Multiple regions within the promoter of the murine *Ifnar-2* gene confer basal and inducible expression

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The (murine) type I interferon (IFN) receptor, *muIfnar-2*, is expressed ubiquitously, and exists as both transmembrane and soluble forms. In the present study we show that the gene encoding *muIfnar-2* spans approx. 33 kb on mouse chromosome 16, and consists of nine exons and eight introns. The three mRNA splice variants resulting in one transmembrane (*muIfnar-2c*) and two soluble (*muIfnar-2a/2a'*) mRNA isoforms are generated by alternative RNA processing of the *muIfnar-2* gene. Treatment of a range of murine cell lines with a combination of type I and II IFN showed that the *muIfnar-2a* and *-2c* mRNA isoforms were up-regulated independently of each other in L929 fibroblasts and hepa-1c1c7 hepatoma cells, but not in M1 myeloid

leukaemia cells. Analysis of the 5' flanking region of *muIfnar-2* using promoter–luciferase reporter constructs defined three regulatory regions: a region proximal to exon 1, conferring high basal expression, a distal region conferring inducible expression, and a negative regulatory region between the two. These data represent the first promoter analysis of a type I IFN receptor and, taken together with our previous data demonstrating high expression levels and dual biological functions for *muIfnar-2a* protein, suggests that the regulation of *muIfnar-2* isoform expression may be an important way of modulating type I IFN responses.

Key words: gene structure, interferon, promoter.

INTRODUCTION

The type I interferons (IFNs) are naturally produced regulators of cell growth and differentiation in the haematopoietic and immune systems, and provide the first defence against viral infections [1]. All type I IFNs, which include at least 17 α subtypes, single β , τ , κ and ω isotypes, and the recently identified cytokine, limitin, have similar biological activities [2–4] and bind to a common cell-surface-receptor complex consisting of two transmembrane proteins, type I IFN receptor (IFNAR)-1 and IFNAR-2 [5]. In addition, IFNs have significant anti-tumour activities and serve specific functions, such as activated T-cell survival factors, activators of natural killer cell cytotoxicity, inhibitors of macrophage and lymphocyte proliferation, and inducers of macrophage differentiation, antibody production and cell-surface-antigen expression [6].

IFNAR-2 is a member of the class II cytokine receptor family [7], which also includes the type I and II IFN receptors IFNAR-1, IFNGR1 and IFNGR2, interleukin (IL)-10R1, IL-10R2, IL-22R and tissue factor (a membrane receptor for the coagulation protease factor VII) [8]. These receptors have a common D200 extracellular domain region of approximately 200 amino acids, which has a conserved tertiary structure, cysteine pairs at both N- and C-termini, and can be distinguished from the class I receptors by the absence of the distinctive Trp-Ser-Xaa-Trp-Ser motif present in that class of receptor [9]. We have previously cloned two murine (*mu*)*Ifnar-2* cDNAs in mice corresponding to transmembrane (*muIfnar-2c*) and soluble (*muIfnar-2a*) isoforms [10]. The predicted polypeptide sequence of *muIfnar-2a* was identical with the extracellular domain of the transmembrane

form, except for 11 additional amino acids at the C-terminus. A minor soluble isoform (*muIfnar-2a'*) of *muIfnar-2* has also been cloned [11], but the biological significance of this transcript is unknown since it was expressed at very low levels relative to the other two transcripts.

High levels of a 45 kDa protein, IFN-binding protein, corresponding to soluble *muIfnar-2* have been detected in normal mouse serum [12]. We also demonstrated, using transfected cDNAs of either the soluble or transmembrane isoforms of *muIfnar-2*, that the soluble *muIfnar-2* protein was the product of a specific mRNA transcript, and not the consequence of proteolytic cleavage of the transmembrane receptor. Our further studies showed that purified recombinant *muIfnar-2a* competitively inhibited IFN activity in reporter assays, and in anti-proliferative and antiviral assays in primary cells. Surprisingly, in primary thymocytes from *Ifnar-2*^{−/−} mice, recombinant *Ifnar-2a* formed a complex with IFN at the cell surface, presumably with *Ifnar-1*, and transmitted a partial anti-proliferative signal. The potential agonistic and antagonistic activities of soluble *muIfnar-2* could thus have a major impact on modulating responses to endogenous and exogenous IFNs. This finding importantly demonstrated a novel aspect of IFN signalling, where the cytoplasmic domain of *muIfnar-2c* was not essential for a biological effect. Both *muIfnar-2* mRNA transcripts were expressed ubiquitously; however, the mRNA transcript for soluble *muIfnar-2a* was more abundant than that encoding transmembrane *Ifnar-2c* in the majority of tissues. The ratio of *muIfnar-2a*:*muIfnar-2c* varied from > 10:1 in the liver and other organs, to < 1:1 in tissues involved in haemopoiesis, e.g. bone marrow macrophages [10,12], suggesting that the two

Abbreviations used: EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAS, γ -activation sequence; IFN, interferon; IL, interleukin; ISGF3, IFN-stimulated gene factor 3; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; (*mu*)/(*hu*)IFNAR, (murine/human) type I IFN receptor; poly(A)⁺, polyadenylated; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

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receptor isoforms were regulated independently in a tissue-specific manner, which is likely to have important consequences on the biological actions of IFNs.

Despite the importance of IFNAR isoforms, little information exists to date on the modulation of IFN receptor expression, and no IFNAR promoter has been identified in any species. The structure and chromosomal localization of the *mulfnar-2* gene have not yet been described, and relatively little is known about how the specific mRNA isoforms are generated. Information on the molecular mechanism of expression and regulation of *mulfnar-2* could provide insights on the function of each isoform *in vivo*, and how each contributes to type I IFN-mediated activity in different cell types.

As a major step towards understanding *mulfnar-2* transcriptional regulation, we report here the structure and complete nucleotide sequence of the *mulfnar-2* gene, including 8.5 kb of 5' flanking sequence and the characterization of the molecular basis of the alternatively spliced forms of *mulfnar-2*. We also demonstrate independent regulation of the *mulfnar-2* mRNA isoforms in different cell types in response to treatment with type I and II IFN, and identify regions in the promoter of the *mulfnar-2* gene that control basal and inducible mRNA expression.

EXPERIMENTAL

Isolation and subcloning of the *mulfnar-2* gene

A murine 129SvJ genomic library in λ Fix II (Stratagene, La Jolla, CA, U.S.A.) was screened with a 303 bp *mulfnar-2* cDNA probe spanning nt positions 507–809 of *mulfnar-2c* cDNA. To obtain further λ genomic clones spanning the extreme 5' end of the *mulfnar-2* gene, the same library was subsequently probed with a 588 bp *NotI*–*HindIII* fragment corresponding to intron 1 of *mulfnar-2* obtained from a bacteriophage clone isolated following the initial library screening. All probes were labelled with [32 P]dCTP using a random primer labelling kit (Roche, Castle Hill, Australia) to specific radioactivities of $> 6.3 \times 10^8$ c.p.m./ μ g. Bacteriophage plaques were transferred to Hybond-N membranes (Amersham, Little Chalfont, Bucks., U.K.) in duplicate, and hybridized under standard conditions. Filters were then washed under high-stringency conditions [$0.5 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS at 65 °C. Positive plaques were identified, and after three further rounds of purification phage DNA was prepared using standard procedures. Phage DNA was digested with restriction enzymes and mapped by Southern blot analysis using specific oligonucleotide probes end-labelled with [32 P]ATP (Amersham) and T4 polynucleotide kinase (Promega). Specific restriction fragments were then subcloned into pBluescript KS (+/–).

Nucleotide sequence analysis of the *mulfnar-2* gene

Sequencing of subcloned restriction fragments of genomic clones was performed on plasmid templates using ABI PRISM BigDye Terminator (v3) chemistry and analysed on an ABI 377 DNA sequencer (PE Applied Biosystems, Scoresby, Australia). Exons and intron–exon boundaries were sequenced on both DNA strands; introns were sequenced on one strand.

5'- and 3'-SMARTTM rapid amplification of cDNA ends (RACE)

5'- and 3'-SMARTTM RACE-ready cDNA libraries were generated according to the manufacturer's recommendations (Clontech) using 1 μ g of polyadenylated [poly(A)⁺] mRNA from normal mouse thymus, liver or placenta. 5'- and 3'-RACE

Table 1 Oligonucleotides used for 5'- and 3'-SMARTTM RACE PCR

Name	Sequence (5' → 3')	Used for
AR2-1	5'-GCGGAAGGGTCAGAGGTAAGGCATGCTG-3'	3' RACE
AR2-2	5'-GTGAGGAGGGGGAAGCCGAGGAATA-3'	3' RACE
AR2-3	5'-GTCTCGTGAATCCCCCTCTGGGATG-3'	5' RACE
AR2-4	5'-AAGCTGAGGAGACCGACGGCAGAGA-3'	5' RACE
AR2-5	5'-CCGGAGGCCAAGGGTCCCA-3'	5' RACE
AR2-6	5'-AGCCAGGCGAAAGGGACGGC-3'	5' RACE
AR2-7	5'-TCTCTAGGCTCGCAGACACCACAAGACA-3'	5' RACE
TE-1	5'-CCACAGCCCCCTTCAATGCCCTGTT-3'	3' RACE
TE-2	5'-GGCAGTCTTTCCACCGACAGCACCA-3'	5' RACE
TE-3	5'-AAGGCTTCACTGCTTTCCA-3'	5' RACE
TE-4	5'-CCCAGCAGGCTCTGCTTCTAAAGGCTTC-3'	3' RACE
TE-5	5'-TGTCAGGCACTCAGAATCAAACGGATGGA-3'	3' RACE
TE-6	5'-GATTCCCCACAGGGTCACTGGGTCTCC-3'	5' RACE
TE-7	5'-AGGTACATACGCGTGCAAGTTTGGTGGACA-3'	3' RACE
TE-8	5'-TCAGAACTCTGCTTCAGCCCTGCTGT-3'	5' RACE

reactions were performed according to the manufacturer's recommendations (Clontech) using 100 units of Advantage 2 polymerase, 0.2 pmol of universal primer mix and 0.2 pmol of gene-specific primer (see Table 1) in a final volume of 50 μ l. The PCR reactions were amplified under the following conditions: 5 cycles of 94 °C (5 s) and 72 °C (3 min); 5 cycles of 94 °C (5 s), 70 °C (10 s) and 72 °C (3 min); and 20 cycles of 94 °C (5 s), 68 °C (10 s) and 72 °C (3 min). To obtain specific RACE products, primary RACE PCR products were diluted 1:50 with 10 mM Tris/EDTA buffer, and amplified further with Advantage 2 polymerase using 0.2 pmol of 'nested' gene-specific primer (see Table 1) and 0.2 pmol of 'nested' universal primer (Clontech) under the following conditions: 94 °C (5 s), 68 °C (10 s) and 72 °C (3 min) for 20 cycles. PCR products were analysed by agarose gel electrophoresis, transferred to GeneScreenPlus nylon membranes, and their specificity was determined by hybridization with an internal oligonucleotide. Positive RACE PCR products were gel-purified, cloned into pGEM-T (Promega) and sequenced.

Statistical and sequence analysis

Differences between means were calculated using Student's *t* test analysis. Transcription-factor-binding site searches were performed using Signalscan (<http://bimas.dcrn.nih.gov/molbio/index.html>) [13], Search Launcher (<http://searchlauncher.bcm.tmc.edu>) [14], TFSEARCH (<http://molsun1.cbrc.aist.go.jp/research/db/TFSEACRH.html>) [15], MatInspector Release Professional (<http://genomatix.gsf.de/matfam>) [16], MacVector version 7.1 (Oxford Molecular Ltd.), and Omiga version 1.1 (1996–98) (Oxford Molecular Ltd.). Global alignment of the human and mouse genomic sequence of conserved synteny was performed with the program AVID (N. Bray, A. Fabrikant, J. Lord, J. Schwartz, I. Dubchak and L. Pachter, unpublished work) using a window size of 100 bp and a conservation level of 70% and the results were viewed with the program VISTA [17,18] (<http://www-gsd.lbl.gov/vista/>). A higher-resolution comparison of the conserved non-coding sequence upstream of exon of the *mulfnar-2* gene was performed using ClustalW (MacVector version 7.1; Oxford Molecular Ltd.). Global alignments were also carried out with the program Dialign2 [19] (<http://genomatix.gsf.de/dialign2>) with the threshold for diagonals (T) set to 0. *Homo sapiens* chromosome 21 sequence (GenBank[®] accession number NT011512) and the chicken class II cytokine receptor genomic sequence (GenBank[®] accession

Figure 1 The organization of the murine *lfnar-2* gene

(A) Schematic representation of the *mulnar-2* gene. Black boxes and a horizontal line represent exons (1–9) and introns (1–8) respectively. The translation initiation codon (ATG) is indicated. Above are the *mulnar-2c* protein domains encoded by their respective exons. Below are the names and corresponding locations of the overlapping genomic clones (horizontal bars), and a restriction map of the *mulnar-2* locus (B, *Bam*HI; E, *Eco*RI; H, *Hind*III). Grey and hatched boxes represent expressed sequence tags EST1 and EST2 respectively, lying in and around the *mulnar-2* genomic locus. (B) Intron–exon boundaries of the murine *lfnar-2* gene.

number AF082667) were obtained from <http://www.ncbi.nlm.nih.gov>. Identification of transcribed nucleotide sequences and repeat sequences in the genomic sequence was performed using the NIX application (<http://menu.hgmp.mrc.ac.uk/menu-ubin/Nix/Nix.pl>).

Cell culture and IFN treatments

Murine L929 cells were grown in Dulbecco's modified Eagle's medium. Hepa-1c1c7 cells (A.T.C.C.) were cultured in α -minimum essential medium without nucleosides, and M1 murine myeloid leukaemia cells were cultured in RPMI medium. All media was supplemented with 10% (v/v) fetal calf serum and 1% penicillin/streptomycin (Life Technologies, Gaithersburg, MD, U.S.A.). Recombinant murine IFN α 4 produced in NS1 cells was a gift from D. Gewert (Wellcome Laboratories,

Beckenham, Kent, U.K.; present address, BioLauncher Ltd., Cambridge, U.K.). Recombinant murine IFN α 1 was produced in *Pichia pastoris* (S. Trajanovska, C. M. Owczarek and P. J. Hertzog, unpublished work). Recombinant IFN γ was obtained from PeproTech Inc. (Rockhill, NJ, U.S.A.). Other cytokines were obtained from Immunex Corp. Cells were grown to 70–80% confluence, then treated with or without a combination of 1000 units/ml murine IFN α 4 and murine IFN γ for 3, 9 and 24 h. Cells from each treatment group were then harvested by trypsin treatment, washed with PBS and snap-frozen in liquid N₂.

Isolation and Northern blot analysis of poly(A)⁺ mRNA

Poly(A)⁺ mRNA from L929 cells, M1 cells and Hepa-1c1c7 cells was prepared essentially as described previously [20]. Northern blots of poly(A)⁺ mRNA were prepared and hybridized as

described previously with a range of ^{32}P -labelled probes, including *mulfnar-2* cDNA, a 537 bp genomic DNA fragment corresponding to *mulfnar-2* intron 1 (accession no. AF367979; nt 18027–18563); 585 bp or 506 bp cDNA fragments encoding the extracellular and cytoplasmic domains respectively of *mulfnar-1* (a gift from Dr G. Uzé, at the Institut de Génétique Moléculaire, CNRS, Montpellier, France), and then washed with $0.2 \times \text{SSC}/0.1\%$ (w/v) SDS at 65°C . Blots were finally probed with a ^{32}P -labelled 1.2 kb *PstI* fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to normalize the different samples for RNA loading. Filters were exposed to a Phosphor screen, and quantitative analysis was performed using a Fuji BAS 1000 PhosphorImage analyser and MacBAS v2.5 software (Fuji, Melbourne, Australia).

Generation of luciferase reporter constructs and stable transfections

In order to analyse the 5' flanking region of the *mulfnar-2* gene for promoters, the following restriction fragments from λ clone 2A-1 (see Figure 1A) were subcloned into a pGL3-Basic vector (Promega) that also contained the neomycin-resistance gene from pMCIneo inserted into the *SalI* restriction site for stable cell line generation: 11 kb *HindIII*-cleaved fragment (nt 539–11638; pGL3Neo-H), 3.65 kb *SacI*-cleaved fragment (nt 6370–10027; pGL3Neo-S), 3.1 kb *SmaI/HindIII*-cleaved fragment (nt 8508–11638; pGL3Neo-SH) and 1.7 kb *BglII*-cleaved fragment (nt 7881–9580; pGL3Neo-B) (see accession no. AF367979 for numbering). Before transfection, all constructs were linearized with *KpnI*. L929 cells were seeded at approx. $(1-3) \times 10^5$ cells/well in a six-well plate in complete growth media, and cultured at 37°C (in the presence of 5% CO_2) until 50–80% confluent. To 100 μl of serum-free media was added 2 μg of linearized DNA, and in another 100 μl of media was added 25 μl of LIPOFECTAMINETM reagent (Invitrogen). The two solutions were mixed gently, and incubated at room temperature for 45 min. The mixture was then added to the cells pre-rinsed with serum-free media, and the volume was made to 2 ml with media. After 18 h incubation, as described above, the media was replaced with complete media and the cells incubated for a further 48 h. At this time, the cells were split 1:10 and 1:20 into 20 cm^2 dishes, G418 (or Geneticin; 600 $\mu\text{g}/\text{ml}$; Invitrogen) was added and the L929 cells were selected for 2 weeks until single colonies appeared. To confirm the presence of each transfected construct in the clones, PCR was performed using *Taq* polymerase (Promega), and specific primers on cells were lysed at 55°C for 1 h, and then at 95°C for 10 min, in a mixture of 5 μl of $10 \times \text{Taq}$ polymerase/1 μl of 4.5% (v/v) Nonidet P40/1 μl of 4.5% (v/v) Tween 20 (ICN) and 0.5 μl of 10 mg/ml Proteinase K (Roche Diagnostics).

Luciferase reporter assays

Aliquots of stably transfected cells (1×10^5) were added to each well of a 24-well plate (Falcon, Lincoln Park, NJ, U.S.A.). Following 21 h incubation at 37°C (5% CO_2) in RPMI or Dulbecco's modified Eagle's medium, the media were removed and to each well was added 1000 units/ml $\text{muIFN}\alpha 4$ and 1000 units/ml $\text{muIFN}\gamma$. For basal promoter analysis, the plates were left untreated for 30 h at 37°C . The plates were incubated at 37°C (5% CO_2) for a further 9 h, and then gently washed with PBS. The adherent cells were then lysed for 15 min with 300 $\mu\text{l}/\text{well}$ of $5 \times$ reporter lysis buffer (Promega) and 20 μl of lysate was analysed on a Lumat LB9501 Luminometer (Berthold, Pforzheim, Germany) for luciferase activity using a luciferase assay kit according to the manufacturer's recommendations (Promega).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye assay for quantification of viable cell numbers

The assessment of viable cell number was performed by an adaptation of the method described by Loveland et al. [21]. To each well of a 24-well plate of adherent cells/media was added 50 μl of 2 mg/ml MTT, and the cells were incubated at 37°C for 4 h. The media/MTT was then removed, and to the cells was added 150 $\mu\text{l}/\text{well}$ DMSO. The absorbance of each well was then read within 30 min at 595 nm using a Bio-Rad 3550 Microplate reader (Bio-Rad).

RESULTS

Structure of the *mulfnar-2* gene

In order to determine the molecular origin of the different mRNA splice variants of *mulfnar-2*, and to study the promoter of the *mulfnar-2* gene, we analysed a total of 41 306 bp of mouse genomic DNA (submitted to GenBank[®] under accession nos. AF367979 and AF440786) covering all the information contained in the published *Ifnar-2* cDNAs [10] (GenBank[®] accession nos. AF013486, AF013274, Y09865, Y09813 and NM010509). The *mulfnar-2* gene contains 9 exons and 8 introns shown in Figures 1(A) and 1(B). The 5'-flanking sequence of the *mulfnar-2* gene lacked a recognizable TATA or CAAT box, making it similar to the 5'-flanking regions of other *IFNAR* genes, e.g. human (*hu*)*IFNAR-2* [22] and *huIFNAR-1* [23]. Exon 1 is approx. 75% GC-rich, and contains most of the 5'-untranslated region (UTR). Exon 2 contains the initiating codon (ATG) and encodes the rest of the 5' UTR and part of the signal peptide, the remainder of which is encoded by exon 3. The ligand-binding SD100A and SD100B domains of the extracellular domain of *mulfnar-2* are encoded by exons 4 and 5, and exons 6 and 7 respectively. Exons 8 and 9 encode the transmembrane and cytoplasmic domains of *mulfnar-2c* respectively. In addition, exon 9 encodes the nucleotide sequence of *mulfnar-2a'* produced by alternative splicing (see below), and contains the common 3' UTRs of both *mulfnar-2a'* and *mulfnar-2c*. Exon 7' is a 3' continuation of exon 7, and encodes the sequence specific to the *mulfnar-2a* mRNA isoform, as well as its own 3' UTR. The sizes of the introns range from 0.9 kb (intron 3) to 10.8 kb (intron 1).

The *mulfnar-2* genomic sequence was analysed for the presence of repetitive elements, such as B1 and B2 repeats, since these have been implicated in modulating gene expression [24–26]. As summarized in Table 2, the most common elements were short interspersed repetitive elements ('SINEs'), long interspersed repetitive elements ('LINEs') and long terminal repeats ('LTRs'), with frequencies of 9.3%, 4.47% and 7.64% respectively. The G + C content of the *mulfnar-2* genomic sequence was also determined (45.65%), making it similar to the current reported average content for mouse genomic DNA (45.6%) [27].

The intron–exon boundaries of the *mulfnar-2* gene (Figure 1B) conform to the 5'-donor and 3'-acceptor consensus rule GT-AG [28,29]. The three possible codon disruption phases are present in the *mulfnar-2* gene splice junctions (Figure 1B). Thus introns 6 and 8 disrupt exons between amino acids (phase 0), introns 1, 2, 3, 5 and 7 interrupt a codon between the first and second nucleotide (phase 1) and intron 4 disrupts a codon between the second and third nucleotide (phase 2). The '1-2-1-0-1' intron phase pattern between exons 3 and 7, which code for the fibronectin III domains of the extracellular domain of *mulfnar-2*, is conserved in all cytokine receptor family members with these domains [30].

Some differences were found between the sequences of the coding exons in the *mulfnar-2* gene and the published cDNA

Table 2 Distribution of repetitive elements within the *mulfnar-2* Locus

Abbreviations: SINE, short interspersed repetitive element; LINE, long interspersed repetitive element; LTR, long terminal repeat.

Repeat	No. of elements	Percentage (%) of total sequence
SINE	26	9.30
B1	13	3.58
B2-B4	12	5.60
ID	0	0.00
MIR	1	0.12
LINE	5	4.47
LINE1	5	4.47
LINE2	0	0.00
L3/CR1	0	0.00
LTR elements	8	7.64
MaLR	5	3.28
ERVL	0	0.00
ERV-class I	1	0.61
ERV-class II	1	2.39
DNA elements	5	1.78
MER1	2	0.63
MER2	3	1.15
Total interspersed repeats	44	23.19

sequences. The following differences were observed in the *Ifnar-2* genomic sequence compared with the *mulfnar-2c* cDNA sequence [11]: an A → C transversion at nt 698 resulting in a change of lysine → glutamate (extracellular domain, SD100B). A C → T transition at nt 1046 results in a proline → serine transition (exon 8, cytoplasmic domain), but this is not in any Box 1/Box 2 motifs or other known functional domains of the cytoplasmic domain [31]. Two other nucleotide differences (A → G, nt 1195; and A → T, nt 1351) resulted in no amino acid substitutions, and there was a deletion at nt 2156 in the 3'-UTR of *mulfnar-2c* [11]. Six nucleotide differences (A → G, nt 1659; C → A, nt 2308; C → G, nt 2362; C → A, nt 2415; T → G, nt 2435; A → G, nt 2465), two nucleotide insertions (G at nt 1921 and C at nt 2428) and four nucleotide deletions (C at nt 1964, C at nt 2011, G at nt 2369 and A at nt 2444) were found in the 3'-UTR of the *mulfnar-2c* cDNA sequence [10] compared with the genomic sequence. These sequence differences probably represent either polymorphisms or strain differences. In addition, four to eight nucleotides at the 5'-end of the cDNA sequences were not

present in the corresponding genomic sequence, and are probably artefacts generated during the construction of the cDNA libraries.

Chromosomal localization of the *mulfnar-2* gene

Since the 3'-end of the human *IFNAR-2* gene lies less than 0.5 kb from the 5'-end of the human *IL-10R2* gene on human chromosome 21q22.1 [32], we sought to determine whether the same arrangement existed for mouse *Il-10r2* gene, previously localized to *MMU* (mouse) chromosome 16 [33], and the *mulfnar-2* gene. PCR amplification of murine genomic DNA using primers corresponding to exon 9 of *mulfnar-2* and exon 1 of *mul-10r2* generated a 2 kb fragment that contained exon 9 of *mulfnar-2*, exon 1 of *mul-10r2* plus intervening sequence (GenBank® accession no. AF440786). The *mulfnar-2* gene is therefore localized on *MMU* chromosome 16 at a distance of 61.0 cM and the 3'-end is located approx. 722 bp from the 5'-end of the *mul-10r2* gene. In addition, the synteny of genes for the cytokine receptor cluster (*IFNAR-2*–*IL-10R2*–*IFNAR-1*–*IFNGR2*) is conserved among human, mouse and chicken species (Figure 2).

Generation of *mulfnar-2* mRNA isoforms by alternative splicing of the *mulfnar-2* gene

Comparison of the *mulfnar-2* genomic sequence with the cDNA sequences of the *mulfnar-2* isoforms indicated that the variant mRNA isoforms of *mulfnar-2* arose by alternative splicing of the *mulfnar-2* gene. Figure 3(A) shows the pattern of alternative splicing that generates the three *mulfnar-2* mRNA isoforms, *Ifnar-2a*, *-2a'* and *-2c*. All three isoforms share exons 1–7. However, *mulfnar-2a* ignores a splice donor site situated at the exon 7/7' junction, and reads through into exon 7', which is effectively an extension of exon 7 (Figure 3A). Exon 7' also contains a stop codon and polyadenylation recognition sequence specific for the *mulfnar-2a* transcript. *Mulfnar-2a'* uses the splice site at the exon 7/7' boundary, but skips exon 8 and splices into exon 9, resulting in a frame shift and a premature stop codon (Figure 3A). *Mulfnar-2c*, which encodes a transmembrane protein, also recognizes the exon 7/7' splice donor site, and splices into exon 8, then exon 9 (Figure 3A). This transcript uses a different stop codon to *mulfnar-2a'*, although both transcripts use the same polyadenylation sequence at the 3' end of exon 9. The pattern of alternative splicing used to generate the mRNA isoforms of *mulfnar-2* is different from that used to generate the

Figure 2 Comparison of the class II cytokine receptor locus among humans, mice and chickens

Comparison of the human, mouse and chicken class II cytokine receptor loci showing the conserved synteny of the *IFNAR-2*, *IL-10R2*, *IFNAR-1* and *IFNGR-2* genes (shown in grey-shaded boxes). Arrows indicate the direction of transcription and numbers indicate the distances between adjacent genes (cen, centromere; tel, telomere). Human gene sequences were deduced from the *H. sapiens* chromosome 21 working draft sequence (GenBank® accession number NT011512) obtained from <http://www.ncbi.nlm.nih.gov/>. Murine gene sequences were obtained from GenBank® accession numbers AF367979 and AF440786 and from the Celera database (www.celera.com), and chicken sequences were from GenBank® accession number AF082667.

Figure 3 Patterns of alternative splicing of the *mulfnar-2* and *HuIFNAR-2* mRNA isoforms

(A) Alternative mRNA splicing patterns of the *mulfnar-2* isoforms. Soluble *mulfnar-2a* is produced by splice-site suppression at the exon 7/7' boundary, which causes translation of 12 unique amino acids at the C-terminus (shown by the arrow). Soluble *mulfnar-2a'* is generated by exon skipping from exon 7 to 9, which causes a frame-shift and a premature stop codon 11 amino acids later (shown by dashed-dotted lines). Splicing from exon 7 to 8, and then to 9, generates the transmembrane *mulfnar-2c* isoform (indicated by the broken lines). poly(A)⁺ signals are also indicated, with asterisks. (B) Alternative mRNA splicing patterns of the *HuIFNAR-2* isoforms. Soluble *HuIFNAR-2a* is generated by splicing from exon 7 into a splice acceptor site (sa1) within exon 9 (continuous line) and a poly(A)⁺ site 1 (*1). Transmembrane *HuIFNAR-2b* (dashed-dotted lines) uses exons 7, 8 and a different splice acceptor site (sa2) in exon 9, and either poly(A)⁺ sites 1 or 2 (*1, *2). Transmembrane *HuIFNAR-2c* (dashed lines) also uses exons 7 and 8, but uses sa2 and poly(A)⁺ site 2.

single soluble and two transmembrane mRNA isoforms of *huIFNAR-2* (*huIFNAR-2a*, *-2b* and *-2c* respectively; Figure 3B).

5'- and 3'-RACE was performed on expressed sequence tag 1 (EST1; GenBank® accession no. AI451113), located in intron 7 of the *Ifnar-2* gene from position 32846 to 33123, using a thymus RACE library and the primers TE-5 to TE-8 (Table 1) to see whether it constituted part of another *mulfnar-2* alternative mRNA splice form. The 278 bp EST1 did not include any *mulfnar-2* coding sequence, had no significant open reading frames, and is of unknown function.

Regulation of *mulfnar-2* expression

To determine whether the mRNA expression levels of the *mulfnar-2* mRNA isoforms could be regulated by stimuli such as pathogenic organisms or cytokines, murine L929 cells were treated separately with a variety of agents, including muIL-4, muIL-6, muGM-CSF (where 'GM-CSF' represents granulocyte/macrophage colony-stimulating factor), muIFN α 1, muIFN α 4, muIFN γ , lipopolysaccharide ('LPS'), Semliki Forest virus, encephalomyocarditis virus and Sendai virus. Northern blot analysis was then performed on poly(A)⁺ mRNA isolated from these cells using an *Ifnar-2* cDNA probe (results not shown). The mRNA transcripts encoding *mulfnar-2a* and *mulfnar-2c* were then quantified relative to GAPDH. In all cases, *mulfnar-2* expression levels were not significantly affected by these factors in this cell type. Since biological fluids contain mixtures of cytokines, and since IFN α and IFN γ have been reported to act synergistically [34], we treated the cells with a combination of IFN α and IFN γ . Northern blot analysis of murine L929 cells treated with a combination of 1000 units/ml muIFN α 4 and 1000 units/ml muIFN γ for up to 24 h (Figure 4A) showed that the levels of *mulfnar-2a* mRNA increased 2-fold after 9 h of treatment ($P < 0.01$), and 3-fold after 24 h ($P < 0.01$) (Figure 4B). The levels of *mulfnar-2c* mRNA achieved a maximal increase of 4.5-fold after 9 h of treatment ($P < 0.0001$), after which they

decreased. In contrast, the mRNA levels of the major 4.2 kb *mulfnar-1* transcript remained unchanged (Figures 4A and 4B). The difference between *mulfnar-2a:Gapdh* and *mulfnar-2c:Gapdh* was significant at time points of 3 h ($P < 0.005$) and 9 h ($P < 0.001$).

To investigate whether the changes in *mulfnar-2* mRNA expression levels in response to a combination of type I and type II IFN occurred in other murine cell types, the experiment was repeated in murine myeloid leukaemia M1 cells and murine hepatoma 1c1c7 cells. No significant changes in *mulfnar-2* isoform mRNA expression levels occurred after treatment of M1 cells with a combination of 1000 units/ml muIFN α 4 and 1000 units/mL muIFN γ for 24 h (Figure 4C). However, the treatment of hepa-1c1c7 cells with the same factors resulted in a 3-fold increase in *mulfnar-2a* mRNA expression after 9 h treatment, which then returned to normal after 24 h (Figure 4D). The *mulfnar-2c* mRNA transcript increased 4-fold after 3 h treatment, then returned to normal after 24 h. Levels of *mulfnar-1* mRNA expression did not change in either M1 or hepa-1c1c7 cells over time.

Determination of the full-length cDNA sequence of *mulfnar-2*

In order to examine the promoter of the *mulfnar-2* gene, it was first necessary to determine the transcriptional start site. 5' RACE analysis [35,36] was performed using mouse liver, placenta and thymus SMART™ RACE libraries and the AR2-3, AR2-4, AR2-5 and AR2-6 primers (Table 1). This generated an additional 116 bp of 5' sequence that was identical and co-linear with the genomic sequence. Sequence analysis of the cloned RACE products identified a major transcriptional start site at nt position 8509 (now defined as +1 when referring to promoter-reporter constructs) and multiple minor sites at nt positions 8520, 8528, 8530 and 8532. Extensive further 5'-RACE experiments, including some in the presence of high-temperature reverse transcriptase (results not shown), failed to yield additional sequence. BLAST analysis was used to identify an EST from GenBank® in the 5'-flanking region of the *mulfnar-2* gene (EST2; nt 5366–6098, GenBank® accession nos. BE691888 and BE623785). 5'- and 3'-RACE was therefore performed out using a mouse-thymus SMART™ RACE library and the TE-1, TE-2, TE-3 and TE-4 primers (Table 1) to determine whether EST2 was part of the *mulfnar-2* gene. A total of 1976 bp of contiguous sequence was isolated, corresponding to nt 5797–7772 of the *mulfnar-2* genomic sequence. No *mulfnar-2* cDNA sequences were present in this sequence, although four repetitive elements were present within the extended EST2 sequence. Analysis of the potential open reading frames encoded by this sequence revealed a putative protein of only 64 amino acids with no homology with any known protein.

5'-RACE analysis from liver and thymus libraries using the AR2-4 and AR2-7 primers (Table 1) also produced three PCR products of 1.5, 1.6 and 1.75 kb that corresponded to *mulfnar-2* transcripts that read directly into intron 1 from the 5' end of exon 2. These PCR products could represent unspliced transcripts or genomic contamination of the RACE libraries. However, Northern blot analysis of murine spleen poly(A)⁺ mRNA using a 537 bp fragment within this sequence resulted in a weakly hybridizing transcript that was of similar size to the minor 9 kb *mulfnar-2* transcript previously reported [10] (results not shown). The significance of this is unknown, but it may represent a minor *mulfnar-2* mRNA isoform.

3'-RACE was also performed using a mouse thymus SMART™ RACE library and the primers AR2-1 and AR2-2 (Table 1). PCR products of 850, 780 and 650 bp were obtained.

Figure 4 Regulation of *mulfnar-2* and *mulfnar-1* mRNA transcripts by types I and II IFN

(A) Representative Northern blot analysis of poly(A)⁺ mRNA from L929, M1 and Hepa-1c1c7 cells treated with 1000 units/ml murine IFN α 4 and 1000 units/ml murine IFN γ for 0, 3, 9 and 24 h, showing the distribution of *mulfnar-2* and *mulfnar-1* mRNA isoforms. The blots were sequentially hybridized with a full-length *mulfnar-2c* cDNA probe, a probe encoding the extracellular domain of *mulfnar-1* and a GAPDH probe. Quantitative analysis of Northern blot expression patterns from murine IFN α 4-treated L929 cells (B), M1 cells (C) or Hepa-1c1c7 cells (D) of *mulfnar-2a* mRNA (black bars), *mulfnar-2c* mRNA (grey bars) and the 4.2-kb *mulfnar-1* mRNA (bars shaded with diagonal lines) transcript relative to GAPDH. Northern blots were sequentially hybridized with *mulfnar-2c*, *mulfnar-1* and GAPDH cDNA probes, as described above, and exposed to a Phosphor screen. The radioactivity in each hybridizing band was quantified using a PhosphorImager analyser with MacBAS v2.5 software. Data are expressed as the means $\pm$ S.E.M for triplicate experiments, or as the means of duplicate experiments. *P* values were calculated using Student's *t* test analysis. (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.0001).

Sequence analysis of the 850 bp product revealed an extra 215 bp of *Ifnar-2a'/-2c* cDNA sequence in exon 9 [11] that terminated 117 bp 3' of the genomic sequence. The 780 bp and 650 bp products corresponded to previously identified *mulfnar-2* cDNA 3' ends. PCR analysis of genomic DNA indicated that this sequence was co-linear with the genomic sequence (refer to GenBank[®] accession number AF440786). The published length of the *Ifnar-2a* cDNA is 1044 bp, and *Ifnar-2a'/-2c* cDNA is 2708 bp. The additional cDNA sequence generated by 5'- and 3'-RACE resulted in a composite *mulfnar-2a* cDNA of 1161 bp and a *mulfnar-2c* cDNA of 3041 bp. Re-examination of the mRNA mobilities of *mulfnar-2* transcripts on Northern blots using RNA molecular-size standards indicated that the

soluble *mulfnar-2a* transcript migrated at approx. 1.2 kb, and the *mulfnar-2a'* and *mulfnar-2c* transcripts migrated at approximately 3.2 kb, rather than the 1.5 kb and 4.0–4.5 kb reported previously [10,11]. The lengths of the *mulfnar-2* cDNAs are now consistent with their respective mRNA transcript sizes, given the level of resolution on a Northern blot of poly(A)⁺ RNA.

Analysis of the *mulfnar-2* regulatory regions

To identify regions of DNA that control basal and inducible *mulfnar-2* expression, specific restriction fragments from the 5'-flanking region of the *mulfnar-2* gene, exon 1 and part of intron 1 were inserted into a vector containing a luciferase reporter gene

Figure 5 Analysis of the *mulfnar-2* 5' flanking region

(A) Schematic representation of the *mulfnar-2* promoter reporter constructs used for the analysis of the *mulfnar-2* 5'-flanking region. Exon 1 (E1) is denoted by a black box, and numbers are relative to the major transcriptional start site. (B) Basal promoter activity of the *mulfnar-2* 5'-flanking region. The five promoter-reporter constructs (above) were stably transfected into murine L929 cells, and clones from each were cultured for 30 h. Cells were lysed, and the luciferase activity was measured. Data are expressed as the means $\pm$ S.E.M relative light units of luciferase activity from replicate experiments ($n = 5$). (C) Inducibility of luciferase expression by *mulfnar-2* promoter-reporter constructs following treatment of stably transfected L929 cells with 1000 units/ml $\text{muIFN}\alpha 4$ and 1000 units/ml $\text{muIFN}\gamma$ for 9 h. Cells were then lysed and analysed for luciferase activity. Data are expressed as the means $\pm$ S.E.M fold induction of luciferase activity from replicate experiments ($n = 4$). P values for all data points were calculated using Student's t test analysis against V8 (a, $P < 0.05$; b, $P < 0.1$; c, $P < 0.0001$; d, $P < 0.0005$; n.s., not significant). MTT assays were also performed to normalize for the cell number.

and a neomycin-resistance gene (pGL3Neo). The numbering of the 5'-flanking sequence is relative to the experimentally determined transcriptional start site at 8509 (+1). Plasmids pGL3Neo-H (nt -7968 to +3131), pGL3Neo-SH (nt -1 to +3131), pGL3Neo-S (nt -2137 to +1520) and pGL3Neo-B (nt -626 to +1073) (Figure 5A) were linearized with *KpnI*, and stably transfected into murine L929 cells. An empty pGL3 vector (pGL3Neo-V) was also transfected. From each transfected construct, 40 clones were isolated and assayed for luciferase activity. Since the majority of clones derived from a particular transfected construct had similar luciferase activities, two to four clones from each construct were selected for further analyses. All four pGL3Neo-SH stable cell lines gave no significant increase in luciferase expression compared with all the pGL3Neo-V clones, suggesting that the 5' region of intron 1 on its own does not regulate basal *mulfnar-2* expression (Figure 5B) in L929 cells. A significant increase of up to 520-fold ($P < 0.0001$) in luciferase activity was detected in all four pGL3Neo-B clones tested compared with V8 (the highest expressing pGL3Neo-V clone) (Figure 5B), suggesting that the region at -626 to -1 (positions 7881-8508 of the *mulfnar-2* genomic sequence) controlled basal *mulfnar-2* expression. One of the pGL3Neo-H stable transfectants (H15) showed an increase of approx. 4.5-fold luciferase activity ($P < 0.005$) compared with V8 (Figure 5B). None of the pGL3Neo-S clones showed any significant increase in luciferase activity compared with the vector controls (Figure 5B), suggesting that an element that can negatively regulate basal *mulfnar-2* expression is located between -2137 to -626.

The region of the *Ifnar-2* 5'-flanking region responsible for the $\text{IFN}\alpha/\text{IFN}\gamma$ induction of *Ifnar-2* mRNA expression in L929 cells was investigated next. A representative clone from each stably transfected promoter-reporter construct (S4, SH5, V8,

H15 and B22) was treated with a combination of 1000 units/ml $\text{muIFN}\alpha 4$ and 1000 units/mL $\text{muIFN}\gamma$, incubated for 9 h and luciferase activity was assayed. No significant increases in luciferase activity were observed following treatment of the V8 control, S4 or SH5 stable cell lines (Figure 5C). However, a 1.9-fold increase in luciferase activity was observed in the B22 cell line ($P < 0.0005$) compared with the V8 control, and a 3.9-fold increase was observed in the H15 cell line ($P < 0.0001$), indicating that there are additional elements in the region -7968 to -2137 controlling inducible *Ifnar-2* mRNA expression in L929 cells.

The 5'-flanking region of the *mulfnar-2* gene was examined for the presence of transcription-factor-binding sites. Figure 6 shows the position of potential binding sites for the transcription factors Oct-1 (Octamer binding factor-1), the homeodomain factor Nkx-2.5, the CCAAT/enhancer binding protein C/EBP α , AP-1, GATA-1, c-Myb, COUP-TF, c-Rel, IFN regulatory factor (IRF)-1/IRF-2 and the IFN-stimulated response element (ISRE) and γ -activation sequence (GAS) elements. Putative binding sites for the transcriptional repressors COUP-TF and IRF-2 [37] were present in the putative negative regulatory region -2137 to -626. Most significantly, five ISREs and 11 GAS elements [6] were located within the IFN-responsive -7969 to -2137 region (Figure 5), suggesting that one or more of these may be responsible for the observed sensitivity to type I and II IFN.

Comparisons of human and mouse non-coding genomic sequences in regions of conserved syntenicity have previously been successful in identifying elements regulating gene expression [38]. These regulatory elements can be found in non-coding regions, and have > 70% identity over at least 100 bp. Alignment of the genomic locus containing the *huIFNAR-2* gene (GenBank® accession no. NT011512), including 8.5 kb of 5'-flanking region and mouse genomic sequence containing the *mulfnar-2* gene plus

Figure 6 Putative transcription-factor-binding sites in the region proximal to exon 1 of the *mulfnar-2* gene

Binding sites for a number of transcription factors (boxed and labelled) predicted by one or more analysis programs in the 5' flanking region of the *mulfnar-2* gene proximal to exon 1 (bold uppercase). The *Bgl*II and *Sma*I restriction sites used in the generation of promoter-reporter constructs are underlined. The sequence is numbered relative to the major transcriptional start site (shown in italics).

8.5 kb of 5'-flanking sequence, was performed using the programs Dialign2 [19] and AVID (using a window size of 100 bp and a conservation level of 70%) (N. Bray, A. Fabrikant, J. Lord, J. Schwartz, I. Dubchak and L. Pachter, unpublished work) with similar results obtained. The chicken genomic *Ifnar-2* sequence was not analysed, since it only spanned exons 2 to 9. Visualization of only the AVID alignment was performed using VISTA [17,18] (Figure 7A). Only segments with a similarity of > 50% are plotted. As can be seen, exons 1, 2 and 3 were poorly conserved, whilst exons 4 to 9, with the exception of exon 6, were conserved. There were also conserved regions in introns 2, 3, 4 and 6. A 204 bp conserved non-coding sequence with 75% identity between the human and mouse sequences was located 1.6 kb upstream of the putative transcriptional start site of *mulfnar-2* (from -1590 to -1387) in the region that appeared to negatively regulate basal *mulfnar-2* expression (Figure 7B). In addition to a conserved TopoII consensus sequence, the 204 bp conserved non-coding sequence contained the following conserved transcription factor binding sites: MEF-2, TFIID, NFE1 and GATA-1 (Figure 7B).

DISCUSSION

Our previous studies have demonstrated the existence of a soluble, secreted form of the type I IFN receptor chain *mulfnar-2*, encoded by an alternatively spliced transcript [10]. More recent studies determined that this soluble receptor (*mulfnar-2a*)

was an abundantly expressed protein (> 10 µg/ml) in normal murine serum and other biological fluids, was generated by alternative splicing rather than by proteolytic cleavage of the extracellular domain of transmembrane *mulfnar-2c*, and had the ability to bind type I IFN [12]. The agonistic and antagonistic actions of soluble *mulfnar-2a* indicate that it could be a key *in vivo* regulator of the actions of IFNs, and that the modulation of soluble *mulfnar-2a* expression levels could have profound biological consequences. Other studies demonstrated that the transcript encoding soluble *mulfnar-2a* was generally more abundantly expressed than that encoding transmembrane *mulfnar-2*, and that the ratio of soluble (*mulfnar-2a*) to transmembrane (*mulfnar-2c*) transcripts varied from > 10:1 in the liver to < 1:1 in bone-marrow macrophages [12]. This observation suggested independent regulation of the two *mulfnar-2* isoforms and potential tissue-specific requirements for soluble *mulfnar-2*. In order to understand the regulation of *mulfnar-2a* and *mulfnar-2c* mRNA expression we have characterized the *mulfnar-2* gene, determined the molecular basis for the generation of its alternative mRNA splice forms and identified key regulatory elements. We have also extended our initial observations and demonstrated that the levels of the two *mulfnar-2* mRNA isoforms could be independently regulated upon stimulation with a combination of type I and II IFN.

The gene encoding *huIFNAR-2* has been previously described and, as is the case for *mulfnar-2*, multiple mRNA isoforms of *huIFNAR-2* [22,39] are generated by alternative splicing (*huIFNAR-2a*, encoding a soluble form lacking both transmembrane and cytoplasmic domains; *huIFNAR-2b*, encoding a truncated non-functional cytoplasmic form; and *huIFNAR-2c*, encoding a full-length functional transmembrane form). Although the proteins encoded by these isoforms are similar, the patterns of alternative splicing used to generate the murine and human *IFNAR-2* isoforms differ, perhaps due to genetic divergence. To date, however, a murine equivalent of *huIFNAR-2b* has not been described. In the type I IFN-unresponsive cell line that lacks IFNAR-2c (U5A), signalling could be reconstituted with IFNAR-2c, but not with IFNAR-2b, suggesting that the latter may be a non-functional isoform or dominant negative receptor [22,40]. However, it is apparent that all but 18 amino acids of the cytoplasmic domain of *huIFNAR-2b* are encoded by an *Alu* cassette in its antisense orientation that is incorporated by alternative splicing into the open reading frame of the cytoplasmic domain [41]. Since *Alu* sequences are not present in rodents, it is likely that this *huIFNAR-2* mRNA isoform is specific to primates. The generation of a soluble *mulfnar-2* mRNA isoform by splice site suppression appears to be a rare phenomenon among cytokine receptors, which use either exon skipping, such as the soluble murine interleukin-5 receptor α -chain [42], or soluble-specific exons, such as the soluble leukaemia inhibitory factor ('LIF') receptor α -chain [43]. However, other genes encoding different classes of receptors have similar alternative splicing patterns, e.g. the rat Fos-responsive gene *Fit-1* [44], the murine *Leptin receptor* [45] and human *CCR5* [46].

Type II IFN or IFN γ has many overlapping biological activities, but no amino acid sequence similarity with any of the type I IFNs and binds to a different receptor [6]. These overlapping biological functions, such as antiviral and anti-proliferative activities, occur through the common use of transcription factors, such as STAT1 [6] and IRF-9 (p48) [10,47], which bind to elements within the promoters of IFN-stimulated genes. In a process known as 'gamma priming' [34,48–50], stimulation of IFN-insensitive cells by IFN γ results in increased mRNA and protein levels of STAT1, STAT2 and p48/IRF9 [components of IFN-stimulated gene factor 3 (ISGF3)] via GAS

Figure 7 Alignments of the human and murine *Ifnar-2* genomic regions of conserved synteny

(A) Global alignment of murine *Ifnar-2* and human *IFNAR-2* genes. Numbers on the y-axis represent the percentage identity of nucleotides in a 100-bp window. Numbers on the x-axis indicate the nucleotide position from the beginning of the mouse *Ifnar-2* genomic sequence (GenBank® accession number AF367979) plus 194 bp of additional 3'-flanking sequence (GenBank® accession number AF440786). Conserved regions where the average identity is > 70% are highlighted below the curve (pink, conserved non-coding region; blue, conserved exon; turquoise, UTR). (B) Detailed alignment of the conserved non-coding region from nt positions 6917 to 7120 (–1590 to –1387 relative to the transcriptional start site). Identities are shaded in pink; gaps introduced during the alignment are also indicated by the dashes. Conserved topoisomerase II (Topo II) and transcription-factor-binding sites are indicated.

Figure 8 Model of the elements/regions within the *Ifnar-2* 5'-flanking region involved in the expression and regulation of *Ifnar-2* transcripts

(A) Regulatory region 1 (nt -626 to -1) provides high basal transcriptional activity and low inducibility by IFN. Element(s) within regulatory region 2 (nt -2137 to -626) can repress promoter activity. EST2 (shaded box), B1 repeat elements (white boxes) and a conserved non-coding sequence with homology with human *IFNAR-2* (box shaded with diagonal lines) are present within this region. Regulatory region 3 (nt -7968 to -2137) provides inducibility by type I and II IFN. Putative ISREs (circles) and GAS elements (diamonds) are indicated. Exon 1 of *mulfnar-2* is indicated by a black box, and the site and direction of transcription initiation is marked by an arrow. (B) Model of regulation of *mulfnar-2* mRNA expression by type I and type II IFN. Treatment of L929 cells with either IFN γ or IFN α/β is unable to cause an increase in *mulfnar-2* mRNA levels. However, stimulation of cells with IFN γ results in increased mRNA, protein levels and DNA-binding activation of ISGF3. IFN α/β is then able to use the increased ISGF3 to stimulate transcription of *mulfnar-2* via ISRE and GAS elements in the promoter of the *mulfnar-2* gene. Initially there is an up-regulation of *mulfnar-2c*, resulting in modulation of biological signals. Subsequently, in a negative feedback loop, up-regulation of *mulfnar-2a* occurs.

elements in the promoters of these genes, and enhanced DNA-binding activation of ISGF3 in response to IFN α . This priming or synergy between IFN γ and IFN α might be an important means of ensuring a rapid or amplified response to pathological stimuli.

We therefore determined whether a combination of IFN α and IFN γ might regulate *mulfnar-2* expression levels as a means of amplifying a response. Treatment of L929 cells and hepa-1c1c7 cells with a combination of IFN α and IFN γ , but not either cytokine alone, caused the levels of all *mulfnar-2* transcripts to be significantly up-regulated over time. Promoter-reporter deletion constructs of the 5' flanking region of *Ifnar-2* stably transfected into L929 cells demonstrated that a 600 bp region proximal to the 5' end of exon 1 (regulatory region 1; Figure 8a) conferred high (> 500-fold) basal luciferase activity compared with vector controls, and that a region distal to this contained strong negative regulatory elements (regulatory region 2, Figure 8a). A 2-fold increase in luciferase activity following treatment of cells containing regulatory region 1 with combined IFN α/γ was also observed, consistent with the increase in *mulfnar-2a* mRNA after similar treatment. A distal region (regulatory region 3; Figure

8a) was able to induce a greater increase in luciferase activity (approx. 4-fold) after IFN $\alpha/4$ and IFN α/γ treatment, which was also consistent with the increase in *mulfnar-2c* mRNA levels seen following similar treatment. Given the dominance of the soluble *Ifnar-2a* mRNA transcript [10,12] and its reduced ability to be induced by IFN α/γ compared with *Ifnar-2c*, it is possible that regulatory region 1 may contain an *Ifnar-2a*-specific promoter, and that regulatory region 3 may contain an *Ifnar-2c*-specific promoter (Figure 8a). This may be a situation similar to the bovine growth hormone receptor, whereby different isoform mRNA levels are achieved through the utilization of isoform-specific promoters [51]. In addition, we propose the following model as an explanation for the ability of L929 cells to up-regulate *mulfnar-2* mRNA levels only to a combination of IFN α/γ . This model (Figure 8b) utilizes the above process of gamma priming, whereby IFN α or IFN γ cannot up-regulate *mulfnar-2* mRNA levels by themselves, but the increased levels of ISGF3 components resulting from IFN γ stimulation allows (in this case the preferential) up-regulation of transmembrane *mulfnar-2c* mRNA in response to type I IFN (presumably by the binding of ISGF3 to ISRE elements present within regulatory

region 3; Figure 8b). The up-regulation of IFNARs on the surface of a cell could then lead to increased biological activity in response to IFN α . Because of the kinetics of *mulfnar-2* mRNA isoform induction, it is reasonable to suggest that the later induction of soluble *mulfnar-2a* mRNA acts as a classical negative feedback loop whereby ligand (type I IFN) induces the expression of an inhibitor of itself. The up-regulation of *mulfnar-2* mRNA levels by co-stimulation with IFN α and IFN γ implies further evidence for an additional level of molecular cross-talk between these two cytokines designed to amplify biological responses.

Regulation of cytokine receptor mRNA isoform expression and correlation with disease pathogenesis has been described for several cytokine receptor systems that have soluble and transmembrane forms, including the soluble IL-6 receptor [52] and the soluble leptin receptor [53]. Interestingly, responsiveness of eosinophils to the growth-and-survival factor IL-5 is thought to be controlled by the expression levels of the transmembrane IL-5R α mRNA isoform relative to the soluble (antagonistic) IL-5R α alternative splice form. Relatively high levels of transmembrane IL-5R α mRNA, and hence increased IL-5-responsiveness, has been implicated in the pathogenesis of asthma and eosinophilic diseases, and recent studies have shown that IL-5 ligand can induce a switch towards generating transmembrane IL-5R α mRNA rather than soluble IL-5R α mRNA [54]. In contrast with work carried out in mice [10,12] a comprehensive study of huIFNAR-2 mRNA expression in normal adult tissue has not yet been described, and hence there is limited information on the relative expression levels of *IFNAR-2* mRNA transcripts in normal physiological situations. However, recent data have demonstrated synergistic up-regulation of *huIFNAR-2* mRNA in liver cells treated with a combination of IFN α and IFN γ [55]. The data presented here demonstrates further that the levels of *mulfnar-2a* and *mulfnar-2c* mRNA can be regulated independently, and that this regulation occurs in a temporal and cell-type-specific manner. Interestingly, recent studies have suggested that high serum levels of soluble huIFNAR-2 suppressed the effectiveness of IFN therapy in patients with chronic hepatitis C [56], suggesting that, for the formation of an effective type I IFN-mediated response, the antagonistic effects of *Ifnar-2a* may need to be neutralized by a concomitant increase in *Ifnar-2c* expression. The studies described here have added a new dimension to analyses of the mouse *Ifnar-2* and human *IFNAR-2* gene loci, and have increased the number of levels and the complexity that exists for modulating responses to endogenous and exogenous type I IFN via the type I IFN receptor.

Ms S. Kitsoulis, Ms K. Portbury and Ms J. Fenner (of this laboratory) are thanked for assistance with cell culture and RNA preparations, Ms S. Trajanovska (also of this laboratory) for library screening, and Mr S. Bardill (Wellcome Trust Sequencing Centre, Monash Institute of Reproduction and Development) is thanked for assistance with sequencing. This work was supported by a grant from the National Health and Medical Research Council of Australia. M.P.H. is the recipient of an Australian Postgraduate Award. The U.K. Medical Research Centre Human Genome Mapping Project (Hinxton Hall, Cambridge, U.K.) is acknowledged for provision of the NIX analysis program.

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Received 15 January 2002/18 March 2002; accepted 9 April 2002

Published as BJ Immediate Publication 9 April 2002, DOI 10.1042/BJ20020105