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-1ĉ1ĉ1 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 isoatypes, 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 muIfnar-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 muIfnar-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 muIfnar-2 transcriptional regulation, we report here the structure and complete nucleotide sequence of the muIfnar-2 gene, including 8.5 kb of 5′ flanking sequence and the characterization of the molecular basis of the alternatively spliced forms of muIfnar-2. We also demonstrate independent regulation of the muIfnar-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 muIfnar-2 gene that control basal and inducible mRNA expression.

**EXPERIMENTAL**

**Isolation and subcloning of the muIfnar-2 gene**

A murine 129SvJ genomic library in λFix II (Stratagene, La Jolla, CA, U.S.A.) was screened with a 303 bp muIfnar-2 cDNA probe spanning nt positions 507–809 of muIfnar-2 cDNA. To obtain further λ genomic clones spanning the extreme 5′ end of the muIfnar-2 gene, the same library was subsequently probed with a 588 bp NotI–HindIII fragment corresponding to intron 1 of muIfnar-2 obtained from a bacteriophage clone isolated following the initial library screening. All probes were labelled with [32P]dCTP using a random primer labelling kit (Roche, Castle Hill, Australia) to specific radioactivities of > 6.3 × 10^6 c.p.m./µg. 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 × SSC (where 1 × 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 [32P]ATP (Amersham) and T4 polynucleotide kinase (Promega). Specific restriction fragments were then subcloned into pBluescript KS (+/−).

**Nucleotide sequence analysis of the muIfnar-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′-SMART™ rapid amplification of cDNA ends (RACE)**

5′- and 3′-SMART™ RACE-ready cDNA libraries were generated according to the manufacturer’s recommendations (Clontech) using 1 µg of polyadenylated [poly(A)]+ mRNA from normal mouse thymus, liver or placenta. 5′- and 3′-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 genespecific primer (see Table 1) in a final volume of 50 µl. 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 GeneScreen Plus 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.dccn.nih.gov/molbio/index.html) [13], Search Launcher (http://searchlauncher.bcm.tmc.edu) [14], TFSEARCH (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html) [15], MatInspector Release Professional (http://genomatix.gsf.de/matinfa) [16], MacVector version 7.1 (Oxford Molecular Ltd.), and Omega 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 muIfnar-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

**Table 1 Oligonucleotides used for 5′- and 3′-SMART™ RACE PCR**

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<thead>
<tr>
<th>Name</th>
<th>Sequence (5′ → 3′)</th>
<th>Used for</th>
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</thead>
<tbody>
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<td>AR2-1</td>
<td>5′-GCCAGAGGCTCAAGGGTAAAGGCACTGCTG-3′</td>
<td>3′ RACE</td>
</tr>
<tr>
<td>AR2-2</td>
<td>5′-GTTGAGGGGAGGAGAAGGCACTGCTG-3′</td>
<td>3′ RACE</td>
</tr>
<tr>
<td>AR2-3</td>
<td>5′-GCTGCAGGTACCTCCCTGGGTAGT-3′</td>
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<td>AR2-5</td>
<td>5′-CCGGAGCCGAGTGGTCCAA-3′</td>
<td>5′ RACE</td>
</tr>
<tr>
<td>AR2-6</td>
<td>5′-ACGGAGGACAGAAGGCACTGCTG-3′</td>
<td>5′ RACE</td>
</tr>
<tr>
<td>TE-1</td>
<td>5′-CCGAGCCCCCACTAATGGCTGCTTG-3′</td>
<td>3′ RACE</td>
</tr>
<tr>
<td>TE-2</td>
<td>5′-GCGACTTCCTCCAAGGACAGA-3′</td>
<td>3′ RACE</td>
</tr>
<tr>
<td>TE-3</td>
<td>5′-AAAGGCTTCAGTCTCCATCA-3′</td>
<td>3′ RACE</td>
</tr>
<tr>
<td>TE-4</td>
<td>5′-CCGAGGCTCCTGCTGTTAAGGCTCTGC-3′</td>
<td>3′ RACE</td>
</tr>
<tr>
<td>TE-5</td>
<td>5′-TTCGAGACCTAGAAATCAAGAAATGAGA-3′</td>
<td>3′ RACE</td>
</tr>
<tr>
<td>TE-6</td>
<td>5′-GACCGCCAGAGGCTTCAGTCTGCTGCCTG-3′</td>
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<td>TE-7</td>
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<td>TE-8</td>
<td>5′-TCAGAATCTCCTGCTGCTCACCGCTGCT-3′</td>
<td>5′ RACE</td>
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Regulation of Ifnar-2 expression

Figure 1 The organization of the murine Ifnar-2 gene

(A) Schematic representation of the murine Ifnar-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 Ifnar-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 Ifnar-2 locus (B, BamHI; E, EcoRI; H, HindIII). Grey and hatched boxes represent expressed sequence tags EST1 and EST2 respectively, lying in and around the Ifnar-2 genomic locus. (B) Intron-exon boundaries of the murine Ifnar-2 gene.

<table>
<thead>
<tr>
<th>3′ Splice Acceptor Site</th>
<th>Exon (bp)</th>
<th>5′ Splice Donor Site</th>
<th>Intron (bp)</th>
<th>Codon Phase</th>
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<tr>
<td>tctctttcaggtgtaagcGag</td>
<td>1 (293)</td>
<td>ggtctgatcctgtactct</td>
<td>1 (10,781)</td>
<td>N/A</td>
</tr>
<tr>
<td>V S A S</td>
<td>2 (93)</td>
<td>tggcagtaaggtgtaagagtct</td>
<td>2 (1,529)</td>
<td>1</td>
</tr>
<tr>
<td>G Y P D</td>
<td>3 (42)</td>
<td>gcttattgaggtgagcagacg</td>
<td>3 (918)</td>
<td>1</td>
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<tr>
<td>tttttcccagctcttagcagagca</td>
<td>4 (124)</td>
<td>cagcagcagctgtaagtc</td>
<td>4 (1,976)</td>
<td>2</td>
</tr>
<tr>
<td>K D E</td>
<td>5 (173)</td>
<td>pankaagtagtagtagtagag</td>
<td>5 (3,605)</td>
<td>1</td>
</tr>
<tr>
<td>A P L E</td>
<td>6 (158)</td>
<td>vkkkkkgagagagagagag</td>
<td>6 (1,970)</td>
<td>0</td>
</tr>
<tr>
<td>H E P K</td>
<td>7 (157)</td>
<td>gqesagagagagagagagagag</td>
<td>7 (5,306)</td>
<td>1</td>
</tr>
<tr>
<td>G L S E</td>
<td>8 (128)</td>
<td>pmvlcagagagagagagagagagagag</td>
<td>8 (4,365)</td>
<td>0</td>
</tr>
<tr>
<td>N F R H</td>
<td>9 (1,872)</td>
<td>ccttcttgctcctggctctggc</td>
<td>9 (1,872)</td>
<td>1</td>
</tr>
</tbody>
</table>

* As determined by 5′ and 3′ RACE  
* Splicing occurs between exons 7–8 or exons 7–9

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-bin/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 minimum essential medium without nucleosides, and M1 murine myeloid leukaemia cells were cultured in RPMI medium. All media was supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Life Technologies, Gaithersburg, MD, U.S.A.). Recombinant murine IFNα4 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 IFNα2 was produced in Pichia pastoris (S. Trajanowska, C. M. Owczarek and P. J. Hertzog, unpublished work). Recombinant IFNγ 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 IFNα4 and murine IFNγ 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 N﻿.

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 ³²P-labelled probes, including muIfnar-2 cDNA, a 537 bp genomic DNA fragment corresponding to muIfnar-2 intron 1 (accession no. AF367979; nt 18027–18563); 585 bp or 506 bp cDNA fragments encoding the extracellular and cytoplasmic domains respectively of muIfnar-1 (a gift from Dr G. Uzę, at the Institut de Génétique Moléculaire, CNRS, Montpellier, France), and then washed with 0.2 × SSC/0.1% (w/v) SDS at 65 °C. Blots were finally probed with a ³²P-labeled 1.2 kb PsiI 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 muIfnar-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; pGGL3Neo-H), 3.65 kb SacI-cleaved fragment (nt 6370–10027; pGGL3Neo-S), 3.1 kb Smal/HindIII-cleaved fragment (nt 8508–11638; pGGL3Neo-SH) and 1.7 kb BglII-cleaved fragment (nt 7881–9580; pGGL3Neo-B) (see accession no. AF367979 for numbering). Before transfection, all constructs were linearized with KpnI. L929 cells were seeded at approx. (1–3) × 10⁵ cells/well in a six-well plate in complete growth media, and cultured at 37 °C (in the presence of 5% CO₂) 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 LIPOFECTAMINE™ 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 cm² dishes, G418 (or Geneticin; 600 µg/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 ×  Taq polymerase/1 µl of 4.5% (w/v) Nonidet P40/1 µl of 4.5% (w/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 × 10⁶) 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% CO₂) in RPMI or Dulbecco’s modified Eagle’s medium, the media were removed and to each well was added 1000 units/ml muIFNα and 1000 units/ml muIFNγ. For basal promoter analysis, the plates were left untreated for 30 h at 37 °C. The plates were incubated at 37 °C (5% CO₂) for a further 9 h, and then gently washed with PBS. The adherent cells were then lysed for 15 min with 300 µl/well of 5 × 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).

**RESULTS**

**Structure of the muIfnar-2 gene**

In order to determine the molecular origin of the different mRNA splice variants of muIfnar-2, and to study the promoter of the muIfnar-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 muIfnar-2 gene contains 9 exons and 8 introns shown in Figures 1A and 1B. The 5’-flanking sequence of the muIfnar-2 gene lacked a recognizable TATA or CAAT box, making it similar to the 5’-flanking regions of other IFNAR genes, e.g. human (ha)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 muIfnar-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 muIfnar-2c respectively. In addition, exon 9 encodes the nucleotide sequence of muIfnar-2a produced by alternative splicing (see below), and contains the common 3’ UTRs of both muIfnar-2a and muIfnar-2c. Exon 7 is a 3’ continuation of exon 6, and encodes the sequence specific to the muIfnar-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 muIfnar-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 muIfnar-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 muIfnar-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 muIfnar-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 muIfnar-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 muIfnar-2 gene and the published cDNA.
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

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 mulf-10r2 generated a 2 kb fragment that contained exon 9 of mulfnar-2, exon 1 of mulf-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 mulf-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, -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

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

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<tr>
<th>Repeat</th>
<th>No. of elements</th>
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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.
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 SMART RACE library and the primers TE-5 to TE-8 (Table 1) to determine whether EST2 was part 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.

![Figure 3 Patterns of alternative splicing of the muIfnar-2 and HuIFNAR-2 mRNA isoforms](image)

(A) Alternative mRNA splicing patterns of the muIfnar-2 isoforms. Soluble muIfnar-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 muIfnar-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 muIfnar-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.

Regulation of muIfnar-2 expression

To determine whether the mRNA expression levels of the muIfnar-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), muIFNα, muIFNγ, muIFNγ, 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 muIfnar-2a and muIfnar-2c were then quantified relative to GAPDH. In all cases, muIfnar-2 expression levels were not significantly affected by these factors in this cell type. Since biological fluids contain mixtures of cytokines, and since IFNα and IFNγ have been reported to act synergistically [34], we treated the cells with a combination of IFNα and IFNγ. Northern blot analysis of murine L929 cells treated with a combination of 1000 units/ml muIFNα and 1000 units/ml muIFNγ for up to 24 h (Figure 4A) showed that the levels of muIfnar-2-2a 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 muIfnar-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 muIfnar-1 transcript remained unchanged (Figures 4A and 4B). The difference between muIfnar-2a:Gapdh and muIfnar-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 muIfnar-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 leukemia M1 cells and murine hepatoma 1c1c7 cells. No significant changes in muIfnar-2 isoform mRNA expression levels occurred after treatment of M1 cells with a combination of 1000 units/ml muIFNα and 1000 units/ml muIFNγ for 24 h (Figure 4C). However, the treatment of hepa-1c1c7 cells with the same factors resulted in a 3-fold increase in muIfnar-2a mRNA expression after 9 h treatment, which then returned to normal after 24 h (Figure 4D). The muIfnar-2c mRNA transcript increased 4-fold after 3 h treatment, then returned to normal after 24 h. Levels of muIfnar-1 mRNA expression did not change in either M1 or hepa-1c1c7 cells over time.

Determination of the full-length cDNA sequence of muIfnar-2

In order to examine the promoter of the muIfnar-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 muIfnar-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 muIfnar-2 gene. A total of 1976 bp of contiguous sequence was isolated, corresponding to nt 5797–7772 of the muIfnar-2 genomic sequence. No muIfnar-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 muIfnar-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 muIfnar-2 transcript previously reported [10] (results not shown). The significance of this is unknown, but it may represent a minor muIfnar-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.
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.
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) 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 *muIfnar-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 *muIfnar-2* genomic sequence) controlled basal *muIfnar-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 one or more of these may be responsible for the observed sensitivity to type I and II IFN.

The region of the *Ifnar-2* 5′-flanking region responsible for the IFNα/IFNγ 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 muIFNα4 and 1000 units/ml IFNγ, 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 Ifnr-2 mRNA expression in L929 cells.

The 5′-flanking region of the *muIfnar-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/EBPα, 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 synteny 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 *muIfnar-2* gene plus...
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 the membrane-bound muIfnar-2, and had the ability to bind type I IFN [12]. The agonistic and antagonistic actions of soluble muIfnar-2a indicate that it could be a key in vivo regulator of the actions of IFNs, and that the modulation of soluble muIfnar-2a expression levels could have profound biological consequences. Other studies demonstrated that the transcript encoding soluble muIfnar-2a was generally more abundantly expressed than that encoding transmembrane muIfnar-2, and that the ratio of soluble (muIfnar-2a) to transmembrane (muIfnar-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 muIfnar-2 isoforms and potential tissue-specific requirements for soluble muIfnar-2. In order to understand the regulation of muIfnar-2a and muIfnar-2c mRNA expression we have characterized the muIfnar-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 muIfnar-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 muIfnar-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

Figure 6 Putative transcription-factor-binding sites in the region proximal to exon 1 of the muIfnar-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 muIfnar-2 gene proximal to exon 1 (bold uppercase). The Apgll and Sma1 restriction sites used in the generation of promoter-reporter constructs are underlined. The site 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 sequence obtained using Dialign2 [17,18] 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 muIfnar-2 (from -1590 to -1387) in the region that appeared to negatively regulate basal muIfnar-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, TFID, NFIE 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 muIfnar-2, encoded by an alternatively spliced transcript [10]. More recent studies determined that this soluble receptor (muIfnar-2a)
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.
Regulation of \textit{muIfnar-2} expression

\textbf{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 indicated. Exon 1 of \textit{muIfnar-2} is indicated by a black box, and the site and direction of transcription initiation is marked by an arrow. \textbf{B} Model of regulation of \textit{muIfnar-2} mRNA expression by type I and type II IFN. Treatment of L929 cells with either IFN\textsubscript{c} or IFN\textsubscript{a/b} is unable to cause an increase in \textit{muIfnar-2} mRNA levels. However, stimulation of cells with IFN\textsubscript{c} results in increased mRNA, protein levels and DNA-binding activation of ISGF3. IFN\textsubscript{a/b} is then able to use the increased ISGF3 to stimulate transcription of \textit{muIfnar-2} via ISRE and GAS elements in the promoter of the \textit{muIfnar-2} gene. Initially there is an up-regulation of \textit{muIfnar-2c}, resulting in modulation of biological signals. Subsequently, in a negative feedback loop, up-regulation of \textit{muIfnar-2a} occurs.

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

We therefore determined whether a combination of IFN\textsubscript{a} and IFN\textsubscript{c} might regulate \textit{muIfnar-2} expression levels as a means of amplifying a response. Treatment of L929 cells and hepa-1C1C7 cells with a combination of IFN\textsubscript{a} and IFN\textsubscript{c}, but not either cytokine alone, caused the levels of all \textit{muIfnar-2} transcripts to be significantly up-regulated over time. Promoter-reporter deletion constructs of the 5'-flanking region of \textit{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\textsubscript{a/}\textsubscript{c} was also observed, consistent with the increase in \textit{muIfnar-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\textsubscript{a/4} and IFN\textsubscript{a/\gamma} treatment, which was also consistent with the increase in \textit{muIfnar-2c} mRNA levels seen following similar treatment. Given the dominance of the soluble \textit{Ifnar-2a} mRNA transcript [10,12] and its reduced ability to be induced by IFN\textsubscript{a/\gamma} compared with \textit{Ifnar-2c}, it is possible that regulatory region 1 may contain an \textit{Ifnar-2a}-specific promoter, and that regulatory region 3 may contain an \textit{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 \textit{muIfnar-2} mRNA levels only to a combination of IFN\textsubscript{a/\gamma}. This model (Figure 8b) utilizes the above process of gamma priming, whereby IFN\textsubscript{a} or IFN\textsubscript{c} cannot up-regulate \textit{muIfnar-2} mRNA levels by themselves, but the increased levels of ISGF3 components resulting from IFN\textsubscript{c} stimulation allows (in this case the preferential) up-regulation of transmembrane \textit{muIfnar-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 IFNz. Because of the kinetics of muIfnar-2 mRNA isomorph induction, it is reasonable to suggest that the later induction of soluble muIfnar-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 muIfnar-2 mRNA levels by co-stimulation with IFNz and IFNγ 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-5Rα mRNA isoform relative to the soluble (antagonistic) IL-5Rα alternative splice form. Relatively high levels of transmembrane IL-5Rα 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-5Rα mRNA rather than soluble IL-5Rα 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 synergetic up-regulation of huIFNAR-2 mRNA in liver cells treated with a combination of IFNz and IFNγ [55]. The data presented here demonstrates further that the levels of muIfnar-2a and muIfnar-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 Medical Research Council Summer Studentship. M.P.H. is supported by 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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