Nrf3* gene, comprising the sequences coding for the bZIP domain, by the neomycin-cytosine deaminase cassette of the targeting vector. *Nrf3* gene exons are represented by open boxes, and the exon encoding the Nrf3 bZIP domain is shown. The targeting vector contains 7.5 and 3.2 kb of Nrf3 gene homologous genomic sequences on the 5′ and 3′ sides of the neomycin-cytosine deaminase cassette, respectively. The probe used for Southern analysis and the expected fragments from EcoRI/SpeI digests of wild-type and knockout genomic DNA are indicated. tk, thymidine kinase; neo, neomycin; cda, cytosine deaminase. (B) Generation of $Nrf3^{-/-}$ mice. Southern blot analysis of $Nrf3^{+/-}$ mouse matings yielding offspring with homozygous wild-type (wt) and heterozygous and homozygous mutant (null) genotypes. ko, knockout.

[^{32}P]dCTP (Amersham) and the Klenow fragment of DNA polymerase I according to standard methods (Roche). Membranes were stripped in 0.1% sodium dodecyl sulfate at 95°C.

Phenotypical characterization. Hematological parameters were measured with the ADVIA 120 hematology analyzer (Bayer Diagnostics, Tarryton, N.Y.) and analyzed with a software package specific to mice. Biochemical parameters were measured by use of standard biochemical assays on a Hitachi 917 analyzer (Roche Diagnostics, Indianapolis, Ind.).

Viral infections. All mouse infections were done by intraperitoneal injection of 2×10^5 PFU of the Armstrong CA 1371 strain of lymphocytic choriomeningitis virus (LCMV). Stocks of the Armstrong CA 1371 strain of LCMV were grown and quantified as previously described (2). Mice were sacrificed on days 8 and 30 post-LCMV Armstrong infection.

Flow cytometry and intracellular staining for IFN-γ. Intracellular staining for gamma interferon (IFN-γ) was used to quantify virus-specific T cells as previously described (3). Briefly, 10^6 splenocytes were cultured in vitro with or without peptide stimulation in the presence of brefeldin A (Golgistop) (BD Pharmingen, La Jolla, Calif.) for 5 h, followed by surface and intracellular stain. CD8 T cells were stimulated with the dominant major histocompatibility complex (MHC) class I-restricted LCMV peptide NP396-404 (0.2 μg/ml), and the CD4 T cells were stimulated with the MHC class II-restricted LCMV peptide GP61-80 (2 μg/ml). The CD8 (clone 53-6.7), CD4 (clone RM4-5), and IFN-γ (clone XMG1.2) antibodies were purchased from BD Pharmingen.

Quantification of LCMV-specific ASC. LCMV-specific antibody secreting cells (ASC) were quantified by using a previously described enzyme-linked immunospot assay method (4). Lysate from LCMV-infected BHK cells was used as

antigen. Biotinylated goat anti-mouse immunoglobulin G [IgG](γ) antibodies were used as detecting antibodies (Caltag Laboratories, San Francisco, Calif.). Horseradish peroxidase-conjugated Avidin D was purchased from Vector Laboratories (Burlingame, Calif.).

Generation of compound CNC transcription factor null mice. Mixed C57BL/6J-129S6 background $Nrf3^{-/-}$ animals were first crossed to $Nrf2^{+/-}$ mice (>90% C57BL/6J generated through a backcross of 129X1 mice to C57BL/6J mice), a kind gift from Paul Ney, St. Jude Children's Hospital, Memphis, Tenn. Double heterozygote $Nrf3^{+/-}/Nrf2^{+/-}$ mice were then used to obtain compound $Nrf3^{-/-}/Nrf2^{-/-}$ animals. To generate $Nrf3^{-/-}/p45^{-/-}$ mice, offspring with an $Nrf3^{-/-}/p45^{+/-}$ genotype on a C57BL/6J-129S6 background were mated (57).

RESULTS

Chromosomal mapping of the *Nrf3* gene. The identification of the *Nfe2l3* gene encoding Nrf3, a new member of CNC family, has been described (32). Nrf3 dimerizes with the small bZIP Maf protein MafK, and the resulting heterodimers bind to NF-E2/MARE (Maf recognition element)-type DNA-binding sites present in the regulatory enhancer and promoter regions of numerous genes (6, 32). To determine the chromosomal localization and to perform gene targeting of the mouse *Nrf3* (*Nfe2l3*) locus, we isolated a 15-kb genomic fragment. The
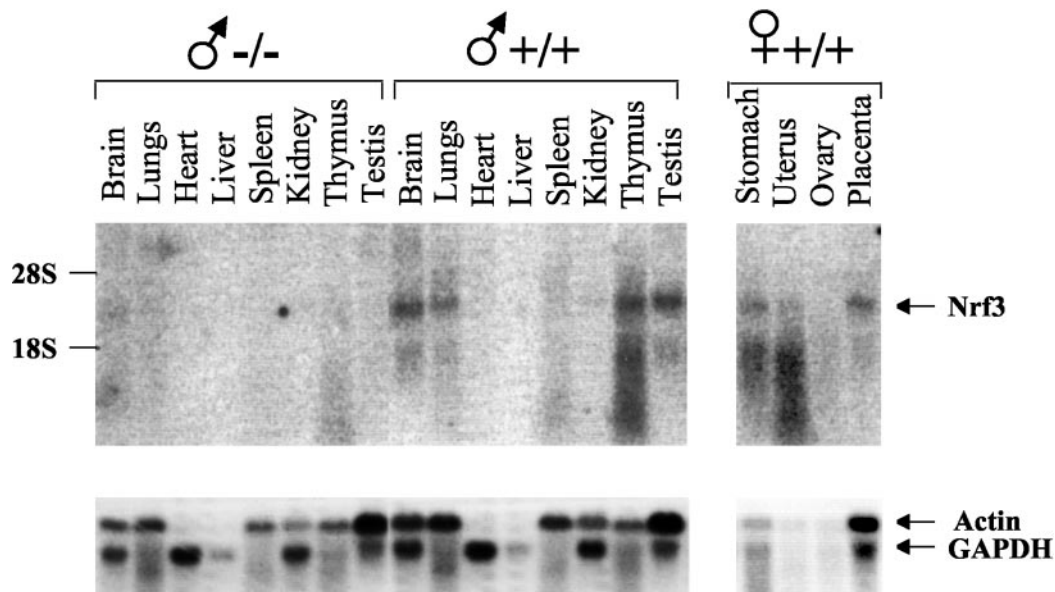
FIG. 3. *Nrf3* transcript analysis of wild-type and gene-targeted mice. Northern blot analysis showing *Nrf3* mRNA levels in various tissues in wild-type and *Nrf3* null mice was done. Ten micrograms of total RNA was used per lane. The same blot was probed with *actin*- and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase gene)-specific control probes.

clone comprised three exons of the mouse *Nrf3* gene, which were identified by DNA sequencing. We performed FISH analysis using the 15-kb genomic fragment as a probe and unequivocally mapped the mouse *Nrf3* gene to chromosome 6, region B2-B3, a region syntenic to that of its human homolog (26) (Fig. 1). We confirmed this result by performing genetic mapping using interspecific backcross panels from the Jackson Laboratory (53; data not shown). Our mapping results confirm unpublished radiation hybrid localization data (Jackson Laboratory, MGI:1339958). As has been reported for other CNC transcription factor loci (10, 32, 40, 41, 63), the mouse *Nrf3* gene colocalizes with members of the *Hox* and *collagen* gene families.

Gene targeting of the mouse *Nrf3* locus. To perform targeted disruption of the mouse *Nrf3* locus, we constructed a targeting vector replacing two exons of the gene by vector sequences (Fig. 2A). This replacement results in an aberrant *Nrf3* transcript lacking the regions coding for the CNC homology, the basic DNA-binding, and the leucine zipper dimerization domains. The absence of the DNA-binding and dimerization domains should yield a nonfunctional Nrf3. Following electroporation of the targeting construct into mouse TC1 ES cells, we obtained 3 out of 103 correctly targeted clones. The microinjection of two of these clones into mouse blastocysts resulted in high-level chimeric (>80 to 90%) offspring. Germ line transmission of the *Nrf3* knockout allele has been achieved with mice generated from one of the original ES cell clones, and the resulting *Nrf3*^{+/−} mice were bred to obtain *Nrf3*^{−/−} offspring (Fig. 2B). The genotypes of the offspring corresponded well to the expected Mendelian ratio of 25% homozygous wild-type, 50% heterozygous, and 25% homozygous knockout mice, suggesting that Nrf3 null mice developed normally and reached adulthood.

***Nrf3* gene expression pattern in wild-type and knockout mice.** It has been shown that human *NRF3* is highly expressed in the placenta and Burkitt's lymphoma cell lines and at lower levels in the heart, brain, lung, kidney, leukocytes, colon, small intestine, thymus, and spleen (32). We performed Northern blot analysis of mouse tissues (Fig. 3). Murine *Nrf3* mRNA is expressed in a wide variety of tissues, although at different levels. The levels of expression are high in the brain, thymus, testis, and placenta, and medium levels are observed in the uterus, stomach, and lung. Transcript levels are low in the kidney, and minimal or no expression is found in the heart, liver, spleen, and ovary. We hypothesize that Nrf3 may perform multiple roles in different tissues in the mouse. Interestingly, the *Nrf3* gene tissue expression pattern in the mouse differs slightly from that reported in humans (32), indicating that Nrf3 might have distinct functions in both mammalian species. To confirm the absence of functional *Nrf3* transcripts in gene-targeted mice, we performed Northern analysis comparing *Nrf3*-targeted and wild-type animals. As shown in Fig. 3, transcripts are not present in *Nrf3*^{−/−} mice.

Phenotypic analysis of Nrf3-deficient mice. We performed a phenotypic analysis of the mice deficient for Nrf3. The general behavior including feeding and mating of *Nrf3* null mice does not seem to be altered under nonchallenging conditions compared to that of their wild-type littermates. These mice also have a normal life span and do not develop any observable abnormalities. We analyzed *Nrf3*^{−/−} animals with respect to their gross anatomy and multiple blood parameters, including red blood cell numbers, white blood cell numbers, and erythrocyte cellular indices (mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and mean cell hemoglobin concentration) (data not shown). In addition, we examined several biochemical parameters in plasma (glucose, blood urea nitrogen, cholesterol, triglyceride, aspartate transaminase, alanine transaminase, lactate dehydrogenase, iron, total bilirubin, and direct bilirubin) (data not shown). No differences between *Nrf3* null mice and
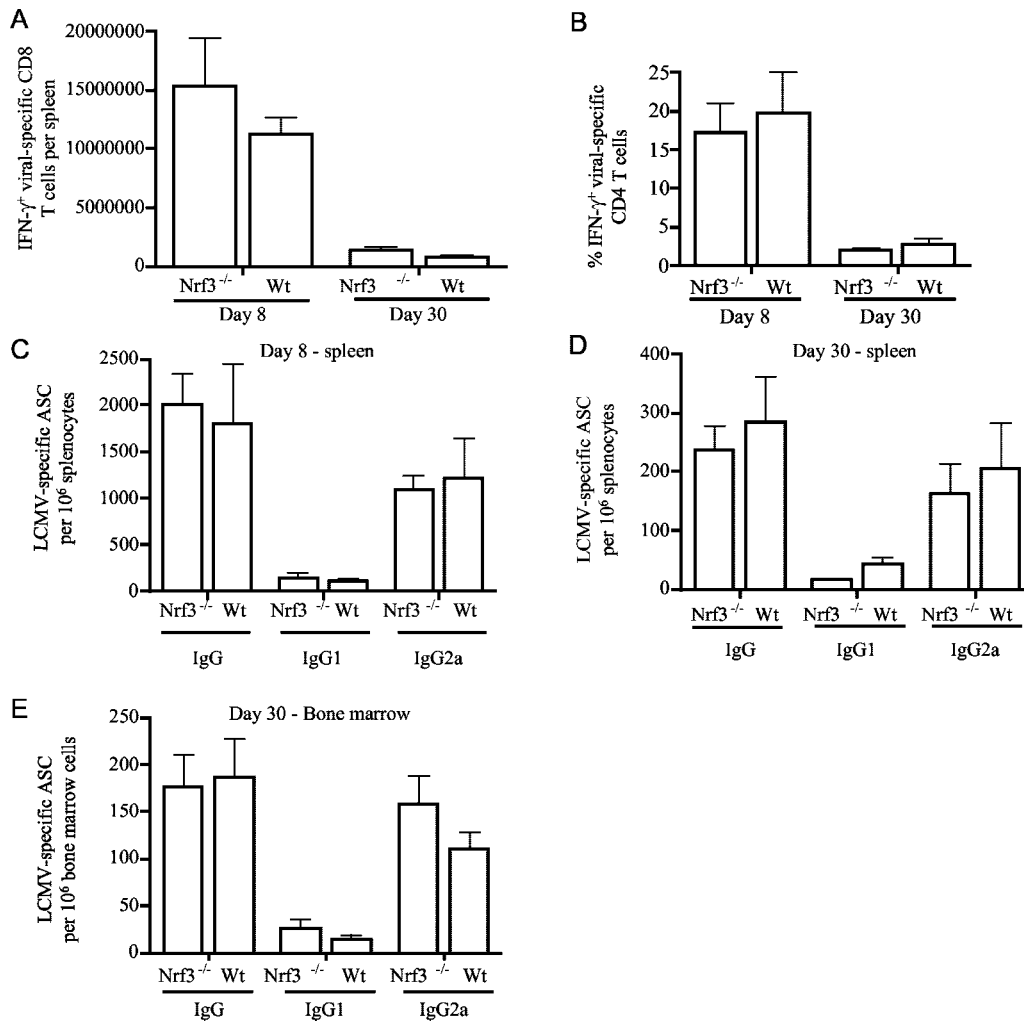
FIG. 4. Response of wild-type and *Nrf3* null mice to LCMV infection. *Nrf3*$^{-/-}$ mice and control mice have comparable T- and B-cell responses to LCMV. IFN-γ production by virus-specific CD8 (A) and CD4 (B) T cells was determined on days 8 and 30 postinfection. Spleen cells from day 8 or day 30 infected mice were stimulated in vitro for 5 h with either MHC class I-restricted epitope NP396-404 (A) or MHC class II-restricted epitope GP61-80 (B). The number of LCMV-specific ASC was determined at day 8 (C) and day 30 (D) in the spleen and on day 30 in the bone marrow (E). LCMV-specific IgG, IgG2a, and IgG1 was determined by an enzyme-linked immunospot assay. The data shown represent a mean of four mice per group. Wt, wild type.

wild-type littermates were found. As it is possible that the loss of Nrf3 function may be compensated for by the overexpression of other CNC transcription factors, we analyzed the expression levels of other family members in the *Nrf3*$^{-/-}$ mice. We found that the transcript levels of the closely related family members *p45*, *Nrf1*, and *Nrf2* appear not to be altered in the *Nrf3* null mice in the tissues analyzed in Fig. 3 (data not shown).

Response of *Nrf3* null mice to viral infection. The murine *Nrf3* gene is expressed at higher levels in the murine thymus, testis, placenta, lung, and brain. Since the animals are housed in a pathogen-free environment, a role for Nrf3 in thymic function and immune defense mechanisms may have remained undetected. Thus, as *Nrf3* is highly expressed in the thymus, we wanted to determine if Nrf3 plays a role in the adaptive immune response. To determine this, we infected *Nrf3*$^{-/-}$ and control mice with acute LCMV. The CD8, CD4, and B-cell

responses to acute LCMV infection have been well characterized (1, 51, 58). The peak CD8, CD4, and B-cell response to LCMV occurs on day 8 postinfection. As shown in Fig. 4, we did not detect any significant differences between the *Nrf3*$^{-/-}$ and control mice at day 8. The numbers of virus-specific CD8 (Fig. 4A) and CD4 (Fig. 4B) T cells were comparable between the *Nrf3*$^{-/-}$ and control mice. We also found no difference in the B-cell response to LCMV. The amounts of plasma or ASC secreting LCMV-specific IgG, IgG2a, the dominant isotype, or IgG1 (Fig. 4C) were equivalent. To determine whether the Nrf3 deficiency had any effect on T-cell memory development, we sacrificed mice 30 days post-LCMV infection. As shown in Fig. 4, both the control and *Nrf3*$^{-/-}$ mice had comparable numbers of virus-specific CD8 (Fig. 4A) and CD4 (Fig. 4B) T cells at this later time point. The numbers of LCMV-specific ASC in both the spleen and bone marrow were also comparable at day 30 postinfection (Fig. 4D and E). Our data suggest
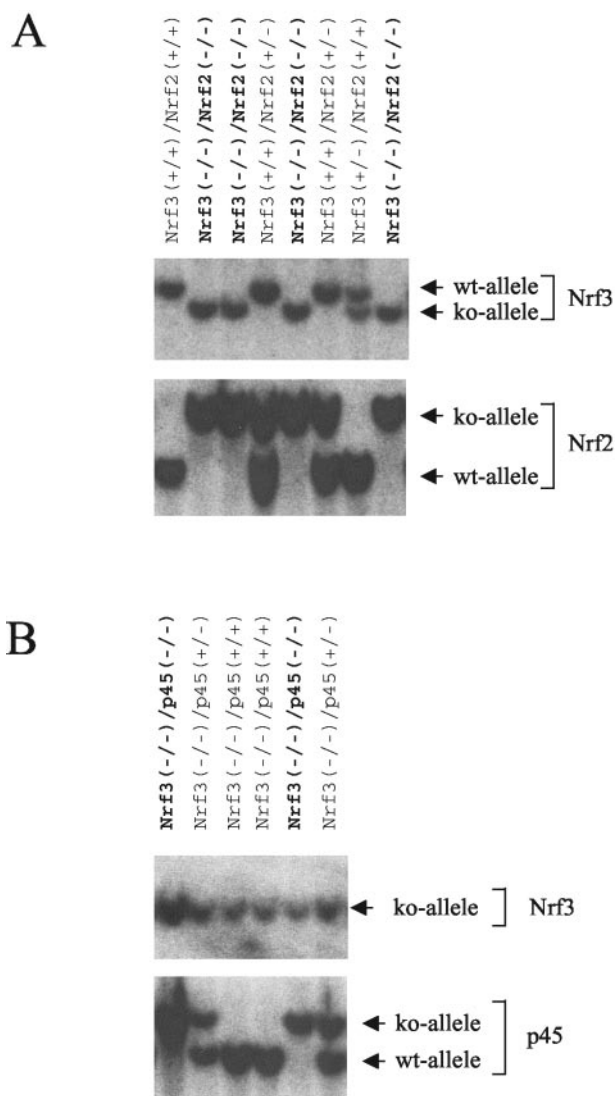
FIG. 5. Generation of compound $Nrf3^{-/-}/Nrf2^{-/-}$ and $Nrf3^{-/-}/$ $p45^{-/-}$ null mice. Southern blot analyses of $Nrf3^{+/-}/Nrf2^{+/-}$ mice matings (A) and $Nrf3^{-/-}$ and $p45^{+/-}$ mice matings (B) are shown. Double knockout (ko) animals are indicated in boldface type. wt, wild type.

that *Nrf3* null mice do not show major differences in their viral response. In summary, our phenotypic analysis of *Nrf3* null mice suggested functional redundancy among CNC family proteins.

Generation of compound $Nrf3^{-/-}/Nrf2^{-/-}$ and $Nrf3^{-/-}/$ $p45^{-/-}$ mice. To determine whether the mild phenotype of *Nrf3* null animals is due to functional redundancy among CNC transcription factors, we generated compound null animals deficient for Nrf3/Nrf2 and Nrf3/p45. Southern blot analysis confirmed the genotype of the offspring of these matings (Fig. 5) (38, 57). We found that $Nrf3^{-/-}/Nrf2^{-/-}$ mice develop normally and are viable and fertile, as we obtained live compound null animals at a rate at least equal to the expected Mendelian ratio (Table 1). In additional matings, we obtained some $Nrf3^{-/-}/p45^{-/-}$ animals that survived to adulthood (Table 1).

We thus conclude that the absence of the *Nrf3* gene does not cause additional lethality in these compound mutant mice.

DISCUSSION

To further characterize the role of the Nrf3 transcription factor in mammalian gene expression, we isolated a mouse genomic clone comprising three exons of the *Nrf3* locus. We used this clone to determine the chromosomal localization of the murine *Nrf3* gene by FISH analysis and genetic mapping. As shown in Fig. 1, we unequivocally localized the mouse *Nrf3* gene to chromosome 6, region B2-B3, confirming unpublished radiation hybrid data (MGI:1339958). The mouse *Nrf3* maps close to the murine *Hoxa* cluster, a map location syntenic to the region containing the human *Nrf3* locus on chromosome 7 p15-7 p14 (bacterial artificial chromosome clone no. RG119C02) (32). This mapping result further strengthens the hypothesis, proposed previously (10, 32), that the genetic loci of *p45*, *Nrf1*, *Nrf2*, and *Nrf3* have evolved from a single gene. Mouse and human versions of these genes colocalize to a cluster of genes comprising members of the *collagen* and *Hox* gene families (10, 32, 40, 41, 63). The *p45* (human, 12q13.1-13.3; mouse, 15), *Nrf1* (human, 17q21.3; mouse, 11), *Nrf2* (human, 2q31; mouse, 2), and *Nrf3* (human, 7p14-15; mouse, 6) genes map to each of the four known *hox* clusters, *hoxC*, *hoxB*, *hoxD*, and *hoxA*, respectively. Duplication and subsequent diversification of each family member may allow for the fine-tuning of CNC-dependent transcription in mammals.

To investigate the in vivo role of Nrf3, we performed gene targeting by homologous recombination in the mouse. We found that *Nrf3* heterozygote matings generated offspring in the expected Mendelian ratio. Gross anatomy and behavior also appeared normal. It has been shown that p45 NF-E2 plays crucial roles in blood cell function, particularly in platelet biogenesis and erythroid cell-specific gene expression (31, 55–57). However, analysis of common blood parameters and chemistry values of $Nrf3^{-/-}$ mice did not reveal any significant changes. We bred *Nrf3* null mice, females and males, and found that both sexes were fertile and that females were able to bear normal-sized litters. Thus, although *Nrf3* is highly expressed in the placenta and testis, and at medium levels in the uterus, its function can be substituted in these tissues. It was previously proposed that Nrf3, in conjunction with Nrf2, may have a role in keratinocyte function (8), but we did not observe any skin abnormalities in $Nrf3^{-/-}$ animals. We observed a limited number of animals for up to 22 months, but no abnormalities were noticeable with advancing age. Thus, *Nrf3* null mice developed normally and are indistinguishable from their wild-type littermates when held in a sterile environment. It is possible that Nrf3 is absolutely required only in adult mice, and a phenotype may be exposed only under challenging conditions. We speculated that Nrf3 may play a role in the adaptive immune response due to its high expression in the thymus, but viral insult with the LCMV model (1, 51, 58) did not expose differences between wild-type and *Nrf3* knockout animals. The mixed C57BL/6J-129S6 genetic background of these mice might contribute to alleviate the phenotype, but $Nrf3^{-/-}$ mice in a pure inbred 129 background still develop and grow normally (A. Derjuga and V. Blank, unpublished observations). In

TABLE 1. Viability of compound Nrf3$^{-/-}$/Nrf2$^{-/-}$ and Nrf3$^{-/-}$/p45$^{-/-}$ mice[c]

Genotype	Alive (%)	Dead (%)	Expected Mendelian ratio (%)
Nrf3$^{+/-}$/Nrf2$^{+/-}$ matings[a]			
Nrf3$^{+/+}$/Nrf2$^{+/+}$	5.8		6.25
Nrf3$^{+/+}$/Nrf2$^{+/-}$	14.5		12.50
Nrf3$^{+/+}$/Nrf2$^{-/-}$	4.4		6.25
Nrf3$^{+/-}$/Nrf2$^{+/+}$	14.5		12.50
Nrf3$^{+/-}$/Nrf2$^{+/-}$	21.7		25.00
Nrf3$^{+/-}$/Nrf2$^{-/-}$	5.8		12.50
Nrf3$^{-/-}$/Nrf2$^{+/+}$	2.9		6.25
Nrf3$^{-/-}$/Nrf2$^{+/-}$	15.9		12.50
Nrf3$^{-/-}$/Nrf2$^{-/-}$	14.5		6.25
Nrf3$^{-/-}$/p45$^{+/-}$ matings[b]			
Nrf3$^{-/-}$/p45$^{+/+}$	33.3	0	25.0
Nrf3$^{-/-}$/p45$^{+/-}$	46.0	2.3	50.0
Nrf3$^{-/-}$/p45$^{-/-}$	16.1	2.3	25.0

[a] Offspring viability of Nrf3$^{+/-}$/Nrf2$^{+/-}$ matings. A sample of 69 animals (nine litters) is shown.
[b] Offspring viability of Nrf3$^{-/-}$/p45$^{+/-}$ matings. A sample of 87 animals (12 litters) is shown.
[c] The observed survival of the animals through adulthood, as well as the expected Mendelian ratio of genotypes, is indicated.

summary, our analysis showed that *Nrf3* null mice exhibit no obvious phenotype.

The mild phenotype of *Nrf3* null mice suggested functional redundancy by closely related transcription factors. We speculated that other CNC proteins, because of their structural homologies, similar DNA-binding specificities, and overlapping expression pattern (45), might compensate for the loss of Nrf3. We found that *p45 NF-E2*, *Nrf1*, and *Nrf2* transcript levels appear to be unchanged in *Nrf3* null mice (data not shown), but compensation for the absence of Nrf3 may not necessarily require higher expression levels. Several other CNC family members have been analyzed by gene targeting. Although *p45 NF-E2* and *Nrf1* null mice show severe phenotypes, *Nrf2*$^{-/-}$ and *Bach1*$^{-/-}$ mice develop normally and are fertile (14, 16, 24, 35, 57, 59). Compound *Nrf2*$^{-/-}$/*p45*$^{-/-}$ mice do not exhibit defects beyond those seen with the loss of p45 alone (38). The absence of Bach1 leads to a high level of expression of heme oxygenase 1 (59). Subsequent analyses of Nrf2-deficient mice showed that this protein is important for liver function in various responses to stress-inducing agents, including antioxidants or drugs such as acetaminophen (13, 17, 19, 23, 29, 36, 42). As *Nrf3* is not expressed in the liver (Fig. 3), an analogous function for Nrf3 in the liver is unlikely. However, Nrf3 may be important for similar responses to stress or other insults in other tissues. To test the hypothesis of functional redundancy among CNC proteins, we generated compound *Nrf3*$^{-/-}$/*Nrf2*$^{-/-}$ and *Nrf3*$^{-/-}$/*p45*$^{-/-}$ mice. Unexpectedly, these compound knockout mice are viable (Table 1). We have recently obtained compound *Nrf3*$^{-/-}$/*Nrf2*$^{-/-}$/*p45*$^{-/-}$ mice, and preliminary observations suggest that some of the triple-compound knockout mice also survive (Derjuga and Blank, unpublished). In conclusion, the absence of Nrf3-dependent transcription in Nrf2 or p45 animals does not cause added lethality, as would be expected if these factors were functionally redundant. It may be that the functions of Nrf3/ Nrf2 or Nrf3/p45 are compensated for by the remaining CNC proteins, Nrf1, Bach1, and Bach2, or by unrelated factors. Thus, the generation of different combinations of CNC transcription factor null animals may be required to obtain insights into Nrf3 function. In addition, future experiments exposing the generated single and compound knockout mice to challenging and stressful conditions may help uncover the role of Nrf3 in vivo.

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