Targeted Disruption of the CP2 Gene, a Member of the NTF Family of Transcription Factors*

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The NTF-like family of transcription factors have been implicated in developmental regulation in organisms as diverse as Drosophila and man. The two mammalian members of this family, CP2 (LBP-1c/LSF) and LBP-1a (NF2d9), are highly related proteins sharing an overall amino acid identity of 72%. CP2, the best characterized of these factors, is a ubiquitously expressed 66-kDa protein that binds the regulatory regions of many diverse genes. Consequently, a role for CP2 has been proposed in globin gene expression, T-cell responses to mitogenic stimulation, and several other cellular processes. To elucidate the in vivo role of CP2, we have generated mice nullizygous for this CP2 allele. These animals were born in a normal Mendelian distribution and displayed no defects in growth, behavior, fertility, or development. Specifically, no perturbation of hematopoietic differentiation, globin gene expression, or immunological responses to T- and B-cell mitogenic stimulation was observed. RNA and protein analysis confirmed that the nullizygous mice expressed no full-length or truncated version of CP2. Electrophoretic mobility shift assays with nuclear extracts from multiple tissues demonstrated loss of CP2 DNA binding activity in the –/– lines. However, a slower migrating complex that was ablated with antiserum to NF2d9, the murine homologue of LBP-1a, was observed with these extracts. Furthermore, we demonstrate that recombinant LBP-1a can bind to known CP2 consensus sites and form protein complexes with previously defined heteromeric partners of CP2. These results suggest that LBP-1a/NF2d9 may compensate for loss of CP2 expression in vivo and that further analysis of the role of the NTF family of proteins requires the targeting of the NF2d9 gene.

Cellular diversity is generated by unique combinations of transcription factors interacting to specify patterns of gene expression in different cell types. This is particularly evident during development where biochemical and genetic studies have identified numerous proteins essential for the processes that govern embryogenesis. Many of these factors are highly conserved in evolution, playing critical roles in organisms as diverse as Drosophila and man. One family of transcription factors that typifies these principles is the NTF-like group of proteins. The founding member of this family is the developmentally programmed Drosophila factor, NTF-1 (neurogenic element binding transcription factor) (1). NTF-1 (also known as Grainyhead or Elf-1) was first identified through its ability to bind a cis element critical for expression of the Dopa decarboxylase gene (2, 3). Subsequently, NTF-1 was shown to bind to promoters of other developmentally regulated genes including Ultrabithorax, fushi tarazu, and engrailed (2). NTF-1 has also been linked to dorsal/ventral and terminal patterning through the formation of multiprotein complexes that influence transcription from the decapentaplegic and tailless genes (4, 5). More recently, tissue-specific isoforms of the protein have been described in Drosophila, and mutation of these isoforms or the ubiquitously expressed gene results in pupal lethality with gross developmental defects (1, 6).

In mammals, two highly related NTF-like genes have been identified. In humans they are known as LBP-1a and CP2 (LBP-1c/LSF), whereas the mouse homologues are referred to as NF2d9 and CP2, respectively (7–10). The human genes are 72% identical in overall amino acid sequence but share higher sequence identity (88%) in the N-terminal halves of the proteins than the C-terminal halves (52%). The homology with the NTF gene is also in the N-terminal region with three amino acid stretches, 148 to 159, 205 to 216, and 233 to 246 showing 66, 75, and 79% identity, respectively. The NTF-like gene family has been shown to have a variety of cellular and developmental functions in human and murine cells. The best characterized member of the family, CP2, was initially identified as a factor that binds to, and stimulates transcription from, the murine γ-fibrinogen promoter and the viral SV40 major late promoter (11, 12). Binding sites for CP2 have also been defined in regulatory regions of the human immunodeficiency virus (HIV) where it acts in concert with YY1 to repress transcription (9, 13–16). In the context of non-viral gene regulation, CP2 has been shown to bind homomerically to the human c-fos, ornithine decarboxylase, c-myc, and DNA polymerase promoters and the murine α-globin and fibrinogen promoters and activate transcription in vitro (17, 18). Binding to the regulatory elements in the fos and ornithine decarboxylase promoters

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** The abbreviations used are: HIV, human immunodeficiency virus; SSP, stage selector protein; kb, kilobase pair; RPA(s), ribonuclease protection assay(s); EMSA(s), electrophoretic mobility shift assay(s); YAC, yeast artificial chromosome; dpc, days post-coitum.
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is modulated by cell growth signals. Mitogenetic stimulation of resting T-cells is associated with rapid phosphorylation of CP2 by the mitogen-activated protein kinase pp44 (extracellular signal-regulated kinase 1) and a consequent increase in its DNA binding activity (17). This modulation suggests that CP2 contributes to the regulation of early response genes and therefore plays a role as a cell growth regulator.

A developmental role for CP2 has been identified in studies of globin gene regulation. In this context, CP2 binds to the stage selector element in the proximal γ-gene promoter as a heteromic complex with a recently cloned fetal/erythroid-specific partner protein, NF-E4 (19, 22, 23). This complex, known as the stage selector protein (SSP), contributes to the preferential recruitment of the β-globin locus control region to the γ-promoter during fetal erythropoiesis (20, 21). SSP binding sites have also been defined in the ε-promoter and in the regions of DNase1 hypersensitivity that constitute the locus control region (19, 22, 23).

Despite the extensive literature examining CP2 function in vitro and in cell lines, the in vivo role of this factor remains unknown. To address this, we have generated a CP2 null mutation in mice by homologous recombination. Mice lacking CP2 expression were examined for defects in growth and development, with a particular emphasis on hematopoiesis, immune, and neural function. We observed no significant abnormality in CP2−/− mice compared with wild type littermates. We have shown through DNA binding and protein-protein interaction studies that the lack of a discernible phenotype may be due to a complete rescue by Nf2d9, the murine homologue of LBPla.

EXPERIMENTAL PROCEDURES

Generation of CP2−/− Mice—We isolated eight CP2 genomic clones by screening a 129-derived ES cell phage library with a full-length mouse CP2 cDNA probe. Duplicate lifts screened with a probe specific for exon 1, containing the initiation ATG, identified one clone with a 12-kb insert that encodes the first two exons and the 5′ untranslated region. Detailed restriction endonuclease mapping of this fragment confirmed the previously reported genomic structure of murine CP2 with the exception of an EcoRI polymorphism detected in the 5′ untranslated region (Fig. 1A) (24). Subsequently, a 6.6-kb NcoI fragment containing the 5′ untranslated region and a portion of the first exon was subcloned into pSL301. A XhoI-SalI fragment containing a phosphoglycerate kinase promoter-regulated hygromycin resistance expression cassette was cloned into a downstream SalI site. Finally, a 3.4-kb SalI-NcoI fragment containing 2.4 kb of the second intron of CP2 and a 1-kb HSV-TK expression cassette fragment (a kind gift of Dr. J. van Deursen) was cloned downstream of this region to provide 3′ homology and a negative selectable marker. This construct, pK01HygTK, was linearized with NotI and transfected by electroporation into RW8 embryonic stem cells (Genomic Systems Inc). The cells were cultured on primary irradiated embryonic STO feeder cells in the presence of 140 μg/ml G418. Cells underwent a homologous transformation on leucine/tryptophan plates (data not shown). Protein interactions are indicated by growth on these plates.

250 amino acids (amino acids 250–500) of CP2 and the corresponding description previously (19).

In Situ Hybridization Studies—Embryo sections were prepared as described previously (25). Briefly, C57BL/6 mice, overdosed with ketamine and xylazine, were perfused intracardially with paraformaldehyde, and the embryos from timed matings were postfixed in a similar solution. Sections of 10–14 μm were cut with a cryostat, mounted on glass slides, and stored at −20 °C. These slides were subsequently probed with sense and antisense riboprobes generated by [32P]UTP labeling from Bluescript plasmids encoding the complete cDNAs of CP2 and Nf2d9 (the latter were a kind gift of Dr. M. Negishi) (10). Specific signals were developed by dipping the slides in NTB2 emulsion (Kodak Scientific Imaging Systems) and exposed at 4 °C for two weeks. The sections were counterstained using 0.1% toluidine blue in distilled water and analyzed by phase-contrast microscopy.

Phenotypic Analysis—Tissues from normal and age-matched heterozygote and homozygote knockout mice were removed and fixed in formalin. Peripheral blood (150 μl) was obtained by retro-orbital puncture and blood cell counts, and erythrocyte parameters were determined utilizing an automated analyzer (Couler). In addition an aliquot was stained with Wright’s Giemsa or methylene blue to study hematopoietic cell morphology and reticulocytes, respectively. Bone marrow hematopoietic progenitors were cultured in methylcellulose in the presence of IL3, erythropoietin, and stem cell factor (Terry Fox Laboratories, Vancouver, Canada). For immunological studies, single cell suspensions were prepared from spleen, lymph node, thymus, and bone marrow and stained with cell type-specific markers for granulocytes (Gr1), T-cells (CD8 and CD4), B-cells (B220 and IAβ), and NK cells (NK1.1). Fluorescence analysis was performed utilizing a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Similar cellular suspensions were utilized to assess the proliferative potential of T- and B-cells, culturing 10^6 cells in the presence of anti-CD3e+/- phorbol 12-myristate 13-acetate, concanavalin A, lipopolysaccharide, phytoge-
RESULTS

Targeting of the CP2 Genomic Locus—To disrupt the murine CP2 gene, a targeting vector was designed that replaced the first untranslated exon and the entire second exon containing the initiation codon and the trans-activation domain with a hygromycin expression cassette (Fig. 1A). In addition, this cassette introduced termination codons in all open reading frames. RW8 embryonic stem cells were electroporated with this construct and selected in hygromycin and FIAU. Southern analysis of resistant clones demonstrated a 9.0-kb EcoRI fragment in addition to the 10.5-kb wild type allele, at a mean frequency of one in 25 clones (data not shown). Four independently targeted clones, with normal karyotypes, were injected into C57BL/6J blastocysts, three of the clones being transmitted through the germ line. Interbreeding of mice heterozygous for the CP2 allele (CP2+/−) resulted in litters of normal size with the expected Mendelian frequency of genotypes. Of 256 total offspring tested, 72 were CP2+/+ (28%), 125 were CP2+/− (49%), and 60 animals (23%) were nullizygous (CP2−/−) for the CP2 allele.

Expression of CP2 in Homozygous CP2−/− Animals—To confirm the loss of CP2 gene expression in nullizygous animals, RNA was prepared from various tissues of both wild type and CP2−/− mice and analyzed by RNase protection analysis. A specific band encoding the 5′ portion of exon 1 and all of exon 2. A 3′ HSV-TK cassette allows for negative selection of hygromycin-resistant clones utilizing FIAU. The lower map delineates the disrupted allele. Homologous recombinants were identified utilizing a unique 5′ probe (hatched box). The sizes of the wild type and disrupted alleles detected by this probe are indicated. B, RNase protection analysis of total RNA isolated from multiple tissues of CP2+/+ and CP2−/− mice. Total RNA was extracted from brain (B), heart (H), kidney (K), lung (L), and spleen (S) of CP2+/+ and CP2−/− animals or from the murine erythroleukemia cell line (MEL). RNA was hybridized to a mixture of CP2- and actin-specific probes adjusted to equal specific activities. Protected fragments are indicated at the right. This experiment is representative of 10 animals assayed. Dashes indicate empty lanes.

Fig. 1. Generation of CP2 null mice. The upper map shows a 12-kb fragment encoding the 5′ end of the murine CP2 locus. The middle map demonstrates the targeting construct, pK01HygTK, a hygromycin expression cassette replacing the 3′ portion of exon 1 and all of exon 2. A 3′ HSV-TK cassette allows for negative selection of hygromycin-resistant clones utilizing FIAU. The lower map delineates the disrupted allele. Homologous recombinants were identified utilizing a unique 5′ probe (hatched box). The sizes of the wild type and disrupted alleles detected by this probe are indicated. B, RNase protection analysis of total RNA isolated from multiple tissues of CP2+/+ and CP2−/− mice. Total RNA was extracted from brain (B), heart (H), kidney (K), lung (L), and spleen (S) of CP2+/+ and CP2−/− animals. Total RNA isolated from multiple tissues was assayed by RT-PCR to detect a splicing event that might produce a functional CP2 transcript. RNA from wild type and CP2−/− tissues was assayed by RT polymerase chain reaction utilizing primers specific to the 3′ end of the mRNA transcript. Although a CP2-specific signal was observed in all wild type tissues tested, no signal was detected from RNA derived from CP2−/− animals (data not shown).

Phenotypic Analysis of CP2−/− Animals—Male and female knockout mice grew normally and were healthy up to 18 months of age. No abnormal behavioral patterns were observed. The fertility of CP2−/− animals was normal, and no increase in morbidity was observed when compared with littermate controls. Careful histopathological examination of brain, spleen, kidney, liver, thymus, lymph nodes, heart, skin, muscle, and bone from CP2−/− animals, performed at 3, 9, and 15 months, was identical to wild type littermate controls (data not shown).

Examination of Hematopoiesis in CP2−/− Animals—CP2 has been implicated in the regulation of several hematopoietic genes, particularly those of the globin loci (7, 19). To determine whether loss of CP2 expression resulted in changes in hematopoiesis, the hematological parameters of CP2−/− mice were assayed and compared with those of wild type littermates. No significant difference in total cell counts, hematocrits, reticulocytes, differential white cell counts, or the α/β-globin ratio was observed (Table I). In addition, the numbers of bone marrow progenitors, as measured by colony-forming unit activity, were similar in CP2+/+ and CP2−/− animals (data not shown). Similar studies of lymphopoiesis were stimulated by recent studies implicating CP2 in the modulation of T-cell proliferative responses (17). However, extensive analysis of T, B, and NK phenotypes, as well as functional assays of B- and T-cell...
function, failed to identify a difference between CP2+/- and CP2-/- cells (data not shown).

It is possible that despite normal adult erythropoiesis, the loss of CP2 expression may affect either \(\alpha\)– or \(\beta\)-globin gene expression during hematopoietic ontogeny. CP2 was initially identified as an \(\alpha\)-globin CCAAT box binding activity, suggesting a possible role in \(\alpha\)-globin gene expression. However, neither \(\zeta\)- nor \(\alpha\)-globin gene expression was perturbed in yolk sac or fetal liver cells (Fig. 2A). We have demonstrated that CP2 is a component of the \(\gamma\)-globin promoter-binding SSP complex and suggested that the \(\gamma\) to \(\beta\) switch in the \(\beta\)-globin subtype may be perturbed in a CP2 null environment. To test this hypothesis, we bred CP2-/- animals with mice transgenic for a \(240\text{-}kb\) YAC containing the human \(\beta\)-globin locus (\(\beta\)YAC). Subsequently, we bred male progeny transgenic for the \(\beta\)YAC with CP2-/- females and examined the expression of both human and mouse \(\beta\)-globin-like genes at several developmental stages. As shown in Fig. 2B, both human and murine \(\beta\)-globin-like gene expression in yolk sac, fetal liver, and bone marrow were identical in CP2+/- and CP2-/- embryos and adult mice, respectively.

**DNA Binding Activity in Extracts from CP2-/- Mice**—To examine CP2 DNA binding site occupancy in the null mice, we prepared crude nuclear extracts from lung, kidney, heart, and liver of wild type and CP2-/- animals and performed EMSA using a double stranded oligonucleotide probe containing the \(\alpha\)-globin CCAAT box (7). Utilizing equal amounts of protein in each lane, a band of similar electrophoretic mobility was observed in all wild type tissues (Fig. 3A, compare lanes 1, 3, 5, and 7). In contrast, extracts from CP2-/- tissues failed to show the band seen with wild type extract and instead showed a DNA-protein complex with a slower migration pattern (Fig. 3A, compare lanes 2, 4, 6, and 8 with 1, 3, 5, and 7, respectively). This result was not dependent on the amount of protein added, as 2- to 8-fold more protein from CP2-/- liver extract incubated with the probe generated an identical band shift (Fig. 3A, compare lane 7 with lanes 8–11).

The lack of an obvious phenotype in the CP2-/- animals coupled with the persistent DNA site occupancy observed with extracts from nullizygous tissues suggested the presence of a ubiquitous CP2-like factor that could compensate for the lack of CP2. One candidate factor was NF2d9, the murine homologue of the human NTF-like gene, LBP-1a (10). Support for this hypothesis was obtained by studying the relative electrophoretic mobilities of CP2 and NF2d9. Both molecules bound the \(\alpha\)-globin CCAAT box and \(\gamma\)-fibrogen probes, the NF2d9-DNA complex having a perceptibly slower mobility (Fig. 3B and data not shown). To determine whether the protein-DNA complex generated with CP2-/- extracts contained NF2d9, we performed competition experiments utilizing excess concentrations of unlabeled oligonucleotides that have been previously shown to bind CP2 and/or NF2d9 (7, 10). These oligonucleotides were capable of ablating both wild type and mutant binding activity (data not shown). We also investigated the ability of monoclonal antisera generated against recombinant NF2d9 to disrup binding activity. This antisera does not cross-react with CP2 as assessed by immunoblotting.3 Addition of the antibody induced a partial supershift of wild type binding activity (Fig. 3C, compare lanes 1 and 3). In contrast, mutant activity was completely supershifted (Fig. 3C, compare lanes 2 and 4). These data suggest that NF2d9 can maintain DNA site occupancy at CP2 binding sites.

**Expression of CP2 and NF2d9 during Mouse Development**—To determine whether the distribution of expression of CP2 and NF2d9 was similar, we performed in situ hybridization on embryo sections using antisense and sense probes specific for each mRNA transcript. Normal embryos were examined at E9.5, E11.5, and E13.5 dpc. Probes were of similar specific activity, and sense probes produced little background signal from embryos probed at all developmental stages (Fig. 4, A and C). However, utilizing an antisense probe, we observed CP2 expression in most tissues at similar levels at E13.5 dpc (Fig. 4B). In contrast, although expression of NF2d9 was observed in all tissues, it was markedly higher in the fetal liver (Fig. 4D, arrow). It is possible that loss of CP2 expression results in up-regulation of NF2d9 expression. To test this

| Parameter measured | CP2+/- mice | CP2-/- mice |
|--------------------|-------------|-------------|
| RCC \(\times 10^9\) cells/ml | 9.52 ± 0.20 | 9.44 ± 0.20 |
| Hematocrit | 48.13 ± 4.67 | 50.83 ± 4.00 |
| Reticulocytes (%) | 2.63 ± 0.47 | 2.77 ± 0.63 |
| MCV | 52.3 ± 4.7 | 55.6 ± 5.46 |
| MCH | 16.49 ± 0.73 | 16.67 ± 0.99 |

**Table I**

Hematological analysis of 18 CP2+/- and 18 CP2-/- animals

RCC, red cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

\(^3 A. T., J. M. C., \text{and S. M. J.},\) unpublished information.
hypothesis, we determined the expression of NF2d9 in the brain, kidney, and heart of CP2+/+ and CP2 null animals. As shown in Fig. 4E, no significant change in the relative expression of NF2d9 was observed in any of these tissues when compared with the actin control.

**LBP-1a Can Functionally Replace CP2 Activity**—The DNA binding, transactivation, and expression patterns of LBP-1a/NF2d9 suggested that this protein could potentially compensate for the loss of CP2. To evaluate this further, we examined whether LBP-1a could fulfill the protein-protein interaction role of CP2. We have recently defined two transcription factors...
that specifically interact with CP2. The first, NF-E4, is the fetal/erythroid-specific component of the SSP. The second, RING1B, is a RING finger domain-containing protein involved in the regulation of CP2-dependent transcription. Utilizing the yeast two hybrid assay system we compared the ability of CP2 and LBP-1a and NF-E4. The experiments were performed using the above methodology with the exception that pACT-Ring1B was substituted for pACT-NF-E4. pACT-Ring1B contains the entire coding region of the Ring1B cDNA fused in frame with the activation domain of GAL4 (amino acids 768–881). A specific interaction between pTD encoding the SV40 T-antigen and pVA3 encoding p53 has been reported previously. Yeast transformants were streaked onto synthetic media plates lacking leucine, tryptophan, and histidine (LTH) to assess potential protein interactions. If yeast two hybrid assay of CP2/LBP-1a and Ring1B. The experiments were performed using the above methodology with the exception that pACT-Ring1B was substituted for pACT-NF-E4. pACT-Ring1B contains the entire coding region of the Ring1B cDNA fused in frame with the activation domain of GAL4 (amino acids 768–881).

**DISCUSSION**

Prompted by studies documenting the importance of the NTF-1 gene in *Drosophila* development, we have examined the effects of gene targeting of the mammalian NTF-like gene, CP2, in mice. These experiments assumed additional importance with the identification of CP2 as a major component of the SSP, a protein complex involved in the regulation of fetal hematopoiesis and with the identification of CP2 as a key factor in the T-cell proliferative response (17, 19). To our surprise, no difference in hematopoiesis, globin chain synthesis, or immunological function between wild type and CP2 null animals was observed. Indeed, the general physiology, behavior, and reproductive capacity of CP2−/− animals was identical to wild type littermates. Examination of the binding activities of nuclear extracts suggested that CP2 consensus binding sites are occupied in CP2−/− animals by NF2d9, a protein highly related to CP2. In addition, we have shown that NF-E4 and RING1B, known heteromeric partners of CP2, also form protein complexes with LBP-1a/NF2d9. The similar patterns of expression of the two highly related genes coupled with the DNA and protein binding data suggests that the lack of a discernible phenotype in the CP2 nullizygous mice may be due to rescue by NF2d9.

Although our data is consistent with redundancy of function in the mammalian NTF-like gene family, it was essential to rule out the possibility that the knockout phenotype was masked by the production of a truncated or alternatively spliced form of CP2. Several lines of evidence suggest that this did not occur. First, RNase protection and RT polymerase chain reaction analysis failed to show evidence of either the 5′ exons 1 and 2 or the 3′ end of the coding sequence. Second, DNA-protein interaction studies demonstrated loss of the CP2 homodimeric band with the appearance of a slower migrating complex, which we attributed to NF2d9. The ability of LBP-1a/NF2d9 to bind to CP2 consensus sites is not surprising. Examination of the amino acid sequence of the respective proteins reveals striking homology in the region that we, and others, have identified as the DNA binding domain (18, 30). Between residues 150 and 291, the core of the binding domain, the two proteins share 90% identity and 96% similarity. Previous studies have demonstrated that LBP-1a and CP2 can bind the CP2 consensus sequence adjacent to the HIV initiation site (9). We have extended that observation, confirming that LBP-1a also binds to the CP2 sites in the SV40 major late promoter and the murine γ-fibrinogen and α-globin promoters. These sequences are archetypal CP2 binding sites in that they consist of a pair of direct repeats (G/A)CTGG spaced by an intervening sequence of variable content, but set length, which restricts protein binding to a single face of the DNA helix (18, 27). It is therefore likely that the other target sites for CP2 will also allow binding of LBP-1a.

The migration pattern we observed with CP2−/− extracts in the EMSA was consistent with a protein-DNA complex containing NF2d9. The slightly slower migration reflects the larger size of the NF2d9 protein and is consistent with previous reports and our observations of the difference in the migration of recombinant CP2 and NF2d9 (Fig. 3B) (9). The ability of antiserum that recognizes only NF2d9 to displace the complex observed in CP2−/− animals, coupled with the partial displacement observed with wild type extract, further supports this conclusion. Previous studies and the results reported here demonstrate the ability of LBP-1a/NF2d9 to functionally compensate for CP2 in its transcriptional roles (9). The activation domains of the two proteins have been mapped by our group to the N-terminal 40 amino acids. In this region, the two factors are 88% identical. We and others have demonstrated transcriptional activity of both CP2 and LBP-1a/NF2d9 in yeast and mammalian cells and in *in vitro* transcription assays (9, 15, 17, 30).

The ability of LBP-1a/NF2d9 to fully compensate for CP2 loss in the context of heteromeric protein interactions was less assured. Sequence comparison of the previously characterized dimerization domain of CP2 reveals that it shares 52% identity and 75% similarity with LBP-1a at amino acid level. Recently, the dimerization domain of CP2 has been refined to a region

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4 S. M. J. and J. M. C., submitted for publication.

5 J. M. C. and S. M. J., submitted for publication.
between residues 266 and 403 (18). In this sequence, the two proteins share 63% amino acid homology and 85% amino acid similarity. Therefore it was not surprising that protein-protein interactions between LBP-1α/CP2d9 and NF2d9 are widely co-expressed, albeit at differing levels. One striking difference was observed in the pattern of expression between the two genes, with NF2d9 being present at significantly higher levels in the fetal liver. Two interpretations of this result are suggested by the known roles of CP2 and NF2d9. First, NF2d9 has been linked to gender-specific expression of the human β-globin locus YAC demonstrate that a distinct fetal stage of human γ-gene expression occurs between days 10.5 and 13.5 (29, 31). As the SSP is involved in the preferential expression of NF2d9 and not CP2 is the primary partner of NF-E4 in the formation of this complex (21). Studies of human globin chain synthesis in NF2d9 nullizygous mice will address this question.

The ability of one highly related gene to compensate for the loss of another in gene-targeting experiments is widely recognized. Redundancy may be observed for all functions of the protein or may be limited to a single organ system (32, 33). As the SSP is involved in the preferential expression of NF2d9 and not CP2 is the primary partner of NF-E4 in the formation of this complex (21). Studies of human globin chain synthesis in NF2d9 nullizygous mice will address this question.

Despite the evidence suggesting that NF2d9 can fully compensate for the loss of CP2 in vivo, it is possible that a subtle CP2-specific phenotype exists in the nullizygous mice. For example, although we have performed extensive phenotypic analysis of this strain, it is possible that we have not identified the tissues or organs that require study. For example, we have closely evaluated the histological features of the central nervous system in the CP2−/− animals, as well as their behavioral patterns in view of the variations in patterns of expression in various parts of the developing brain. Although we have observed no abnormality, it is possible that an abnormal phenotype may become evident with the establishment of the CP2 null genotype in different inbred strains. Our studies documenting the pattern of expression of CP2 and NF2d9 failed to provide clues as to possible organs in which CP2 may be non-redundant. However, they did suggest that NF2d9 may play a key role in fetal liver function. The generation of NF2d9-deficient animals and their interbreeding with the CP2 nullizygous mice will address this issue.

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