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Cloning and characterization of the mouse Interleukin Enhancer Binding Factor 3 (*Ilf3*) homolog in a screen for RNA binding proteins

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Abstract. In a screen for RNA-binding proteins expressed during murine spermatogenesis, we have identified a cDNA that encodes a protein of 911 amino acids that contains two copies of the double-stranded RNA-binding motif and has 80% identity with human Interleukin Enhancer Binding Factor 3 (ILF3). Linkage and cytogenetic analyses localized the *Ilf3* cDNA to a portion of mouse Chr 9, which shows conserved synteny with a region of human Chr 19 where the human ILF3 gene had been previously localized, supporting that we had cloned the murine homolog of ILF3. Northern analysis indicated the *Ilf3* gene is ubiquitously expressed in mouse adult tissues with high levels of expression in the brain, thymus, testis, and ovary. Polyclonal antibodies detected multiple protein species in a subset of the tissues expressing *Ilf3* RNA. Immunoreactive species are present at high levels in the thymus, testis, ovary, and the spleen to a lesser extent. The high degree of sequence similarity between the mouse ILF3 protein and other dsRNA binding motif-containing proteins suggests a role in RNA metabolism, while the differential expression indicates the mouse ILF3 protein predominantly functions in tissues containing developing lymphocyte and germ cells.

Introduction

Several classes of RNA-binding motifs have been described (Burd and Dreyfuss 1994). One class, the double-stranded (ds)RNA-binding motif (dsRBM), is found in a number of proteins that function in embryonic development, growth control, cellular differentiation, and viral defense (St Johnston et al. 1992). The molecular functions of these proteins are presumably quite diverse, since they are localized to different subcellular compartments and different cells types. Furthermore, a number of dsRBM-containing proteins contain other functional motifs, such as deaminase or kinase domains that enable specific macromolecular reactions to be carried out in addition to their RNA-binding role (Clemens and Elia 1997; Maas et al. 1997). To date, no dsRBM-containing protein has shown sequence-specific binding in vitro. However, in vivo experiments with the *Drosophila* protein Staufen, which contains five copies of the dsRBM, indicates it can interact with selected RNAs (Broadus et al. 1998; Ferrandon et al. 1994; Kim-Ha et al. 1991; Li et al. 1997). Most proteins with the dsRNA binding motif have more than one copy, although some copies are non-functional or not required for RNA binding (Bycroft et al. 1995; St Johnston et al. 1992). NMR structural analysis has shown the dsRBM to be a compact structure consisting of two alpha-helices lying across the surface of a three-stranded anti-parallel beta-sheet (Bycroft et al. 1995).

RNA-binding proteins play important roles in RNA metabolism during germ cell development in several organisms (Beck et al. 1998; Eberhart et al. 1996; Ellis and Kimble 1994; Ruggiu et al. 1997; St Johnston et al. 1991). During murine spermatogenesis, post-transcriptional control is required owing to the silencing of global transcription several days before the completion of spermatogenesis. Several RNAs encoding sperm structural proteins and chromosome-associated transition and protamine proteins are synthesized in meiotic or early haploid cells, translationally repressed for several days, and then translated in late-stage spermatids. Translational repression of protamine 1 (*Prm1*) mRNA is required for normal spermatogenesis with premature translation leading to a dominant male sterile phenotype (Lee et al. 1995). Sequences residing in the 3′UTR of the *Prm1* mRNA mediate proper translational repression and activation (Braun et al. 1989; Fajardo et al. 1997).

To identify potential translational regulators of *Prm1* mRNA, an expression cloning screen was employed to identify *trans*-acting factors that bind to its 3′UTR. Here we report the primary characterization of a dsRNA-binding motif-containing protein identified in this screen.

Materials and methods

cDNA cloning. Two overlapping partial cDNAs were isolated from a mouse male germ cell cDNA expression (Mitch Eddy Niehs) library probed with a radiolabeled RNA containing the *Prm1* 3′UTR (Schumacher et al. 1995b). DNA sequencing and database searches led to the identification of mouse expressed sequence tags (ESTs) representing more 5′ regions of the cDNA. Oligonucleotides based on the mouse EST were used to screen a plasmid cDNA library made from total mouse testes (Clontech, Palo Alto, Calif.). To generate the final cDNA sequence contig, PCR primers were designed to amplify a small region from the plasmid cDNA library that was not represented by the cDNA clones. Plasmid cDNA clones and purified PCR fragments were sequenced with Dye-terminator cycle sequencing (Perkin Elmer, Norwalk, Conn.). Database analyses and searches were performed with the BLAST and CLUSTALW internet servers (Altschul et al. 1990; Thompson et al. 1994).

RNA analysis. A multiple-tissue Northern blot containing 15 μg of total RNA from various mouse tissues was generated and probed with previously described methods (Lee et al. 1996). The blot was hybridized with an α ^{32}P-radiolabeled probe generated from a random hexamer labeling reaction with a ~2-kb cDNA insert representing nucleotides 1546–3426 of the mouse *Ilf3* sequence. A skeletal α-actin cDNA probe was used to assess the integrity of the RNA.

Antibody production. A portion of the mouse *Ilf3* cDNA encoding the C-terminal region (amino acids 468–793) was cloned into the pMAL-c2

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number AF098967.

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expression vector. The maltose-binding-protein (MBP)-ILF3 fusion protein was produced and purified as previously described (Lee et al. 1996). Rabbit antiserum was raised with the MBP-ILF3 fusion protein using R & R Rabbitry (Stanwood, Wash.) services.

Western blot analysis. Total protein was prepared from several mouse tissues, quantified, and analyzed via Western blot as previously described (Lee et al. 1996). The anti-ILF3 antiserum was used at a 1:250 dilution.

Chromosomal mapping. Linkage analysis was performed with a restriction fragment length polymorphism on DNA prepared from 94 offspring from (C57BL/6Ei × SPRET/Ei) × SPRET/Ei mice (BSS panel, Jackson Laboratory Backcross DNA Panel Mapping Resource). The oligonucleotides, 5′-GCCAGGCACTCCAGCACTCTCCGC-3′ and 5′-GTCTGTGGATGATGCTGCGATTGTG-3′, were used to amplify a 2.3-kb portion of the mouse *Ilf3* locus. *Stu*I restriction digests of the PCR product allowed the detection of the *Mus musculus* (0.4 kb, 1.0 kb, and 1.3 kb) or *Mus spretus* (1.0 kb and 1.7 kb) allele. This *Stu*I RFLP was used to analyze the BSS panel DNA samples, and the segregation pattern was used to determine the frequency of recombination events between *Ilf3* and known mouse chromosomal markers.

Fluorescence in situ hybridization localization of the mouse gene was performed as previously described with a *Ilf3* 2 kb cDNA probe (Schumacher et al. 1995b).

Results

Cloning of the mouse Ilf3 cDNA. Two partial cDNA clones encoding a dsRNA binding motif-containing protein were cloned in an expression cloning screen intended to identify *Prml* 3′UTR RNA-binding proteins. Database searches revealed several mouse expressed sequence tags (ESTs) that represented additional sequence not present in the two cloned cDNAs (Genbank: AA168925, AA087712 and X84692). An oligonucleotide based on the mouse ESTs was used to screen a testis plasmid cDNA library to identify more 5′ clones. A complete cDNA sequence was generated by PCR. The 3.4-kb cDNA contig contains a predicted open reading frame (ORF) that would encode a 911-amino acid protein with a predicted molecular weight of 98 kDa (Fig. 1).

A non-redundant Genbank database search with the Basic local alignment sequence tool (BLAST) revealed that the predicted ORF is most similar to the Interleukin Enhancer Binding. Factor 3 (ILF3, NF90, MPP4) protein with an 80% amino acid identity (Altschul et al. 1990). This high degree of identity between the human and mouse sequence, as well as their chromosomal locations (discussed below), suggests that the cDNA identified in our screen represents the mouse homolog of human ILF3 gene. Additional similarities with the mouse spermatid perinuclear RNA-binding protein, SPNR, and the *Xenopus* RNA-binding proteins 4F-1 and 4F-2 are very striking over a majority of the coding regions, as shown by the multiple sequence alignment (Fig. 2). A common feature to all these proteins are the two copies of the dsRNA binding motif (St Johnston et al. 1992). The mouse ILF3 protein contains two copies of the dsRBM: amino acid residues 417–478 and 540–601.

Database searches also revealed the presence of bipartite nuclear localization sequence just upstream of the first dsRBM (Dingwall and Laskey 1991). The human ILF3 protein is predominantly localized to the nucleus in HeLa and Jurkat T-cells, suggesting this sequence motif functions in vivo (Kao et al. 1994; Matsumoto Taniura et al. 1996). Significant sequence similarity, up to 65%, between the N-terminal third of mouse ILF3 and a motif found in several zinc finger-containing RNA binding proteins was also found (Meagher et al. 1998).

Differential levels of mouse Ilf3 mRNA and protein in adult mouse tissues. The expression level of the murine *Ilf3* mRNA was evalu-

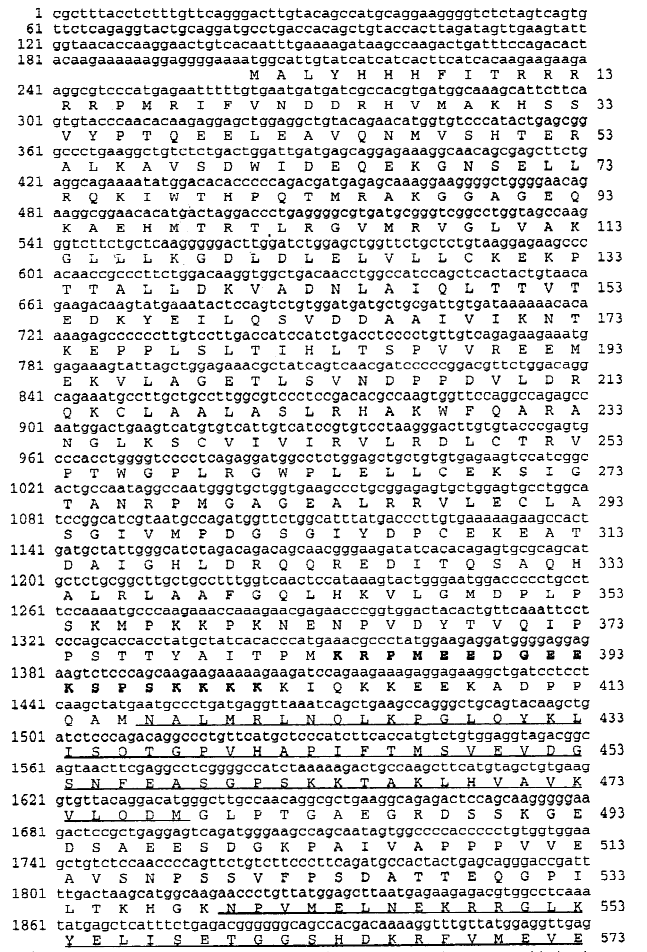


Fig. 1. Sequence of the mouse *Ilf3* cDNA. The nucleic acid sequence of the mouse *Ilf3* cDNA is listed above the predicted protein translation. The two copies of the dsRNA-binding motif are underlined, and the bipartite nuclear localization signal is in bold.

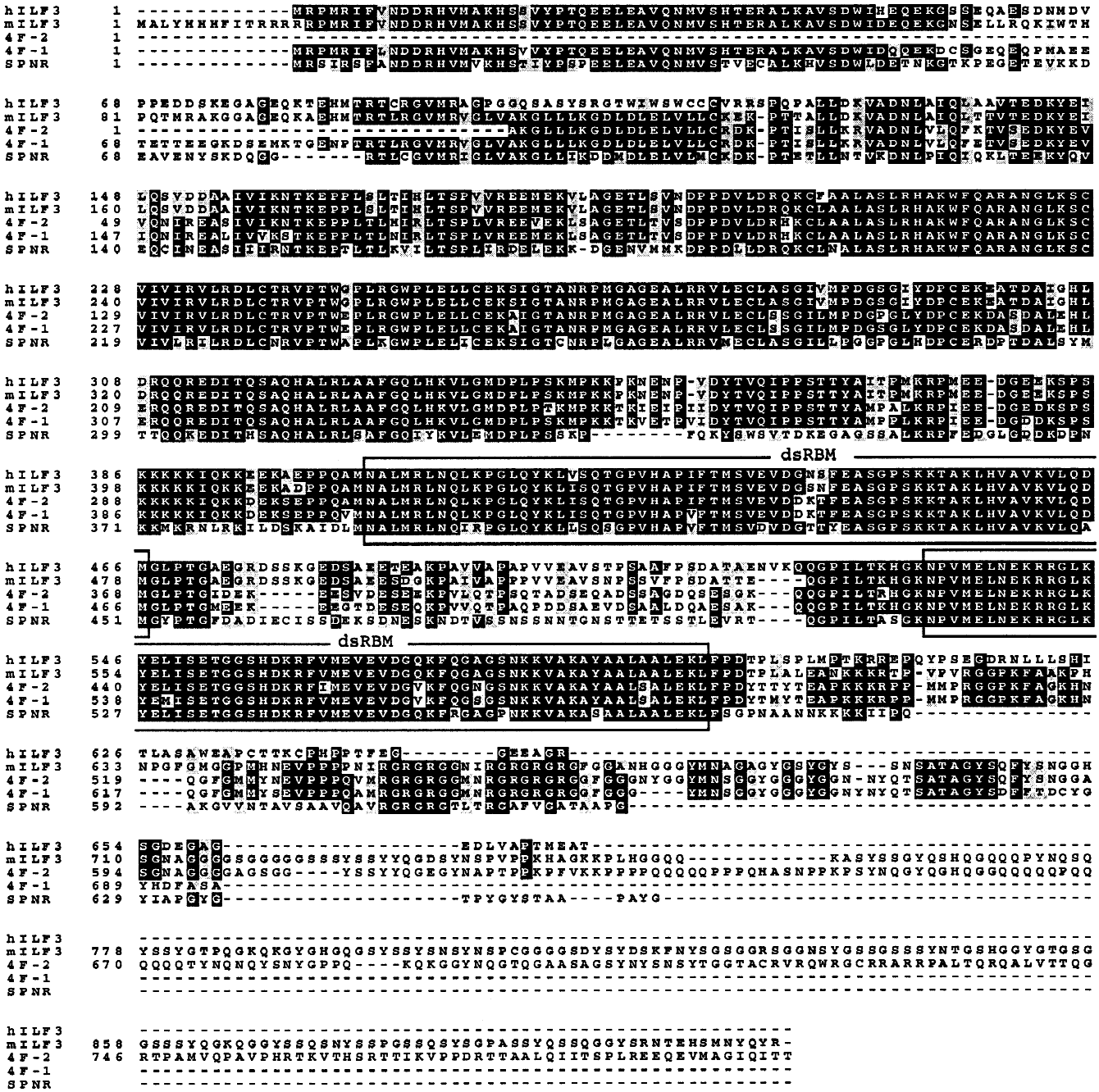


Fig. 2. Multiple sequence alignment of mouse ILF3 and related proteins. The multiple sequence alignment was generated with the CLUSTALW program: human ILF3 (B54857), mouse ILF3 (AF098967), *Xenopus* 4F-1 (I51652), and 4F-2 (I51653), and SPNR (A57284). Amino acids that are identical to the consensus (three or more proteins with an identical amino acid at that position) are shaded in black. Conservative changes with respect to the consensus are shaded in gray. The two copies of the double-stranded RNA binding motif are boxed.

ated by Northern blot analysis of total RNA extracted from multiple adult mouse tissues. A predominant 3.4-kb mRNA was detected in all tissues examined, with the brain, thymus, testis, and ovary showing the highest levels and the liver showing the lowest. Lower abundance mRNA species of different sizes were detected in the thymus, testis, and ovary. Three smaller mRNA species ranging from 1.5 kb to 2.4 kb and a large 6-kb mRNA were expressed at significantly lower levels in several adult tissues (Fig. 3).

In order to examine the mouse ILF3 protein distribution, we generated a rabbit polyclonal anti-ILF3 antiserum using a maltose-binding-protein (MBP)-ILF3 fusion protein. The antiserum was used to probe a multi-tissue Western blot containing the same mouse tissues used in the RNA expression analysis. The antiserum detected two protein species migrating closely with each other at a molecular weight of ~95 kDa; they were highly abundant in the thymus, testis, and ovary and to a much lesser extent in the brain and spleen (Fig. 4). The *Ilf3* cDNA ORF predicts a 98-kDa protein, which is extremely close to the observed size of these immunoreactive bands. The human ILF3 protein has been shown to be phosphorylated in HeLa cell extracts (Matsumoto Taniura et al. 1996). Such post-translational modifications that lead to a slightly re-

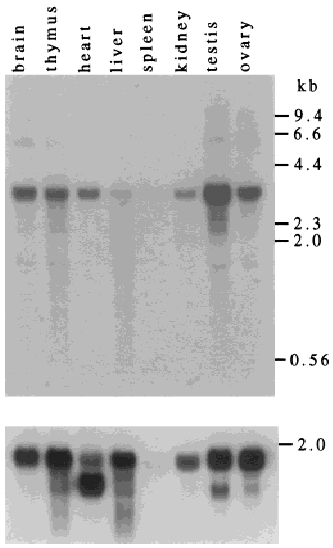


Fig. 3. Northern analysis of the mouse *Ilf3* mRNA expression. A mouse *Ilf3* cDNA was used to probe a Northern blot containing total RNA from mouse tissues (upper panel). The blot was subsequently probed with an α-actin cDNA probe as a control (lower panel).

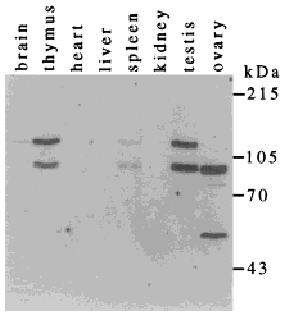


Fig. 4. Western analysis of adult mouse tissues with mouse ILF3 antisera. Total protein was extracted from various mouse tissues and quantified. 300 μg of total protein from each tissue was resolved with SDS-PAGE and subsequently blotted to nitrocellulose. Antiserum raised against a MBP-ILF3 fusion protein was used as the probe.

tarded species have been shown for a variety of proteins and could explain the detection of the two immunoreactive bands migrating at ~95kDa (Peeper et al. 1995). Alternatively, a different start codon could have been selected to generate a slight size variant in the ILF3 protein. A methionine codon in a good Kozak consensus can be found 81 nucleotides downstream of the predicted start codon (Kozak 1981). Selection of this alternative methionine codon would generate a predicted protein of 95 kDa as opposed to 98 kDa. Two prominent immunoreactive bands not predicted were the ~125-kDa species found in the brain, thymus, spleen, and testis, and the ~65 kDa protein found only in the ovary.

Ilf3 maps to mouse Chr 9. Two approaches were taken to map the murine *Ilf3* gene. First, a RFLP in a PCR product representing a portion of the *Ilf3* locus was used to analyze an interspecific backcross panel from The Jackson Laboratory. The analysis indicates the mouse *Ilf3* gene is on Chr 9 between the *Gir* gene and the chromosomal marker *D9Bir7* (Fig. 5 and available at http://www.jax.org/resources/documents/cmdata). Consistent with the above data, fluorescent in situ hybridization (FISH) analysis showed signals present on Chr 9 in the region of A4–A5 with no significant hybridization to other chromosomes (Fig. 6). Together, these experimental results indicate the murine *Ilf3* gene is present as a single copy in the mouse genome.

Discussion

We have identified a dsRNA-binding motif-containing protein from a mouse male germ cell library in an expression cloning screen designed to identify proteins that bind the *Prm1* 3′UTR. Although the mouse *Ilf3* cDNA was identified by its ability to preferentially bind the *Prm1* 3′UTR, the interaction is most likely

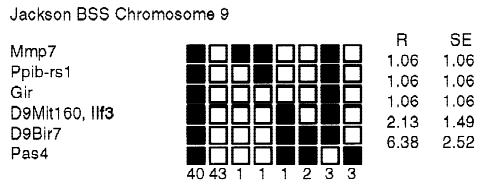
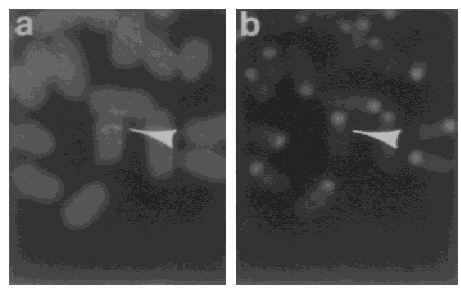


Fig. 5. Haplotype figure from The Jackson Laboratory's BSS interspecific backcross showing part of mouse Chr 9 with loci linked to *Ilf3*. The loci are listed in a proximal to most distal order at the left of the figure. Black and white boxes indicate the C57BL/6Ei and SPRET/Ei alleles respectively, while the number of animals with a given haplotype is listed at the bottom of the column. The percentage recombination (R) between adjacent loci with the associated standard error (SE) is given to the right of the figure. Raw data from The Jackson Laboratory were obtained from "http://www.jax.org/resources/documents/cmdata".



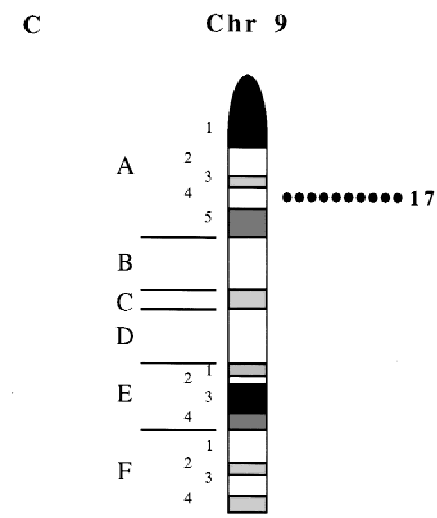


Fig. 6. Mapping the mouse *Ilf3* gene by fluorescence in situ hybridization. Localization of mouse *Ilf3* on mouse Chr 9 A4-A5 with the arrowhead pointing to the hybridization signal **(a)** and DAPI staining of the same chromosome depicted in A to identify banding pattern **(b).** An ideogram of mouse Chr 9 depicting the banding regions on the left and the hybridization distribution from 17 samples on the right **(c).**

non-physiological. Immunocytohistochemical experiments on adult mouse male testes with the ILF3 antiserum detect expression of the protein in spermatocytes and early haploid spermatids, cells that do not express the *Prm1* gene (unpublished data). Additionally, four of the five cDNAs identified in this expression cloning screen encode proteins that contain the dsRBM, a motif that binds double-stranded RNA or single-stranded RNA with structure (Lee et al. 1996; Schumacher et al. 1995a, 1995b). While the *Prm1* 3′UTR does not contain any extensive regions that could form

dsRNA, there are two alternate stem loops that could theoretically form (Lee et al. 1996). These small regions may have led to the fortuitous isolation of dsRBM-containing proteins in our expression cloning screen. Nucleic acid-binding experiments have shown that the human ILF3 protein can bind dsRNA and ssRNA molecules that are predicted to have a significant amount of secondary structure (Liao et al. 1998).

Localization of the mouse *Ilf3* gene by use of an interspecific backcross and FISH are in agreement with each other and indicates the *Ilf3* gene is a single-copy gene located on Chr 9 near *D9Mit160*, a region that shows conserved synteny with a small region of human Chr 19p13.2 (DeBry and Seldin 1996). Low-resolution mapping of the human ILF3 gene has localized it to a broad region of human Chr 19 (19q11-qter and 19p11-p13.1) with human–rodent somatic cell hybrids (Marcoulatos et al. 1998). The high degree of sequence similarity and chromosomal localizations strongly suggest the mouse gene we have identified is the homolog of the human ILF3 gene.

Recently, human ILF3 was found to copurify with the DNA-dependent protein kinase (DNA-PK), a protein complex composed of a catalytic serine-threonine kinase and the heterodimeric DNA-binding cofactor Ku, which is activated upon binding double-stranded DNA ends. Purified recombinant human ILF3 was shown to promote complex formation between DNA-PK and double-stranded DNA ends, implicating ILF3 in the process of DNA double-strand break repair. Mice with a mutation in the gene encoding the DNA-PK catalytic subunit (DNA-PKcs), *Prkdc*, exhibit the severe combined immunodeficiency (scid) phenotype (Blunt et al. 1995). *Prkdc*^{scid} mice are unable to complete the rearrangement of the variable (V), diversity (D), and joining (J) genetic elements, which prevents the production of mature B and T cells. Additionally, cells derived from *Prkdc*^{scid} mutants have been shown to be highly sensitive to ionizing radiation, suggesting the *Prkdc* gene product plays a critical role in V(D)J recombination and DNA double-strand break repair (Biedermann et al. 1991; Fulop and Phillips 1990; Hendrickson et al. 1991). From these observations, one might expect to find abundant levels of ILF3 protein in cells that are carrying out V(D)J recombination and the process of DNA double-strand break repair. We have detected mouse ILF3 protein in the spleen and thymus where B and T cells respectively are carrying out V(D)J recombination (Hertz et al. 1998; Robey and Fowlkes 1994). The high levels of protein in the testis and ovary suggest it could be playing a role in meiotic recombination. The remaining tissues in the organism might not require abundant ILF3 protein levels, as observed in our Western analysis, unless they are subjected to conditions that induce DNA double-strand breaks such as ionizing radiation. The continuous presence of *Ilf3* mRNAs in these other tissues, as suggested by the ubiquitous mRNA expression observed in our Northern analysis, would permit the rapid production of ILF3 protein, allowing the process of DNA double-strand break repair to initiate rapidly.

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