Affinity Purification, Peptide Analysis, and cDNA Sequence of the Mouse Interferon γ Receptor*

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The receptor for mouse interferon γ (IFN-γ) was purified from detergent-solubilized plasma membranes of EL-4, a thymoma cell line which expresses a high number of receptors on its cell surface. The purification was carried out by immunoaffinity chromatography using an anti-receptor monoclonal antibody. The purified receptor was subjected to NH₂-terminal sequence analysis as well as sequencing of endopeptidase-generated peptides. One of the peptides was found to be identical to a portion of the published amino acid sequence of the human IFN-γ receptor deduced from cDNA. This information was utilized to construct a mixed-sequence oligodeoxynucleotide probe which permitted the isolation of a full-length cDNA clone coding for the mouse IFN-γ receptor. The mouse IFN-γ receptor cDNA is comprised of 105 base pairs of the 5'-untranslated region, an open reading frame coding for a 477-amino acid serine-rich protein having calculated M, 52,276, and a 3'-untranslated region of 539 base pairs. The receptor is first synthesized as a preprotein from which a 25-amino acid signal peptide is cleaved. The receptor contains a hydrophobic transmembrane portion near the center of the molecule. Northern blot analysis of various cell lines showed that each contained a single 2.0-kilobase mRNA. A direct correlation between the amount of IFN-γ receptor mRNA and the level of receptor expressed on the cell surface was observed. The mouse and human IFN-γ receptors are structurally similar, showing 51% overall homology in amino acid sequence. Mouse IFN-γ receptor cDNA when inserted in a mammalian shuttle vector and transfected into COS-7 monkey cells was able to direct the expression of specific binding activity for mouse IFN-γ.

Interferons (α, β, γ) are polypeptide hormones involved in the modulation of cellular growth and differentiation, and in the release of certain cytokines, which in turn have a pleiotropic effect on target cells (1-6). IFNs are involved in the induction of an antiviral response in the inhibition of cell proliferation and cellular mobility (7), and in the expression of membrane class I and II antigens, certain cell surfaces receptors (8, 9), and fibronectin (10). IFN-γ and to a lesser extent IFN-α and β are known to be immunomodulatory factors (11). IFN-γ strongly inhibits mitogen activation of B cells, but promotes growth and final differentiation of these into immunoglobulin producing cells (12). In addition, it activates macrophages, boosts cytotoxicity of natural killer cells and stimulates T cell cytotoxicity (13, 14). IFN-γ is synthesized and released from immune effector cells (activated T lymphocytes and NK cells) upon stimulation by various agents (alloantigens, tumors, mitogens, etc.). The biological response to IFN-γ is induced by its binding to a specific receptor on the surface of target cells (11), which is distinct from that recognized by IFN-α and IFN-β (15). IFN-γ receptors are generally expressed at low levels, except on certain tumor cells, which exhibit up to 60,000 receptors/cell (16, 17). The IFN-γ receptor binds noncooperatively to the ligand with high affinity, with Kd ranging from 10⁻⁹ to 10⁻¹¹ M (16, 18-20).

The human IFN-γ receptor was purified by sequential ligand-affinity chromatography and immobilized anti-receptor monoclonal antibodies (21). The receptor consists of two protein species with an apparent M, of 90,000 and 50,000 of which the 50-kDa component was shown to be a proteolytic degradation product of the 90-kDa species. Recently, a cDNA coding for the human IFN-γ receptor was isolated from an expression library using a polyclonal anti-receptor antibody (22). The deduced amino acid sequence revealed structural features typical of cell surface receptors, such as a putative signal peptide and a hydrophobic, transmembrane-like portion.

The mouse IFN-γ receptor has been solubilized by various detergents from plasma membranes of myelomonocytic (WHEI-3) and thymoma (EL-4) cell lines and identified by direct radiolabeled IFN-γ binding (23, 24). Detergent-solubilized membranes showed only a single class of binding activity with values comparable to that obtained using intact cells. IFN-γ binding was demonstrated to be specifically competed by unlabeled IFN-γ, as well as by monoclonal antibodies (23-25). The receptor was shown to be a glycoprotein of 85-95 kDa.

To determine the structure of the mouse IFN-γ receptor...

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1 The abbreviations used are: IFN(s), interferon(s); PBS, phosphate-buffered saline; CHAPS, 3-(cholamidopropyl)dimethylammonio-1-propanesulfonate; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) J05265.

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we have purified the protein on a large scale by affinity chromatography with immobilized monoclonal anti receptor antibodies. Chemical sequencing of peptides obtained by digestion of the purified receptor with a lysine-specific endopeptidase has permitted the construction of an oligodeoxynucleotide probe which was used to isolate a full-length mouse receptor cDNA clone. The mouse and human IFN-γ receptors are in fact structurally similar, showing 51% identity in amino acid sequence.

MATERIALS AND METHODS

**Large Scale Preparation of Membranes—**EL-4 cells were grown in RPMI 1640 containing 5% fetal calf serum, 2 mM glutamine, 50 μM 2-mercaptoethanol, 50 μg/ml gentamicin in the presence of 5% CO₂ to a density of 1–2 × 10^6 cells/ml. Cell cultures were expanded by serial 1:1 dilutions until 40 liters were obtained (6 × 10^6 cells).

The cells were centrifuged by ultracentrifugation and washed with PBS. They were lysed in a hypotonic solution containing 10 mM Tris·HCl, pH 7.5, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM CaCl₂, plus the following proteases inhibitors: phenylmethylsulfonyl fluoride (50 μg/ml), tosylphenylalanine chloromethyl ketone (50 μg/ml), pepstatin (0.07 μg/ml), and leupeptin (0.5 μg/ml) and disrupted further in a Potter homogenizer. Membranes were recovered by sequential centrifugation of the supernatants at 10,000 × g for 7 min, 3,500 × g for 10 min, and 30,000 × g for 30 min. The last centrifugation step was performed after the addition of an on-line 120A PTH analyzer. The supernatant was centrifuged at 100,000 × g. The supernatant was stored at -70 °C. Specific activity (pmoles of receptor/mg of total solubilized protein) was deduced from the binding activity of solubilized membranes using [³²P]labeled IFN-γ as described (24).

**Mouse IFN-γ Receptor Purification—** Rat anti-mouse receptor monoclonal antibody, GR-20 (25), was covalently bound to Amino-link-agarose support according to the protocol supplied by the manufacturer (Pierce Chemical Co., at a concentration of 2 mg/ml of gel). Protein G-purified GR-20 was then a kind gift of Dr. S. W. Russell (University of Kansas Medical Center). Two ml of the GR-20-linked support were incubated batchwise with 50 ml of solubilized membranes on a rotating platform for 2 h at 4 °C. The gel was washed 5 times with 50 ml of PBS containing 8 mM CHAPS, 1% deoxycholate, 0.2% SDS, and the above protease inhibitors, i.e., until the 280-nm absorbance was less than 0.006. The washed gel was then packed in a 1-cm diameter column. The mouse IFN-γ receptor was eluted with glycine·HCl, pH 3, containing 8 mM CHAPS and the above protease inhibitors. The purity of the receptor was assayed by silver staining of a 10–15% gradient SDS-PAGE in a Phast System electrophoresis apparatus (Pharmacia LKB Biotechnology Inc.) and confirmed by Western blotting with GR-20.

**Western Blotting—** Fractions from the immunofluorescence column were subjected to 7.5% SDS-PAGE then transferred for 1 h, 45 min at 250 V onto a nitrocellulose filter. The blot was incubated for 2 h in PBS containing 10% non-fat dry milk and 0.05% Tween 20. The receptor was specifically detected by a 2-h incubation with the GR 20 monoclonal antibody followed by incubation with peroxidase-conjugated anti-rabbit IgG.

**Endoprotease Digestion and Edman Degradation—** About 50 μmol of pure IFN-γ receptor were lyophilized, and then reduced in 100 μl of Tris·HCl, pH 8.5, 1 mg/ml dithiothreitol and 6 μm guanidinium CI. After overnight incubation at room temperature, the sample was pyridylethylated by the addition of 2.1 μl of 10% (v/v) iodoacetamide. After a 3-h incubation at room temperature, the sample was desalted by repeated dilution with 10 mM NaHCO₃ containing 50 μm CHAPS followed by centrifugation on a Centricon 10 filter (Amicon) and then lyophilized. CHAPS was extracted from the lyophilized sample by two additions of 500 μl of 2:1 chloroform/methanol. The precipitated protein was dissolved in 100 μl of 1% acetic acid and lyophilized. Finally, it was dissolved in 75 μl of 0.1 M NH₄HCO₃, pH 8.5, containing 4 μmol urea and digested with 5 μl of Endoprotease I (Boehringer-Mannheim), a lysine-specific endopeptidase, followed by digestion with 40,000 × g for 2 h for the protease digestion (28), by adding 1 μg at zero time and after 8 h. The total incubation time was 24 h. The digest was subjected to reverse-phase HPLC on an Aquapac (RP-300) column (Brownlee Laboratories) in 0.05% trifluoroacetic acid with acetonitrile as mobile phase on a Hewlett-Packard 1090A. Peptides or undigested receptor were sequenced on an Applied Biosystems 470A sequencer equipped with an on-line 120A PTH analyzer.

**Recombinant DNA Procedures—** General methods of recombinant DNA analysis were performed as described (27). Enzymes were purchased from Pharmacia IKB Ribochemistry Inc. Bethesda Research Laboratories, United States Biochemical Corp., and Promega. Oligodeoxynucleotides were synthesized by the phosphoramidite method using an automated DNA synthesizer (Versagen, Applied Biosystems). The termini of the oligonucleotides were purified by HPLC. Labeling at the 5'-end of oligodeoxynucleotides to ~5 × 10^6 dpm/μg was performed using [³²P]ATP (600 Ci/mmole and T4 polynucleotide kinase.

A cDNA library was constructed in XzaplI (29) (Stratagene) with poly(A)+ RNA isolated from the placematic lysosoma cell line JEG-3 kindly provided by Dr. M. G. Kline (National Cancer Institute). Briefly, 5 μg of poly(A)+ RNA was used to synthesize double-stranded cDNA by a modification of a previously described method (30). After blunt-ending the ends of cDNAs with Klenow fragment, EcoRI-Nol adapters (Promega) were ligated to the cDNA and the EcoRI ends phosphorylated with T4 DNA kinase.

The cDNA was ligated to XzaplI vector arms and packaged in vitro using Gigapack Gold reagents (Stratagene). The library was screened by high density plaque hybridization (6 × 10^6 plaques, 4 × 10^15 plaques/15-cm plate) with a 32P-labeled oligodeoxynucleotide probe. The probe was synthesized on the basis of an internal peptide of the mouse IFN-γ receptor and was of the sequence 5'-TGTCGCGGCTGCTGTGTTGTC-3' (where N = A, C, G, and T). Hybridization of the screening plaques was performed at 42 °C in 50% formamide, 5 × SSPE (100 mM NaCl, 0.5 mM sodium citrate, pH 7), 7 × Denhardt's solution (0.02% each bovine serum albumin, polyvinylpyrrolidone, and Ficoll), 1% SDS, 100 μg/ml heat-denatured, sheared salmon sperm DNA, 0.05% sodium pyrophosphate, and 2 × 10^5 cpm of 5'-end-labeled probe. Following a 16-h hybridization, filters were washed in 6 × SSC%, 1% SDS at 49 °C. Phagemids carrying the cDNA inserts were excised from hybridization-positive XzaplI recombinant phage clones by superinfection with R408 helper phage (30). Preliminary analysis of phagemid DNA clones was performed by double-stranded DNA sequencing (31). EcoRI-generated inserts from selected phagemid clones were subcloned into M13mp18 (32) and sequenced by primer extension from synthetic oligodeoxynucleotides. Sequence analysis in both cases was performed by the chain termination method (33) using wedge gelo (34) and [³²P]ATTP Sα as label. Computer assisted analysis of sequence data was performed as described (35).

In Vitro Transcription and Translation—RNA was synthesized in vitro from DNA templates with T7 polymerase using conditions and previously (36). Capping of RNA was performed during the in vitro synthesis by inclusion of GpppG in the reaction (36). The DNA template was removed by treatment with RNase-free DNase (Pharmacia) and the RNA purified by phenol extraction and ethanol precipitation. RNA was translated in vitro using a rabbit reticulocyte lysate system in the presence or absence of canine pancreatic microsomal membranes (Promega) using protocols supplied by the manufacturer.

**Immunoprecipitation—** EL-4 and CV-1 cells were metabolically labeled for 4 h with [³²S]methionine, then solubilized in PBS containing 8 mM CHAPS plus protease inhibitors. Immunoprecipitation of solubilized cells or in vitro translation products was carried out for 2 h at 4 °C with the monoclonal antibody GR-20 covalently bound to Amino-link-agarose. The support was washed as described above, boiled in Laemmli buffer and the eluate subjected to 12.5% SDS-PAGE.

**Transient Expression of Mouse IFN-γ Receptor cDNA in Monkey Cell Lines—** Transfections of cells were performed by the calcium phosphate precipitation method as described (37). 72 h after transfection, [³²P]labeled mouse IFN-γ was added (1 × 10^12 cpm/ml of New England Culture) and the cells were harvested. The [³²P]labeled mouse IFN-γ and incubated for 1 h at 35 °C. After washing the cells 3 × with PBS, 1 ml of trypsin was added, the cells collected, and radioactivity measured in a γ-counter.

**RESULTS**

Mouse IFN-γ Receptor Purification—To purify the mouse IFN-γ receptor on a large scale, we tested the effectiveness of both ligand and monoclonal antibody affinity chromatography. Although mouse recombinant IFN-γ linked to Sepharose 4B retained negligible amounts of receptor activity, immu-
no affinity column chromatography proved to be effective. Recovery of the mouse receptor from the immunoaffinity column was found to be optimal under conditions of mild acid treatment with glycine-HCl buffer, pH 3, containing 8 mM CHAPS. Before elution of the receptor, however, extensive washes under stringent conditions were required (see "Materials and Methods"). Following acid elution, it was necessary to immediately neutralize the preparation to maintain integrity of the receptor. The receptor preparation was found to be predominantly a single band of ~85 kDa by silver staining of SDS-PAGE gels (Fig. 1A). The band itself was somewhat diffuse, typical of a glycosylated protein. A band of the same apparent M, was also recognized with the GR-20 monoclonal antibody by Western blotting (Fig. 1B). From 2 x 10^9 cells, the yield of the receptor purified by this procedure was determined by amino acid analysis to be 150 pmol.

**Amino Acid Sequence Analysis of IFN-γ Receptor**—About 50 pmol of pure receptor were digested with *Achromobacter lyticus* Protease I, a lysine-specific endopeptidase, and the products separated by HPLC. The chromatographic profile consisted of 17 peaks (Fig. 2). Fractions corresponding to four of the peaks were subjected to automated Edman degradation and each of these was found to give a single sequence. The sequence of the peptide in the fraction corresponding to peak 2 showed 100% identity with a stretch of amino acids deduced for the human IFN-γ receptor. The sequences of the peptides in fractions corresponding to peaks 4 and 7 showed 60 and 40% identity, respectively, to the human receptor, whereas peptide from peak 3 showed no similarity. The sequences of these peptides and yield at each cycle are shown in Table I.

The undigested receptor was also subjected to automated Edman degradation to determine the NH₂-terminal amino acid sequence. The sequence obtained was found to match that determined for the peptide from peak 7, which was generated by endopeptidase digestion.

cDNA Cloning and Nucleotide Sequence Analysis—A mouse cDNA library consisting of approximately 6 x 10⁸ recombinant phage, constructed with poly(A)+ RNA from the cell line ABPL-2, was screened at low stringency of hybridization with a 24-base mixed-sequence oligodeoxynucleotide probe. The probe was constructed on the basis of the amino acid sequence of the peptide from peak 2 (see "Materials and Methods"). Seven positive signals were observed on autoradiographs of duplicate plaque lifts (~0.001% abundance). One of these clones, designated λMIR3b1, was found by nucleotide sequence analysis to contain a full-length cDNA. All of the peptide sequences determined for the mouse IFN-γ receptor were in complete agreement with the determined cDNA sequence, thereby verifying the reading frame of the full-length cDNA clone. The remaining clones contained the 3'-noncoding portion together with a truncated coding region. The structural map and sequencing strategy for mouse IFN-γ receptor cDNA are shown in Fig. 3. The cDNA was found to be unique, having no homology to entries in the GenBank other than that of the human IFN-γ receptor (22). A comparison of the nucleotide sequence of the full-length cDNA and deduced protein sequence for the mouse IFN-γ receptor with those of the human receptor is shown in Fig. 4.

The mouse IFN-γ receptor cDNA is comprised of 105 base pairs of the 5'-untranslated region, an open reading frame extending for 1431 base pairs, and a 3'-untranslated region

![Fig. 1. Silver staining and immunoreactivity of monoclonal antibodies with purified mouse IFN-γ receptor.](http://www.jbc.org/)

![Fig. 2. Profile of reverse-phase HPLC separation of lysine-specific endopeptidase cleavage products of mouse IFN-γ receptor.](http://www.jbc.org/)
of 539 base pairs followed by three bases of the poly(A) tail. The poly(A) tail was identified by comparison of the sequence with that of one of the truncated clones, which had 12 adenines at the 3′ end. The cDNA had 49% G + C content, with the 5′-untranslated, coding, and 3′-untranslated regions having 73, 49, and 42% G + C content, respectively. The first is surrounded by the sequence GCAGGAATGG, which matches 7 of 10 positions in the sequence believed to be the proposed start site for translation of the human receptor codons downstream from the first, AUG, which coincides with translationally optimal for translation initiation, GCC(A,G)CCAUGG (39). However, in accordance with the scanning model for translation initiation by the eucaryotic ribosome (39), very little, if any, protein product would be expected to originate from the second AUG. The mouse receptor mRNA has the termination codon UAA and contains a variant polyadenylation signal AGUAAA, located 12 bases upstream from the poly(A) tail, compared to the typical signal, AAUAAA (40).

The open reading frame in the full-length cDNA codes for a 477-amino acid protein having MW = 52,276. The discrepancy between the size of the cloned product and the apparent size of the purified receptor isolated from membranes is apparently the result of the known glycosylated nature of the receptor (25).2 The NH2 terminus of the mature receptor starts at Ala-26. The receptor is therefore synthesized as a preprotein from which a 25-amino acid signal peptide is cleaved. The mouse receptor contains two portions having considerable hydropathic character. One of these is located between residues 5 and 20 at the NH2 terminus, typical of a hydrophobic core of a signal sequence. The other is located at amino acids residues 254–275, suggestive of a transmembrane region.

The mouse and human receptor cDNAs share a number of common structural features. The two cDNAs show an overall similarity of 60%; there is 51% homology between the 5′-untranslated region of the human receptor cDNA and the corresponding portion of the mouse receptor cDNA, 66% homology between the coding regions and 48% homology between the 3′-untranslated regions. Both the mouse and human receptor cDNAs contain a 14-base oligo(dT) block in their 3′-noncoding regions, located at positions 1851-1864 and 1706-1719, respectively. Oligo(U)-rich regions in some mRNAs are apparently capable of binding to the poly(A) tail (41).

The amino acid sequences of the mouse and human receptors as deduced from their respective cDNAs show an overall similarity of 51%. The mouse receptor protein contains a nine-amino acid extension in the NH2-terminal signal sequence which is absent from the human receptor. Despite this extension, there is 88% identity in amino acid residues 286-311 to the corresponding portion of the human receptor (Fig. 4). This stretch also contains a sequence of 12 consecutive identical residues, the longest region of identity between the two molecules.

**Comparison of Mouse and Human IFN-γ Receptors**

![Diagram](http://example.com/diagram.png)

**In Vitro Transcription/Translation**—Capped RNA was synthesized in vitro from the T7 promoter contained on phagemid DNA derived from clone AMIR3b1. The T7 promoter in this phagemid is contained in the polylinker portion of the vector and is located upstream of the 5′ end of the mouse IFN-γ receptor cDNA insert. Prior to transcription, the phagemid was cleaved with HindIII, which cuts the template in the vector portion downstream of the poly(A) tail of the cDNA insert, producing a single 2.2-kilobase transcript (not shown). The length of this transcript is consistent with the distance between the T7 promoter and the site of scission of the phagemid. The RNA was translated in vitro, in the presence or absence of microsomal membranes, and the products were immunoprecipitated with the monoclonal antibody GR-20. The monoclonal antibody recognized a ~75-kDa in vitro translation product derived from lysates supplemented with microsomal membranes (Fig. 5, lane 3). The size of the product corresponded to the lower portion of the MW range of authentic glycosylated mouse IFN-γ receptor immunoprecipitated from EL-4 cells metabolically labeled with [35S]methionine (Fig. 5, lane 2). The monoclonal antibody was unable to recognize the translation product when synthesized in the absence of glycosylating activity (Fig. 5, lane 4).

**Expression of Mouse IFN-γ Receptor in Transfected COS-7 Cells**—For transfection experiments, a 2.2-kilobase PstI-Kpn1 segment from the phagemid, containing the full-length mouse IFN-γ receptor cDNA, was inserted into these same sites in the mammalian shuttle vector pCD-PS (42). Various amounts of DNA from the resulting construct, designated pCD-PS-MIR3b1, were transfected into COS-7 cells and the expression of authentic mouse IFN-γ binding activity was measured 72 h after transfection. Table II shows that specific binding increased with an increasing amount of transfected DNA. No binding was observed with nontransfected COS-7 cells or transfec-tants with the vector itself.

**Northern Blot Analysis**—Poly(A) mRNA was isolated from seven different cell lines known to exhibit varying levels of IFN-γ receptor on their cell surface. The RNAs were subjected to Northern blot analysis using the full-length cDNA as probe under conditions of high stringency of hybridization. As shown in Fig. 6, a single band corresponding to a 2.0-kilobase mRNA was observed in all cell lines tested. The levels of mRNA in the various cell lines was seen to vary strikingly,

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*F. Cofano, S. K. Mouse, S. Tanaka, N. Yuhki, S. Landolfo, and E. Appella, unpublished results.*

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**Fig. 3.** Structural organization and sequencing strategy of mouse IFN-γ receptor cDNA. Depicted is the insert from the clone AMIR3b1, a full-length cDNA clone isolated from a cDNA library constructed with RNA from the plasmacytoid lymphosarcoma cell line ABPL-2. The shaded area depicts the coding region. The double cross-hatched areas indicate the location of the NH2-terminal signal peptide and putative transmembrane regions. The direction and extent of sequences determined by the dideoxy method are shown by horizontal arrows. Open arrowsheads denote the location of restriction enzyme sites: B, BamHI; H, HindIII; and R, EcoRI. The scale at the bottom shows the length in kilobase pairs (kb).

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FIG. 4. Comparison of the nucleotide and deduced protein sequences of mouse and human IFN-γ receptor cDNAs. Alignment of the 5'-untranslated region of the mouse (M) cDNA with the 5' end of the cDNA sequence from human (H) is shown on the first two lines of the diagram. In the coding regions of the two cDNAs (lines 3–54), the comparison is shown in sets of four lines, the upper two lines being an alignment of the deduced protein sequences (single-letter code) and the lower two lines being an alignment of the cDNAs. Dots indicate identical residues. Dashes indicate gaps inserted to maximize alignment between the sequences. Numbers on the left indicate the position in the sequence of the first residue of the corresponding line. In the protein sequences, the NH2-terminal signal peptides and the putative transmembrane regions are underlined. Potential asparagine-linked glycosylation sites are underlined with a dashed line. In the cDNA sequences, the triplets for translation initiation and translation termination, as well as the putative polyadenylation signals, are underlined. Data for the human receptor sequence are from previously published works (22, 38).

Discussion

Previous studies have shown that the mouse IFN-γ receptor is a cell surface glycoprotein having a reported Mr of 85,000–95,000, which specifically binds IFN-γ with high affinity (23–25). A rat monoclonal antibody, GR-20, was developed previously against the mouse IFN-γ receptor which specifically immunoprecipitates the 85–95-kDa glycoprotein and inhibits IFN-γ binding to the purified receptor (25). We utilized this monoclonal antibody for large scale immunoaffinity purification of the mouse IFN-γ receptor enabling us to characterize the receptor at the molecular level.

Mouse IFN-γ Receptor Purification—Large scale purification of the mouse IFN-γ receptor was made feasible by using the thymoma cell line EL-4, known to express a high number of receptors/cell (16), and the zwitterionic detergent, CHAPS, for membrane solubilization. The binding activity of the correlation well with known levels of receptor expression (16, 17).
Comparison of Mouse and Human IFN-γ Receptors

In vitro synthesis of the mouse IFN-γ receptor. Phagemid DNA derived from clone XMIR3bl was linearized with HindIII and used as template to generate capped RNA transcripts of mouse IFN-γ receptor cDNA. Transcription was from the T7 promoter contained in the polylinker portion of the vector located upstream of the 5' end of the cDNA insert. RNA samples were translated in a reticulocyte lysate in the presence of [35S]methionine. Shown is an autoradiograph of a Western blot of proteins immunoprecipitated by monoclonal antibody GR-20 from CHAPS-solubilized cells labeled with [35S]methionine and from in vitro translation reactions. Separation was by 12.5% SDS-PAGE. Immunoprecipitates were from the following sources: Lane 1, solubilized CV-1 monkey cells; lane 2, solubilized EL-4 mouse cells; lane 3, in vitro translation products derived from recombinant mouse IFN-γ receptor transcripts in which the reticulocyte lysate was supplemented with canine pancreatic microsomal membranes; lane 4, as in lane 3 except that translation was performed in the absence of microsomal membranes.

FIG. 6. Northern blot analysis of mRNA from various cell lines. Poly(A)+ RNA was isolated from the following cell lines: Lane 1, EL-4; lane 2, ABPL-2; lane 3, ABPL-4; lane 4, SP2/0 p.87; lane 5, P 194; lane 6, BW 52; and lane 7, Meth A. Electrophoresis was carried out with 10 µg of each RNA in a 0.8% agarose-formaldehyde gel. 32P-Nick-translated mouse IFN-γ receptor cDNA insert was used as probe. Hybridization was at 65 °C under standard conditions using a final wash at 65 °C in 0.1 × SSC containing 0.1% SDS.

Table II
Expression of mouse IFN-γ receptor in transfected COS-7 cells

| DNA    | Specific binding of mouse [35S]-IFN-γ | Specific binding of mouse [35S]-IFN-γ |
|--------|-------------------------------------|-------------------------------------|
|        | Transfection A | Transfection B | Transfection A | Transfection B |
| pCD-PS-MIR3bl |                      |                      |                      |                      |
| 0      | 5 (895-890) | 0 (890-979) | 5 (895-890) | 0 (890-979) |
| 15     | 1033 (1963-930) | 987 (1809-872) | 1033 (1963-930) | 987 (1809-872) |
| 25     | 1118 (2034-917) | 1205 (2164-959) | 1118 (2034-917) | 1205 (2164-959) |
| pCD-PS (control) | 1413 (2391-976) | 1411 (2403-992) | 1413 (2391-976) | 1411 (2403-992) |

*Specific binding is expressed as the difference in counts/min between the total and the nonspecific binding of [35S]-IFN-γ to whole cells, the total and nonspecific binding being shown in parentheses. Nonspecific binding was the amount of [35S]-IFN-γ bound in the presence of a 100-fold excess of unlabeled IFN-γ.

Expression of mouse IFN-γ receptor in transfected COS-7 cells

A single-column immunoaffinity purification step was sufficient to obtain pure IFN-γ receptor. The purified receptor preparation consisted predominantly of the 85-kDa species. However, a minor component of about 200 kDa was occasionally detected by Western blotting. The 200-kDa component was detected with rabbit anti-rat antibodies alone and is most likely the result of the leaching of a small amount of monoclonal antibody from the affinity column. Also, a 60-kDa minor component was detected on silver-stained gels. This component is presumably a degradation product of the receptor since it increases upon prolonged storage of the receptor preparation.

Partial Amino Acid Sequence—The NH2 terminus and lysine-specific endopeptidase-generated peptides of the IFN-γ receptor were subjected to sequence analysis. All of the endopeptidase-generated peptides, which gave a single sequence, were in agreement with the protein sequence deduced from the cDNA clone, thereby providing additional evidence of the purity of the receptor preparation. One of these peptides showed 100% identity to a portion of the deduced amino acid sequence of the human IFN-γ receptor. This peptide was chosen to synthesize an oligodeoxynucleotide probe for screening a cDNA library. Despite the degeneracy of this
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probe, we found that it could be used successfully to detect IFN-γ receptor cDNA clones with no false signals.

Structural Features of the Receptor and Its cDNA—The cloned cDNA was observed to code for a 477-amino acid protein having M, = 52,276. The discrepancy between the size of the cloned product and the reported size of the receptor isolated from membranes is primarily the result of N-linked glycosylation, since incubation of the native receptor with N-glycosidase F decreases the apparent size by approximately 25 kDa (25, 38). The in vitro translation product derived from the cDNA clone was recognized by monoclonal antibody GR-20, but only when synthesized in reticulocyte lysates supplemented with microsomal membranes. The size of the recombinant receptor synthesized under such conditions is consistent with the presence of core glycosylation. Direct evidence that the cDNA codes for the mouse IFN-γ receptor was obtained by transfecting COS-7 monkey cells with a mammalian shuttle vector carrying the full-length cDNA under the direction of the SV40 promoter. The transfectants were observed to specifically bind radiolabeled mouse IFN-γ, whereas the host cells showed no binding activity.

The amino acid sequence of the mouse and human IFN-γ receptors as deduced from cDNA show considerable structural similarities (see Fig. 4). The two proteins show an overall similarity of 51% with some short stretches exhibiting complete homology. Differences between the two proteins were distributed throughout their entire lengths. Also, some stretches in the human receptor were missing in the mouse sequence. One striking observation concerning the mouse and human receptors is that both are rich in Ser residues with Ser and Thr residues in an environment in which basic residues are conserved. These observations suggest that the IFN-γ receptor is typical of transmembrane proteins which in general are characteristically oriented with the NH2-terminal portion being extracellular and the carboxyl-terminal portion being intracellular.

The mouse receptor sequence shows the presence of eight potential asparagine-linked glycosylation sites (Asn-X-Ser/Thr), five in the extracellular portion and three in the intracellular portion. Four of these potential sites are positionally conserved between the mouse and human receptors. There are also numerous potential sites for O-linked glycosylation considering that the receptors are rich in Ser and Thr residues.

Activation of protein kinase C by the addition of phorbol esters to mouse EL-4 cells is known to result in a down regulation of the IFN-γ receptor (43). Additional evidence for the involvement of protein kinase C in the cellular response to IFN-γ comes from the finding that addition of a phorbol ester to human macrophage-like cell lines stimulates the IFN-γ inducible gene γ1, whereas protein kinase C inhibitors suppress its induction as well as the induction of HLA-DR by IFN-γ (44). Thus, protein kinase C may directly phosphorylate the IFN-γ receptor. Protein kinase C phosphorylates Ser and Thr residues in an environment in which basic residues are thought to influence the kinetics and specificity of the enzyme (45). Examination of the mouse IFN-γ receptor sequence reveals a number of potential acceptor sites for this kinase. In fact, the intracellular portion of the receptor contains the majority of the Ser and Thr with adjacent basic residues. Furthermore, Ser residues in the mouse and human receptors are more highly conserved in the intracellular compared to the extracellular portions.

Expression of IFN-γ Receptor mRNA in Various Cell Lines—In general, the IFN-γ receptor is expressed on cell surfaces at a very low levels, only a few thousand molecules per cell (16, 18–20). Notable exceptions are certain tumor cell lines such as EL-4 and the ABPL clones, ABPL-2 and ABPL-4, which exhibit up to 50,000 receptors/cell (16, 18–20). We examined the expression of the mouse IFN-γ receptor mRNA in tumors (all lines of B cell (ABPL-2 and ABPL-4, SP2/0 P81, P194), T cell (EL-4, BW-52), and fibroblastic (Meth A) origins. Extremely high levels of receptor mRNA were found in the cell lines EL-4, ABPL-2 and ABPL-4. In contrast, only minimal levels of receptor mRNA were seen in cell lines SP2/0, P194, BW 52, and Meth A. These observations demonstrate that the neoplastic state itself is not responsible for abnormally high expression of the IFN-γ receptor and its mRNA. Nor is there a correlation between the degree of biological response to IFN-γ and the level of the receptor on the cell surface (16) or the level of its mRNA. In the case of ABPL-2 and ABPL-4, the c-myc proto-oncogene transcript is also known to be highly expressed (17, 46, 47). Both the IFN-γ receptor and c-myc have been mapped to chromosome 10 (17, 48), although it is not known whether or not the two genes are closely linked. The activation of c-myc occurs as a consequence of the insertion of a defective Moloney leukemia virus into an intron of c-myc (46, 47). Viral integration therefore could be involved in enhanced expression of IFN-γ receptor in these cell lines. Alternatively, c-myc could be acting as a trans-activating factor (49), the level of which modulates IFN-γ receptor transcription.

Previously, mouse cells transfected with the human receptor gene were shown to bind to human IFN-γ, although in such a heterologous system, they lacked the capacity to elicit the characteristic cellular response (9). This suggests that additional components besides the receptor will need to be identified in order to elucidate the process of signal transduction. This possibility is currently being explored using the mouse system.

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Addendum—Data similar to that contained herein have been recently presented by Kumar et al. (1989) J. Biol. Chem. 264, 17939–17946 and Gray et al. (1989) Proc. Natl. Acad. Sci. USA 86, 8497–8501 except that the sequence of Gray et al. has Glu in place of Gly at amino acid position 95.

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