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Molecular cloning of *LSIRF*, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE)

Toshifumi Matsuyama, Alex Grossman, Hans-Willi Mittrücker, David P. Siderovski, Friedemann Kiefer, Takatoshi Kawakami[1], Christopher D. Richardson, Tadatsugu Taniguchi[1], Steven K. Yoshinaga[2] and Tak W. Mak*

Amgen Institute, Ontario Cancer Institute and Departments of Immunology and Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, Ontario M4X 1K9, Canada, [1]Institute for Molecular and Cellular Biology, Osaka University, Yamada-oka 1-3, Suita-shi, Osaka 565, Japan and [2]Amgen Inc., Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789, USA

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ABSTRACT

Interferon regulatory factor (*IRF*) genes encode a family of DNA-binding proteins that are involved in the transcriptional regulation of type-I interferon and/or interferon-inducible genes. We report here the characterization of *LSIRF*, a new member of the *IRF* gene family cloned from mouse spleen by the polymerase chain reaction using degenerate primers. *LSIRF* was found to encode a 51 kDa protein that shares a high degree of amino acid sequence homology in the DNA-binding domain with other IRF family members. *LSIRF* expression was detectable only in lymphoid cells. In contrast to other *IRF* genes, *LSIRF* expression was not induced by interferons, but rather by antigen-receptor mediated stimuli such as plant lectins, CD3 or IgM crosslinking. In *in vitro* DNA binding studies, LSIRF was able to bind to the interferon-stimulated response element (ISRE) of the MHC class I promoter. The expression pattern and DNA binding activities suggest that LSIRF plays a role in ISRE-targeted signal transduction mechanisms specific to lymphoid cells.

INTRODUCTION

Regulation of gene expression can occur at several different levels, but the activation of gene-specific transcription factors is considered the most fundamental to this process. One family of transcription factors, the interferon regulatory factors (IRFs), consists of four members: IRF-1, IRF-2, ISGF3γ and ICSBP. All four IRFs are characterized by a strongly conserved N-terminal DNA-binding domain containing a repeated tryptophan motif (1).

Interferon regulatory factors-1 (IRF-1) and -2 (IRF-2) were originally identified by studies of the transcriptional regulation of the human interferon-beta (IFN-β) gene (2,3). cDNA expression studies have demonstrated that IRF-1 functions as a transcriptional activator of IFN and IFN-inducible genes, whereas IRF-2 represses the effect of IRF-1 (4,5). Recent analyses have shown that *IRF-1* can also act as a tumor suppressor gene and *IRF-2* as a possible oncogene (6). *IRF-1* expression is induced by type-I (α/β) and type-II (γ) IFNs (2,7), whereas IRF-2 is both constitutively expressed and induced by type-I IFNs (3).

Interferon-stimulated gene factor-3 gamma (ISGF3γ) is an IFN-γ-inducible protein which associates with ISGF3α subunits activated from a latent cytosolic form by type-I IFNs (8,9). Upon association, this complex has been shown to translocate to the nucleus and bind a specific DNA sequence found in the promoter region of IFN-inducible genes, known as the ISRE (IFN-stimulated response element) (1). Recently, ISGF3α subunits of 91/84 and 113 kDa have been cloned (10,11) and designated as signal transducer and activator of transcription-1 (STAT-1) and -2 (STAT-2), respectively, which are targets of JAK kinase phosphorylation following type-I IFN/IFN-receptor engagement (12,13).

Interferon consensus sequence binding protein (ICSBP) is also an IFN-γ-inducible protein, originally isolated as a protein that recognizes the ISRE motif (also called ICS) of the promoter of murine MHC class I, H-2L^D gene (14). However, unlike IRF-1, IRF-2 and ISGF3γ, ICSBP exhibits a tissue-restricted pattern of expression as it is induced exclusively in cells of macrophage and lymphoid lineages (14). Recent studies have suggested that ICSBP has a similar role to IRF-2 in antagonizing the effect of IRF-1 on the induction of IFN and IFN-inducible genes (15,16). Indeed, the ISREs of interferon-inducible genes overlap *IRF-E*, the DNA sequences recognized by IRF-1 and -2 (17). Very recently, ISGF3γ was shown to bind the *IRF-Es* of the *IFN*-β gene

* To whom correspondence should be addressed

(18). These results clearly suggest some redundancy among IRF family members in their DNA recognitions.

With the aim of isolating new members of IRF family, we screened murine spleen cDNA by the polymerase chain reaction (PCR) using degenerate primers designed to amplify within the conserved DNA-binding domain of IRF family member genes. We then isolated the cDNA and gene for *LSIRF*, a new member of the interferon regulatory factor family. Analysis of both promoter sequence and gene expression have revealed that *LSIRF* is unique to other IRF members, as *LSIRF* expression is not induced by IFNs, and is restricted to the lymphoid lineage. In primary cultured lymphocytes, *LSIRF* is expressed at low levels: however, *LSIRF* is induced to significantly higher levels by antigen-receptor mediated stimuli. Analysis of *in vitro* DNA binding activity shows that LSIRF can bind the ISRE found in the MHC class I promoter. These results suggest that LSIRF is a transcription factor induced in lymphoid lineage following antigenic stimulation, resulting in the regulation of genes having ISREs.

MATERIALS AND METHODS

Cell lines and reagents

Cell lines EL-4, BW5147, HT-2, D10.G4.1, CTLL-2, J558, WEHI231 and P338D1 were obtained from the American Type Culture Collection (ATCC). CB17.51 and FDC-P1 (19) were kind gifts from Drs Christopher Paige and Norman Iscove, respectively. All cell lines and primary cultured cells were maintained in Iscove's medium supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37°C and 5% CO_2. Cell cultures of HT-2 and CTLL-2 were supplemented with 50 U/ml of recombinant murine IL-2 (Genzyme; Cambridge, MA) with 50 μM of 2-mercaptoethanol (2-ME); D10.G4.1 cultures were maintained with 50 U/ml of IL-2 and 50 U/ml of recombinant murine IL-1 (Genzyme) with 50 μM of 2-ME. Natural murine IFN-β was purchased from Lee Biomolecular Research (San Diego, CA), and recombinant murine IFN-γ and murine TNF were obtained from Genzyme. Antibodies against CD3 was obtained from PharMingen (San Diego, CA) and antibodies against IgM were a kind gift from Dr Christopher Paige. Concanavalin-A (ConA), cycloheximide and phorbol myristate acetate (PMA) were purchased from Sigma (St Louis, MO). Cyclosporin A was purchased from Sandoz (Basel, Switzerland).

Isolation of *IRF* gene fragments by PCR

Two degenerate oligodeoxynucleotide primers containing inosine bases (denoted 'I') were designed to amplify an 146 bp span by hybridizing to codons encoding conserved amino acids within the N-terminal, DNA-binding, tryptophan-repeat domain of the IRF family members (1), with the sense primer (5′-ATI CCI TGG AAI CAC GC-3′) corresponding to amino acids IPWKHA (e.g. amino acids 36–41 of murine IRF-1; Swiss-Prot accession no. P15314, or amino acids 38–43 of murine ICSBP; Swiss-Prot accession no. P23611) and the antisense primer (5′-GCA CAI CGI AAI CTI GCC TTC CA-3′) corresponding to amino acids WKTRLRCA (e.g. amino acids 80–87 of murine ICSBP). These two primers were used to amplify a cDNA derived from the spleen of a C57Bl/6 mouse (a kind gift from Dr Kenji Kishihara). PCR was carried out on a programmable thermal cycler (Perkin-Elmer Cetus; Norwalk, CT) in 50 μl of PCR buffer (10 mM Tris-Cl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl) containing 200 μM dNTPs, 2 U *Taq* polymerase and 100 pM of each primer. The reaction was performed for 30 cycles under the following thermal conditions: 94°C for 60 s, 37°C for 60 s and 72°C for 60 s.

PCR amplification products were cloned into the pCRII plasmid using the TA-cloning system (Invitrogen; San Diego, CA). Plasmid DNA from pCRII clones was prepared by alkaline lysis, electrophoresed through 1.5% agarose, and blotted onto Hybond N filter membrane (Amersham; Oakville, Canada) and hybridized with random-primed, ^{32}P-labeled DNA fragments of murine *IRF-1* and *IRF-2* (20) by manufacturer's protocol (Amersham). Among 37 clones, 18 clones hybridized to an IRF-1 cDNA probe and none hybridized to an IRF-2 cDNA probe. Five of the remaining clones were sequenced by the double-stranded Sequenase procedure as described by the manufacturer (USB; Cleveland, OH). One such clone, Spl 5, contained a novel sequence, *LSIRF*, as determined by a GenBank BLAST Search. Probed again with LSIRF, 19 clones hybridized.

cDNA library screening

A 146 bp PCR product with unique sequence was labeled with ^{32}P by random priming (Amersham) and used to screen a mouse IL-4-induced spleen cDNA library (Clontech; Palo Alto, CA) by standard protocols (21), yielding two *LSIRF* cDNA clones lacking the 5′-end of a complete open-reading frame. Plaque purification and preparation of phage DNA were carried out by standard techniques (21). One partial cDNA clone was used to screen a λZAP II mouse spleen cDNA library (Stratagene) and thereby isolate five additional *LSIRF* cDNA clones, spanning the coding region, but no single clone contained the entire open reading frame. An artificial clone PV-1 containing the whole coding region was constructed by PCR 5′ extension of one such clone C7A using the sense primer (5′-GGAATTCCC-AGCCAT-GAACTTGGAGACGGGCAGCCGGGGCTCGAGTTC-GG-3′), and anti-sense primer (5′-GGAATTCACTCTTGGATGGAAG-AATGACG-3′) as well as Vent DNA polymerase (New England Biolabs; Mississauga, Canada). The PCR product was cloned into the *Eco*RI sites of pBSII (Stratagene) and the sequence was verified as correct, forming PV-1.

Genomic library screening

A 630 bp probe was amplified by PCR from one of the cDNA clones of *LSIRF*, C16, using the sense primer (5′-CAGC-CCGGGGTACTTGCCGCTGTC-3′) and the antisense primer (5′-AGACCTTATGCTTGGCTCAATGGG-3′). This 630 bp fragment was labeled with ^{32}P by random priming (Amersham), and subsequently used to screen a genomic library derived from the mouse strain 129/J (a kind gift from Dr Janet Rossant). Two overlapping genomic clones (12 and 15 kb) were isolated and restriction fragments were subcloned into pBSII for sequencing.

Northern blot analyses of *LSIRF* expression

Total RNA from cell culture was prepared as previously described (22), electrophoresed through a 1% agarose/formaldehyde gel and blotted onto Hybond N (21). Blots were hybridized with a random-primed (Amersham), ^{32}P-labeled 1.4 kb cDNA fragment encompassing the entire coding region of *LSIRF* (derived from *Eco*RI digested PV-1), and subsequently washed using conditions modified from Church and Gilbert (23). Blots

were reprobed with β-*actin* (20) or *L32* (24) as previously described. For the multiple tissue blot, total RNA was prepared as previously described (25).

Expression of LSIRF with recombinant baculovirus

The coding sequence for LSIRF (derived from *Eco*RI digested PV-1) was inserted into the pETL (BlueBacII) baculovirus expression vector using previously described methods (26,27).

Western blot analyses of LSIRF expression

Rabbit polyclonal antisera were raised against an LSIRF C-terminal peptide, GYELPEHVTTPDYHR (amino acids 427–441). Cell extracts were prepared from peripheral lymphocytes or Sf9 cells infected with baculovirus, resolved by 8% SDS–polyacrylamide gel electrophoresis (PAGE), and transferred to Immobilon-P membrane (Millipore; Bedford, MA) according to standard protocols (21). Protein blots were first incubated in blocking buffer [4% skim milk and 0.05% Tween 20 in 1 × phosphate buffered saline (PBS)] for 1 h at room temperature, incubated with anti-LSIRF antiserum at a dilution of 1:2000 in diluted blocking buffer diluted 1:1 with 1 × PBS, detected with goat anti-rabbit horseradish peroxidase-conjugated antibodies at a dilution of 1:5000 and developed with chemiluminescence substrate (Amersham).

Electrophoretic mobility shift assay

For nuclear extract preparation, baculovirus infected Sf9 cells were pelleted by low speed centrifugation and were washed twice in PBS. The cells were resuspended in 0.5 ml H-buffer/10^7 cells (hypotonic 'H' buffer: 25 mM HEPES–NaOH pH 8.0, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA and 0.5 mM DTT) and were swollen on ice for 30 min. They were disrupted with 15 strokes of a type B pestle in a dounce homogenizer. The nuclei were pelleted by centrifugation (10 000 r.p.m. for 10 min) in a microfuge at 4°C. They were then extracted by resuspension in 0.5 ml N-buffer/10^7 cells (nuclear extract 'N' buffer: 25 mM HEPES–NaOH, pH 8.0, 400 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol and 0.5 mM DTT) and were placed on ice for 20 min. The nuclei were centrifuged for 15 min at 15 000 r.p.m. in a microfuge (4°C). The supernatant was buffer exchanged using a Centricon 10 microconcentrator (Amicon) in E-buffer (exchange buffer: 25 mM HEPES–NaOH pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 15% glycerol and 0.5 mM DTT). All buffers contained protease inhibitors (0.5 mM PMSF, 0.5 μg/ml pepstatin, 0.5 μg/ml leupeptin and 0.5 μg/ml aprotinin).

For electrophoretic mobility shift assay, an oligonucletide containing the mouse MHC ISRE (ICS) sequence, 5′-TGCAGAAGTGAAACTGAG-3′ (14), and the antisense oligonucleotide were annealed and 5′-end-labeled with [^{32}P]ATP. In each reaction, 25×10^3 c.p.m. (corresponding to 1×10^{-11} moles) of the probe was used. The binding reaction buffer contained 12 mM HEPES–KOH pH 7.9, 30 mM KCl, 60 μM EDTA, 60 μM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N,N′*-tetraacetic acid], 0.3 mM DTT, 2.5% Ficoll, 0.6 μg poly(dI–dC)–poly(dI–dC) (Pharmacia; Uppsala, Sweden) and 0.05% NP-40. The extracts were diluted 8-fold with E-buffer and 0.1 mg/ml bovine serum albumin for use in the binding reaction. For the reactions in Figure 7A, the concentration of the control extract was 0.22 μg/μl, and that of the LSIRF containing extract was 0.14 μg/μl, after dilution. The binding reaction was started with the addition of 1 μl of the diluted extract, for a total binding reaction volume of 6.25 μl, then incubated for 20 min at 23°C. A 9% polyacrylamide gel (0.25 × TBE) was pre-run at 250 V for 2 h before sample application. The protein–DNA complexes were separated from the free probe by electrophoresis for 2 h at 300 V. As indicated in Figure 7, unlabeled competitor DNA was added at a 750-fold molar excess.

RESULTS

Cloning of *LSIRF*, a member of the interferon regulatory factor family

IRF family members have similar N-terminal DNA binding domains with a striking conserved tryptophan repeat (1). Given that tryptophan is encoded by a single codon (TGG), this invariant tryptophan motif was the starting point for the design of degenerate PCR oligonucleotides for cloning new IRF members. Two degenerate primers spanning 146 bp within the IRF family DNA binding domain were used to amplify mouse spleen cDNA. A PCR product with unique sequence, denoted LSIRF, was subsequently used to screen a cDNA library and, in turn, LSIRF cDNA clones were used to isolate genomic clones from a 129/J mouse genomic library (Fig. 1).

The nucleotide and predicted amino acid sequences of *LSIRF*, cDNA and genomic clones were compared with all sequences in the GenBank and Swiss-Prot databases. Significant similarity, at both nucleotide and amino acid levels, was detected between LSIRF and four other IRF family members in the N-terminus (Fig. 2). Among IRF family members, ICSBP is most closely related to LSIRF by deduced protein sequence. N-terminal homology between LSIRF and ICSBP is 83% identity, allowing for a one-amino-acid gap; by comparison, identity between LSIRF and ISGF3γ or IRF-1/IRF-2 in this region is ~65 and 55%, respectively (Fig. 2A). LSIRF protein sequence is also related to ICSBP and ISGF3γ over 95 amino acids in a C-terminal domain [51 and 37% identity, respectively, (Fig. 2B)].

Translation of nucleotide sequences from seven independent *LSIRF* cDNA clones revealed that five clones have an extra glutamine residue at position 164. At present, it is not clear whether there is a functional difference between the two isomers; placement of the extra glutamine is not within the DNA binding domain nor the conserved C-terminal domain shared with ICSBP and ISGF3γ. The plasmid PV-1 has an extra glutamine residue at position 164.

As with other IRF family members, *LSIRF* also has five conserved tryptophan residues in the N-terminal DNA binding domain (Fig. 2A). The spacing between the five tryptophan repeats is highly conserved (from the first to the fifth): 14, 11, 19 and 17/18 amino acids, respectively (Fig. 3)

LSIRF cDNA has an extensive 3′ untranslated region that is A/T-rich relative to the coding region and contains two ATTTA and one TTATTTAT motifs which are found in the mRNA of many cytokines and proto-oncogenes and are thought to confer mRNA instability (28,29) (Fig. 1A).

Organization of the *LSIRF* gene

Nucleotide sequence of genomic clones and comparison with cDNA sequence has revealed that the murine *LSIRF* gene consists

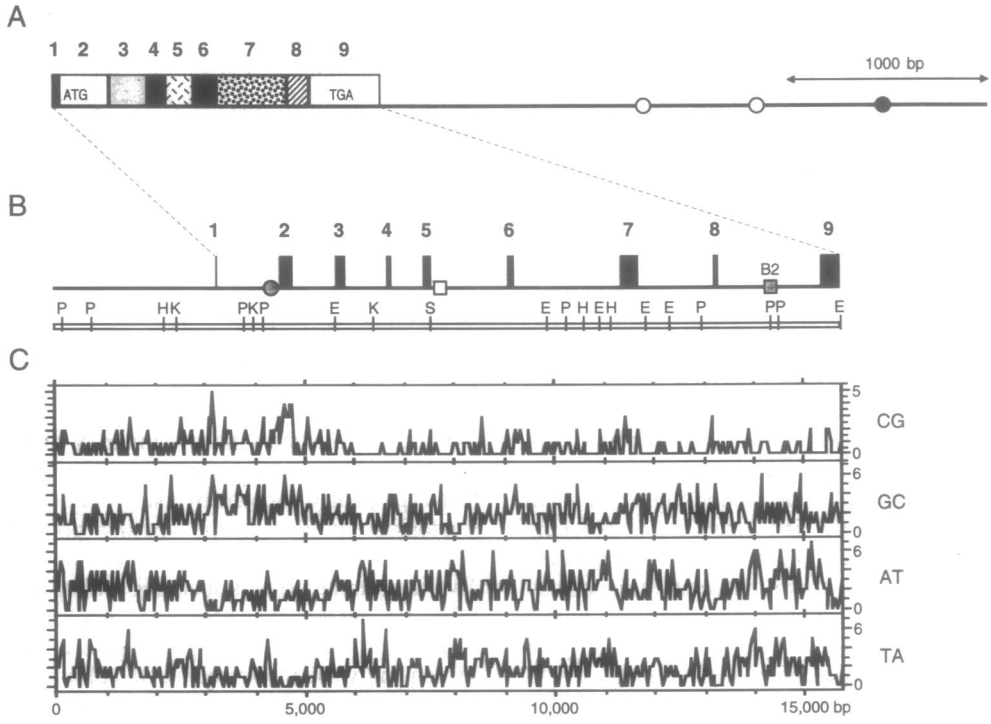


Figure 1. Genomic organization of the murine *LSIRF* cDNA and gene. (**A**) *LSIRF* cDNA (GenBank accession no. U11692). Numbers denote the relative position of exons. Exon 1 corresponds to the 5′ untranslated region, exon 2 contains the putative initiator ATG codon and exon 9 contains the terminator TGA codon. Exons 2 and 3 encode the conserved IRF-family DNA binding domain (Fig. 2A); exons 7 and 8 encode a polypeptide sequence conserved among LSIRF, ICSBP and ISGF3γ (Fig. 2B). Open and closed circles represent locations of ATTTA and TTATTTAT mRNA instability motifs, respectively (26,27). The 3′ boundary of exon 9 represents the 3′ end of the isolated *LSIRF* genomic clone and may indeed extend further into the 3′ untranslated region of the cDNA. (**B**) Exon/intron organization of *LSIRF* gene (GenBank accession no. U20949). Solid, numbered boxes represent exons and horizontal lines represent introns and 5′ flanking region. Shaded circle represents $(AGG)_7$ trinucleotide repeat located 5′ of exon 2. Open square indicates 102 bp region containing multiple T/G-rich repeat elements located 3′ of exon 5A/B. Shaded square denotes a 165 bp region with >80% sequence identity to the murine B2 short interspersed repeat element (SINE). Restrictions sites are denoted below genomic map: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sfi*I. (**C**) Frequency of various dinucleotides within genomic sequence displayed using the GCG programs Window and Statplot (64). Readings were taken in successive 40 nucleotide windows with one nucleotide overlap.

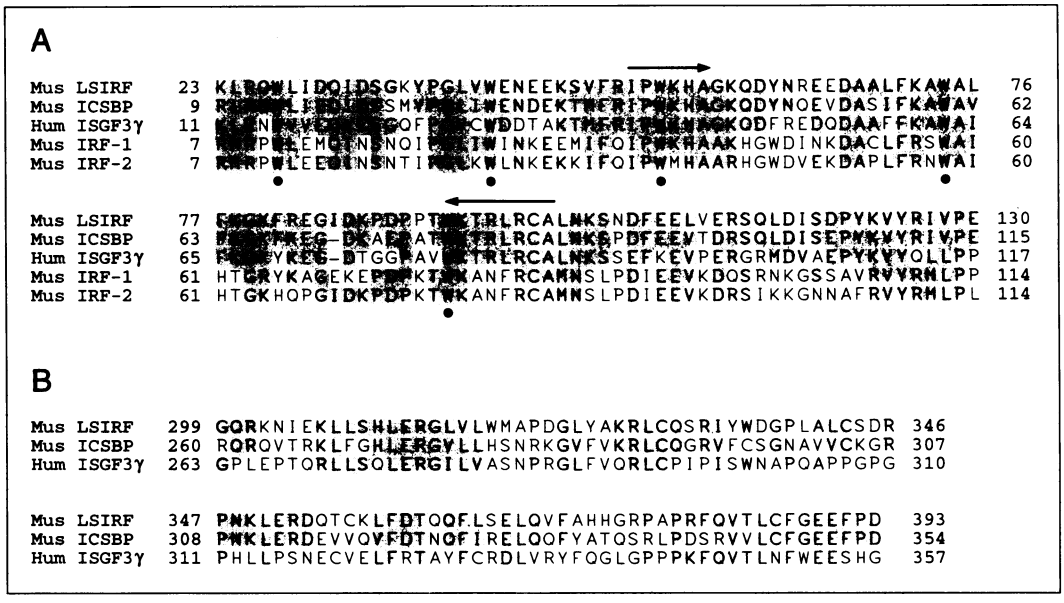


Figure 2. Protein sequence comparisons between LSIRF and other IRF family members. Shading indicates residues with similarity to LSIRF protein sequence. (**A**) Alignment of N-terminal, DNA-binding domain (104 amino acids) conserved between murine LSIRF, murine ICSBP (SwissProt P23611), human ISGF3 γ subunit (SwissProt Q00978), murine IRF-1 (SwissProt P15314) and murine IRF-2 (SwissProt P23906). Circles denote tryptophan residues conserved among IRF family members (1). Arrows indicate relative positions of degenerate PCR primers used to clone *LSIRF* and designed by reverse translation of conserved polypeptide sequences. (**B**) C-terminal region (95 amino acids) of sequence similarity between murine LSIRF, murine ICSBP and human ISGF3 γ subunit.

Figure 3. Structure of 5′ upstream region of *LSIRF* gene. The putative transcription start site is indicated by +1. Exon 1 sequence, identified by comparison of cDNA and genomic *LSIRF* sequences, is represented in lower case. Potential *cis*-regulatory elements within the 5′ upstream region are indicated with italics as follows: GC boxes (Sp1 binding site), NF-IL6-binding sites, AP2-binding site, TCF-1/LEF-1-binding sites, AP1-binding site, NF-κB-binding sites, Ets-1-binding sites, GATA-3-binding sites, PU.1/Spi-1-binding sites, homology with E1-box of immunoglobulin heavy chain gene enhancer (NFmuE1), and homology with immunoglobulin heavy chain gene enhancer core motif (IgH-core). Sequence presented in this figure corresponds to base pairs 1078–3377 of GenBank accession no. U20949.

of at least nine exons that span a minimum of 13 kb (Fig. 1B). The first exon encodes solely 5′ untranslated sequence, whereas exon 2 contains the putative translation start codon (ATG). Exons 2 and 3 encode the conserved IRF-family DNA binding domain (Fig. 2A). Exons 7 and 8 encode the C-terminal polypeptide sequence conserved among LSIRF, ICSBP and ISGF3γ (Fig. 2B), and exon 9 contains the terminator TGA codon. The sizes of each exon and intron as well as the DNA sequence of each exon/intron junction in comparison with *ICSBP* (30) are summarized in Table 1. Consensus splice sequences (GT/AG) are found at every exon/intron junction. As mentioned above, five out of seven *LSIRF* cDNA clones have an extra glutamine residue, resulting from the use of two alternative splice sites, which create two alternative exon 5s [A and B; where B has an extra glutamine codon at the 5′ end (Table 1)].

Several repetitive motifs exist in the *LSIRF* locus (Fig. 1B). There are seven direct repeats of AGG trinucleotide in the first intron, and an extended region of multiple T/G-rich repeats 3′ to exon 5 with a core structure of $(GGT)_6(GCT)_5(GGGGTG-GT)_4GGAG(TGG)_6$. There is also a murine B2 short interspersed repeat element (SINE) (31,32) in the intron between exons 8 and 9. In addition, there are several instances of poly-A and poly-T tracts across the entire genomic sequence. Analysis of dinucleotide frequencies across the genomic sequence indicates a relative enrichment for rare CpG dinucleotides, extending from ~300 bp upstream of the promoter TATA-box to the 3′ end of the second intron: this region is also enriched in GpC dinucleotides and correspondingly depleted in ApT and TpA dinucleotides (Fig. 1C). This relative enrichment for CpG dinucleotides is suggestive of a CpG-island and this may indicate that *LSIRF* is expressed in the germ-line as well as in mature lymphocytes (33).

The 5′ upstream sequence of the *LSIRF* gene

The predicted transcription start site, as illustrated in Figure 3, is based on a weak CAP consensus motif (CANPyPyPy; −1 to +5) described for eukaryotic RNA pol II promoters (34), distance from the TATA box (−38 to −33), and presumed exon size similarity with the murine *ICSBP* gene (30) (Table 1). Analysis of nucleotide sequence up to 2000 bp 5′ of the putative transcription start site revealed a TATA-box and three potential Sp1 sites (34–36), but no CAAT box (37) in the promoter sequence. For comparison, the murine *ICSBP* promoter has an inverted CAAT box (30), and the murine *IRF-1* and *IRF-2* promoters have CAAT boxes but no TATA boxes (38).

Several potential transcription factor binding sites were found in the 5′ upstream region of the *LSIRF* gene: NF-IL6-binding sites (39), AP2-binding site (40), TCF-1/LEF-1-binding sites (41,42),

Table 1. Comparison of murine *LSIRF* exon/intron organization with murine *ICSBP* gene

Exon	Exon size (bp) *LSIRF*	Exon size (bp) *ICSBP*	Percent identity	EXON/.....intron...../EXON	Intron size (bp) *LSIRF*	Intron size (bp) *ICSBP*
1	38+	51	-[a]	GGTGAG/**gt**aaggcc...ctcctc**ag**/CGCACA	1249	~3500
2	248	175	74%	PheLys AlaTrp TTCAAG/**gt**tagcag...gttctt**ag**/GCTTGG	884	~3700
3	187	184	74%	LysLysG lyAla AAAAAG/**gt**aagggg...acacac**ag**/GAGCAA	824	~2000
4	89	89	-	AlaGln ValHis GCCCAG/**gt**atgtgg...gtcagc**ag**/GTTCAT	668 (A)	~1300
				GlnVal ...tctgtc**ag**/CAGGTT	665 (B)	
5A/B	142/145	106	-	GluAsnG lyCys AAAATG/**gt**aaggat...aaatgc**ag**/GTTGCC	1552	~1200
6	108	48	-	LeuSerA spCys TCTCAG/**gt**gagtgt...gttcac**ag**/ACTGCC	2162	~4100
7	354	381	57%	LeuSerG luLeu TATCAG/**gt**aacaca...gttttc**ag**/AGCTGC	1491	~700
8	113	116	60%	AlaHis ValGlu GCTCAT/**gt**gagtac...tattaa**ag**/GTGGAA	2041	~600
9	374+	1700+	-			

[a]Not significantly different than percent identity of *LSIRF* exon sequence to randomized *ICSBP* exon sequence (GCG GAP program). Bold letters identify conserved splice donor/acceptor sequence; underlined letters indicate identity with murine *ICSBP* gene sequence.

AP1-binding site (43), NF-κB-binding sites (44,45), Ets-1-binding sites (46), GATA-3-binding sites (47), PU.1/Spi-1-binding sites (48,49), homology with the E1-box of immunoglobulin heavy chain gene enhancer (50) (NFmuE1) and homology with the immunoglobulin heavy chain gene enhancer core motif (51,52).

LSIRF is preferentially expressed in lymphocytes

Basal expression of *LSIRF*mRNA in various organs was analyzed. As shown in Figures 4A and 5B, faint signals of *LSIRF* mRNA were detected in bone marrow, spleen and lymph node cells, and more weakly in lung and thymus. We have confirmed that *LSIRF* mRNA levels in thymus are much lower than peripheral lymph nodes (data not shown). The size of the *LSIRF* transcript was ~5.5 kb and no additional bands were observed under stringent hybridization conditions. We could not detect any *LSIRF* mRNA in brain, liver, intestine, salivary gland, testis, heart or smooth muscle. The lack of expression of *LSIRF* mRNA in pancreas was comfirmed by RT–PCR (data not shown), because much less total RNA was loaded on that lane of the Northern blot (Fig. 4A). To examine which cell lineages express *LSIRF*, we further studied the mRNA expression levels in established cell lines. In T cell lines, expression was found in CTLL-2, D10.G4.1 and HT-2, but not in EL-4 and BW5147 (Fig. 4B, HT-2 data not shown). The first three cell lines are believed to represent peripheral T cells, whereas EL-4 and BW5147 represent more immature thymocytes (53). Therefore, together with the data from thymus, spleen (Fig. 4A), and lymph nodes (Fig. 5B), we conclude that, in T cell development, *LSIRF* is preferentially expressed in mature T cells. In B cell lines, *LSIRF* is expressed in pre-B cell (CB17.51), B cell (WEHI231) and plasmacytoma (J558) cell lines; among the cell lines tested, J558 exhibited the strongest *LSIRF* expression (Fig. 4B). Embryonic fibroblasts and myeloid [FDC-P1; (19)] and macrophage (P388D1) cell lines did not express detectable *LSIRF* transcript (Fig. 4B).

LSIRF is strongly induced in primary lymphocytes by antigen mimetic stimuli

We examined next the induction of *LSIRF* transcript following several stimuli in primary lymphocytes from spleen or lymph nodes. In a time course experiment, an increase in *LSIRF* mRNA levels appeared 1 h after ConA stimulation, peaked after 2 h, and then declined, although lasting up to 36 h after stimulation (Fig. 5A). *LSIRF* was also strongly induced by CD3 crosslinking (Fig. 5B). Significant inductions were also observed using anti-IgM antibodies and PMA. However, LPS induction of *LSIRF* mRNA was marginal (Fig. 5C). Cycloheximide, a protein synthesis inhibitor, was observed to induce *LSIRF-3* in 1 h and an additive effect was seen with ConA stimulation (Fig. 5D). On the other hand, cyclosporin A significantly inhibited the induction of *LSIRF* by ConA.

All known IRF family members are strongly induced by interferons (1–3,7,14). Thus we studied whether type-I or type-II interferon could induce *LSIRF*. However, *LSIRF* was not induced in lymphocytes by interferons nor by tumor necrosis factor-α (TNF-α) (Fig. 5B). On the other hand, reprobing experiments showed that interferon-inducible genes such as 2′5′ oligoadenylate synthase and IRF-1 were induced by IFN-β and IFN-γ, respectively (data not shown). We studied next the induction of *LSIRF* in embryonic fibroblasts with interferons. Although another member of the IRF family, *IRF-1*, was strongly induced by IFN-β, we could not detect *LSIRF* expression after stimulation of IFNs (data not

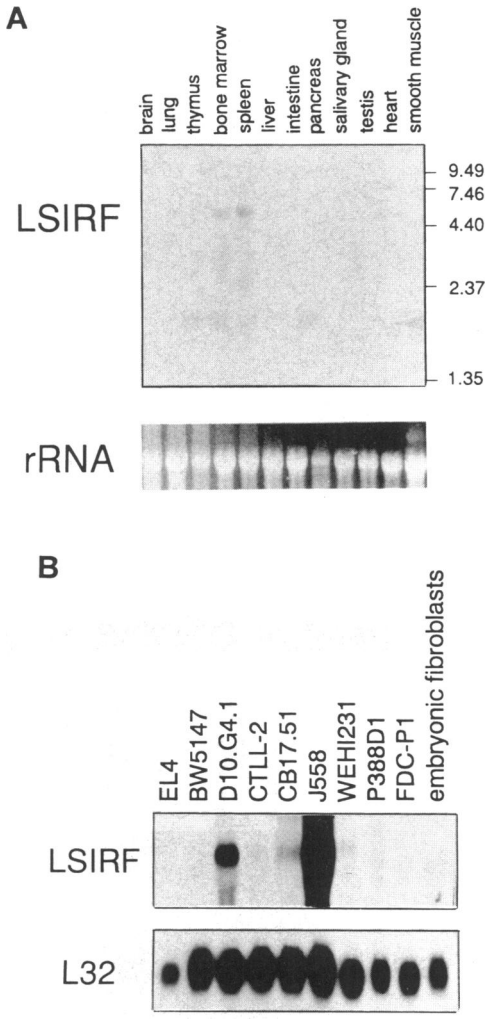
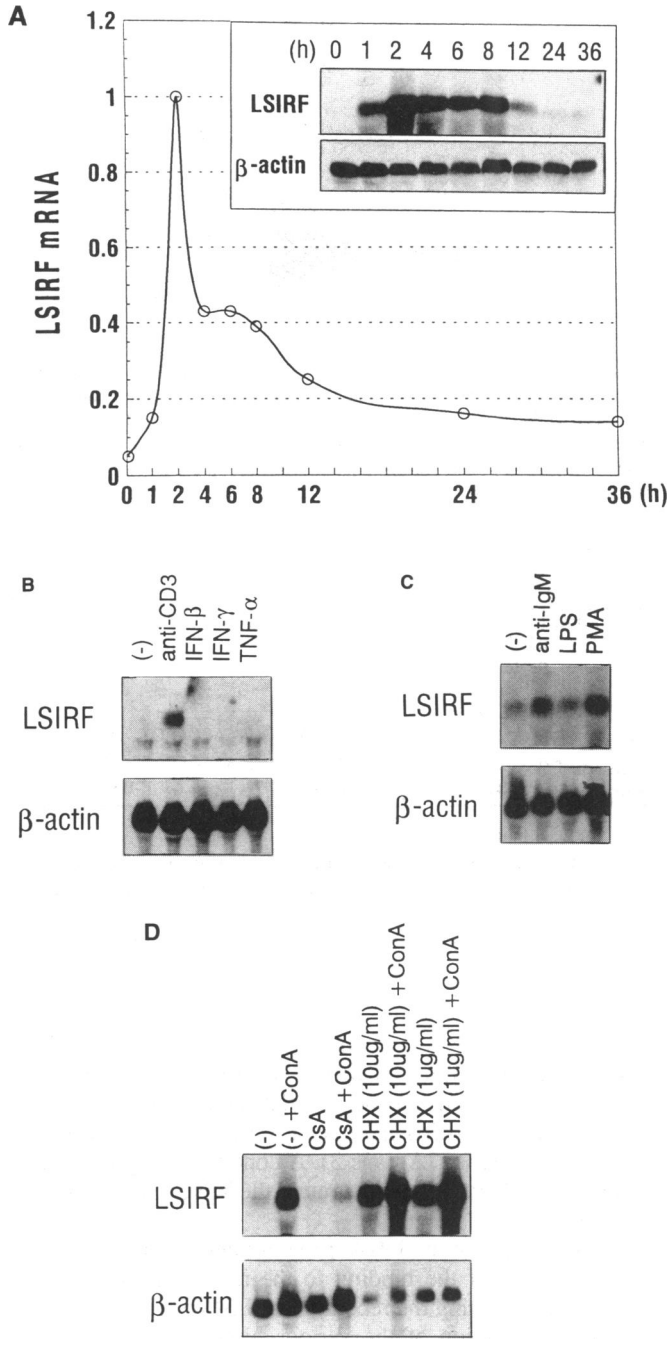


Figure 4. Northern blot analysis of constitutive *LSIRF* expression. (**A**) Multiple tissue blot of *LSIRF* mRNA. Ethidium bromide stained *28S* ribosomal *RNA* is shown to illustrate amount of total RNA. (**B**) *LSIRF* mRNA expression in established cell lines. Hybridization with probe for ribosomal protein *L32* mRNA was performed to control for amount of total RNA.



shown). As mentioned above, *ICSBP* is only expressed in hematopoietic lineages (14). Because *ICSBP* is strongly induced by IFN-β in macrophage cell lines (14), we examined the expression of *LSIRF* by IFNs in the macrophage cell line, P388D1. However, P388D1 did not express *LSIRF* after treatment with either type-I or type-II interferons (data not shown).

DNA binding activity of *LSIRF*

LSIRF protein has a predicted molecular weight of ~51 kDa, which is in good agreement with the estimated size of the product observed by Western blotting of proteins from peripheral T cells stimulated with anti-CD3 antibodies (Fig. 6). The size of the LSIRF protein produced in peripheral T-cells was also similar to the size of the baculovirus-expressed recombinant protein produced in Sf9 cells (Fig. 6).

The DNA binding activity of the recombinant baculovirus-produced LSIRF was determined using an electrophoretic mobility shift assay (Fig. 7A). Because of the redundancy in the DNA

Figure 5. Northern Blot analysis of *LSIRF* induction. To control for total RNA amounts, β-*actin* mRNA is shown below the figures. (**A**) Time course of Con A induction of *LSIRF* mRNAs in splenocytes. Splenocytes were treated with 10 μg/ml of Con A, total RNA was extracted at the times indicated, and 5 μg used for Northern blot analysis (inset). *LSIRF* and β-*actin* mRNA levels were quantitated by densitometry (Image Quant, Molecular Dynamics; Sunnyvale, CA) and relative amounts of RNA normalized by β-*actin* were graphed. (**B**) Induction of *LSIRF* mRNA in lymphocytes. Cells from lymph nodes were treated for 6 h with murine IFN-β (1000 U/ml), IFN-γ (100 U/ml) or TNF (10 ng/ml). For crosslinking experiments, the plate was treated with anti-CD3 antibodies (2 μg/ml) for 3 h before adding lymphocytes. (**C**) Induction of *LSIRF* mRNA in splenocytes. Cells were treated with anti-IgM antibodies (20 μg/ml), LPS (10 μg/ml) or PMA (10 ng/ml) for 6 h. (**D**) The effect of cyclosporin A (CsA) and cycloheximide (CHX) in *LSIRF* mRNA induction. Splenocytes were treated with or without Con A (10 μg/ml) for 6 h. Cyclosporin A (1 mg/ml) was used for 6 h. CHX (1 or 10 μg/ml) was added 30 min before Con A treatment.

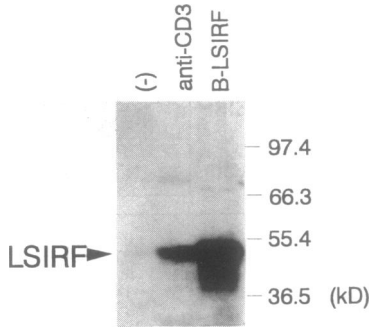


Figure 6. LSIRF cDNA encodes a 51 kDa protein induced by anti-CD3 crosslinking. Peripheral lymphocytes from lymph nodes were left untreated (–) or treated with anti-CD3 antibodies (anti-CD3) for 12 h. Total cell lysates of lymphocytes, or Sf9 cells infected with baculovirus vector carrying LSIRF cDNA (B-LSIRF) were electrophoresed in 8% polyacrylamide–SDS for Western blot analysis.

Table 2. Binding of LSIRF to IFN response motifs

Gene[a]		Sequence		Binding[d]
Mus MHC ISRE wt	-139	TGCAGAAGTGAAACTGAG	-157[b]	+
ISRE m1		----------CTG-		+
ISRE m2		----------CA----		-
ISRE m3		----------GTC-----		-
ISRE m4		GCT----------		+
Mus Igλ B	373	AAAGGAAGTGAAACCAAG	390[c]	+
Mus Igκ E3'	448	TGAGGAACTGAAAACAGA	465[c]	+/-
Hum ISG54 ISRE	-90	GGGAAAGTGAAACTAG	-105[b]	+

[a]Mus, mouse; Hum, human.
[b]Nucleotide positions relative to the transcription start site of the gene.
[c]Nucleotide positions according to numerical system in original references (65,66).
[d]Gel-mobility retardation analysis of binding to LSIRF protein is presented in Figure 7.

recognition among reported IRF family members, LSIRF was also expected to recognize ISRE. It was found that a ^{32}P-labeled DNA probe containing the mouse MHC ISRE sequence (Table 2) was specifically retarded in the gel using an extract from cells that express LSIRF (Fig. 7A, lane 3). A control wild-type baculovirus-infected cell extract did not retard the MHC ISRE probe (Fig. 7A, lane 2).

The specificity of the DNA–protein interaction was analyzed by competition of the binding to the radiolabeled probe with unlabeled DNA using the recombinant LSIRF. Table 2 summarizes the composition of the various binding sites used in the competition experiment. The wild-type MHC ISRE and two mutants of the MHC ISRE, m1 and m4, compete well for binding. In addition, the λB and ISG54 binding sites also compete well for LSIRF binding. On the other hand, the MHC ISRE mutants, m2, m3 and kE3′ do not compete effectively for binding.

The binding activity of native protein to the wild-type MHC ISRE was studied. Nuclear extract of J558 cells showed several distinctive retarded bands that disappeared with the presence of competitor (Fig. 7B, lane 4), indicating that the retarded bands were specific. One of the bands co-migrated with recombinant baculovirus LSIRF (Fig. 7B, lanes 1 and 5) and these two bands were supershifted with anti-LSIRF specific anti-serum (Fig. 7B, lanes 2 and 6), but not by pre-immune serum (Fig. 7B, lanes 3 and 7). This result clearly shows that native LSIRF can bind the wild-type ISRE.

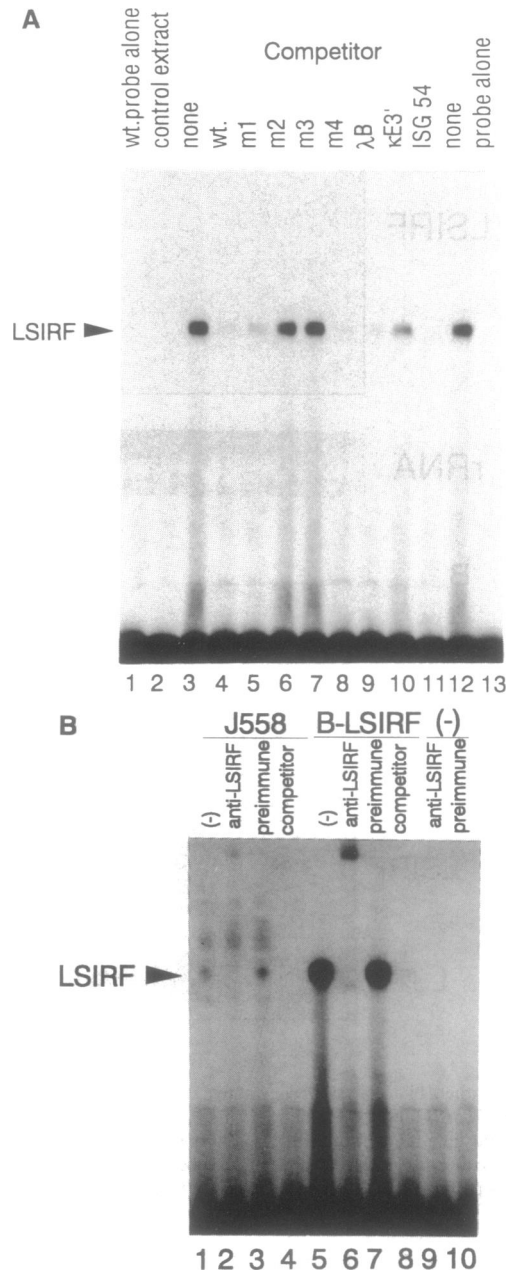


Figure 7. Electrophoretic mobility shift analysis of LSIRF binding of the mouse MHC ISRE. (**A**) Nuclear extracts from control baculovirus-infected cells (lane 2) or from cells infected with baculovirus expressing the LSIRF gene (lanes 3–12) were incubated with a ^{32}P-labeled mouse MHC ISRE probe and the indicated competitor DNA oligos in the binding reaction (also refer to Table 2). Lanes 1 and 13 contain probe alone. (**B**) Nuclear extract from J558 cells (lanes 1–4) or cells infected with baculovirus expressing LSIRF (lanes 5–8) were incubated with ^{32}P-labeled mouse MHC ISRE (lanes 1 and 5), and one of: antibodies against LSIRF (lanes 2 and 6), pre-immune serum (lanes 3 and 7) or 750-fold molar excess competitor of mouse MHC ISRE (lanes 4 and 8). Lanes 9 and 10 contain only the probe, with antibodies against LSIRF or pre-immune serum, respectively.

DISCUSSION

We have cloned, sequenced and expressed a cDNA encoding a novel member of the interferon regulatory factor family, LSIRF. The amino acid sequence of murine LSIRF exhibits significant

similarity to the other IRF family members in the DNA binding domain, including the conservation of the five invariant tryptophan residues. Although all known IRF family members are induced by IFNs, *LSIRF* is not induced by these cytokines, rather strong induction of *LSIRF* was produced by antigen mimetic stimuli. Compared with other family members, LSIRF has unusually restricted tissue distribution, its expression being confined to cells of the lymphoid lineage; in particular pre-B, B, plasmacytoma and peripheral T cells. LSIRF is not found in immature T cells, suggesting a role in T cell effector function rather than T cell development.

For the 5′ region of the LSIRF genome sequence, we have performed a computer search for potential transcription binding sites and found possible *cis*-elements which might regulate the lymphoid specific expression of *LSIRF*. There are motifs for possible lymphoid specific enhancers (four TCF-1/LEF-1 sites) (41,42), B cell immunoglobulin enhancers (IgH core and NFmuE1) (50–52) and T cell specific enhancers (three possible GATA-3 sites and three possible Ets-1 sites) (46,47). There are also possible *cis*-elements which confer inducibility to *LSIRF* expression: three possible PU.1/Spi-1 sites (48,49), three possible NF-IL-6 sites (39), two possible NF-κB sites (44,45), one possible AP-2 site (40) and one possible AP-1 site (43). There are no obvious ISRE (13,54,55), GAS (13,56,57) (IFN-γ activation site), nor pIRE (palindromic IFN response element) (7) within 2000 bp 5′ of the putative transcription start site, supporting our findings that *LSIRF* is not induced by type-I or type-II IFNs. The physiological relevance of these *cis*-elements remains to be determined.

The finding that LSIRF can recognize *in vitro* the ISRE motifs of the MHC class I gene promoter suggests that LSIRF is involved in the regulation of IFN-inducible genes in lymphocytes. Other IRF family members are also expressed in lymphoid cells (2,3,14,20; TK and M. Kitagawa, unpublished results) but they are IFN-inducible. However, in contrast with other IRFs, LSIRF is not induced by IFNs but by antigenic stimuli. Therefore, competition between LSIRF and other IRFs for ISRE may occur when both antigenic and IFN stimuli are present. Following antigen stimulation alone, competition between LSIRF and IRF-1 may occur in T cells, as IRF-1 is also induced in T cells by antigen stimulation. Another model would have LSIRF cooperating with an IFN-induced/activated factor (i.e., other IRF family members, or STAT family factors) to bind the ISRE. In support of this, ICSBP can form a complex with IRF-1 or IRF-2 *in vivo* and *in vitro*, regardless of the presence of the ISRE (58). Also, there is significant C-terminal homology between LSIRF, ICSBP and ISGF3γ in a domain important for the interaction of ISGF3γ with STAT-1 (59), suggesting a possible interaction between LSIRF and a STAT-1. However, from the immunoprecipitation experiment using primary lymphocytes, LSIRF was not associated with STAT-1 (T.M., unpublished results).

In lymphocytes, LSIRF may bind not only the ISRE of IFN-inducible genes, but also the ISRE of genes induced by antigen stimuli and not by IFN. Interestingly, the core motif of MHC class I ISRE (GAAGTGAAAC) is present in the immunoglobulin λ enhancer, λB (60,65). The finding that LSIRF can bind the ISRE motif of λB strongly supports the idea that the target genes of LSIRF are not limited to IFN-inducible genes. It has been shown that PU.1/Spi-1 binds specifically to the Ets motif of the λB domain (61), where the LSIRF binding site is partly overlapped. PU.1/Spi-1 is expressed in B cells and macrophages but not in T cells (49). Therefore, on the λB domain in B cells, there could be interactions between LSIRF and PU.1/Spi-1; LSIRF could be an associated molecule with PU.1/Spi-1 or a competitor of PU.1/Spi-1 for binding to the λB domain. There is a factor proposed to associate with PU.1/Spi-1 for λB binding (61). However, this factor does not seem to be LSIRF, because this factor could not bind the λB domain autonomously (61).

Recently, we reported the creation of mutant mouse strains lacking *IRF-1* or *IRF-2* (20). Type-I IFN production is poorly induced by double-stranded RNA (dsRNA) in *IRF-1*-deficient embryonic fibroblasts; however, induction of type-I IFNs is restored to normal levels by pre-treatment ('priming') with interferon (20). These results suggest the existence of one or more novel IFN-inducible factors ('factor X') that can substitute for IRF-1 function in *IRF-1*-deficient cells; since the IRF-1 binding element (*IRF-E*) in the promoter of type-I IFN genes is essential for induction (62,63), it is likely that factor X is a member of the IRF family or, at least, a factor which binds IRF-E. On this basis, we attempted to clone novel IRF family members. However, LSIRF is unlikely to be 'factor X' because it is not an IFN-inducible factor nor is it expressed in embryonic fibroblasts. Furthermore, LSIRF mRNA is not detected in IRF-1–/– embryonic fibroblasts even after stimulation with IFNs (data not shown), excluding the possibility that LSIRF is aberrantly expressed in IRF-1–/– embryonic fibroblasts, and suggests that further members of the the IRF family may exist.

After submitting this manuscript, we learned that a novel chicken IRF, cIRF-3, was cloned by C. Grant *et al.* (described in this issue). Comparison of predicted amino acid sequence, tissue specificity and inducers of expression suggest that cIRF-3 is not a homologue of LSIRF, but rather they both are entirely new members of the IRF family.

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