Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 15623–15628, December 1998
Medical Sciences

Identification of genes differentially regulated by interferon α, β, or γ using oligonucleotide arrays

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Communicated by George R. Stark, Cleveland Clinic Foundation, Cleveland, OH, October 14, 1998 (received for review August 12, 1998)

ABSTRACT **The pleiotropic activities of interferons (IFNs) are mediated primarily through the transcriptional regulation of many downstream effector genes. The mRNA profiles from IFN-α, -β, or -γ treatments of the human fibrosarcoma cell line, HT1080, were determined by using oligonucleotide arrays with probe sets corresponding to more than 6,800 human genes. Among these were transcripts for known IFN-stimulated genes (ISGs), the expression of which were consistent with previous studies in which the particular ISG was characterized as responsive to either Type I (α, β) or Type II (γ) IFNs, or both. Importantly, many novel IFN-stimulated genes were identified that were diverse in their known biological functions. For instance, several novel ISGs were identified that are implicated in apoptosis (including RAP46/Bag-1, phospholipid scramblase, and hypoxia inducible factor-1α). Furthermore, several IFN-repressed genes also were identified. These results demonstrate the usefulness of oligonucleotide arrays in monitoring mammalian gene expression on a broad and unprecedented scale. In particular, these findings provide insights into the basic mechanisms of IFN actions and ultimately may contribute to better therapeutic uses for IFNs.**

Interferons (IFNs) are a family of related cytokines that mediate a range of diverse functions including antiviral, antiproliferative, antitumor, and immunomodulatory activities (1). While considerable efforts have been devoted to exploit the therapeutic potential of IFNs (2), the IFN system has also served as a model system for studying mammalian signal transduction and transcriptional regulation (3). IFNs bind to their cognate receptors and initiate a signaling cascade, involving the JAK family of tyrosine kinases and STAT family of transcription factors, that leads to the transcriptional induction of the IFN-stimulated genes (ISGs). IFN actions are largely mediated by the proteins encoded by ISGs, the best studied of which include the double-stranded RNA-activated protein kinase (PKR), the 2′-5′ oligoadenylate (2–5A) synthetases, and the Mx proteins (1). The other well characterized category of ISGs includes the signal transducer and activator of transcription (STAT) and IFN regulatory factor (IRF) families of transcription factors, which are involved in the regulation of both ISG and IFN gene expression (1, 3, 4). However, the biological functions for many ISGs, including 6–16, 9–27, and the ISG-54 gene family, remain unclear despite, in some cases, extensive investigation of the 5′ regulatory regions of those genes (5–7).

Recently, oligonucleotide arrays have been used to study the differential expression of mRNAs for cytokine genes in murine T cells after activation, and for the entire complement of more than 6,200 genes from the yeast genome, in yeast cells grown in minimal or rich media, or at different points during cell cycle progression (8–10). Monitoring of gene expression by this method is based on hybridization of labeled RNA populations to high-density arrays of oligonucleotides, synthesized on a glass substrate by a combination of photolithography and conventional oligonucleotide chemistry (11). To identify novel ISGs and obtain a comprehensive profile of differential ISG expression by distinct IFNs, oligonucleotide arrays containing probe sets for more than 6,800 human genes were used to assay for changes in mRNA expression after stimulation of the human fibrosarcoma cell line, HT1080, with either IFN-α, -β, or -γ. Several hundred genes were determined to be IFN-regulated including many of which represented novel ISGs. Significantly, the identification of novel ISGs with known biological functions provides informative insights into the mechanisms of IFN action. In addition, our data from measuring IFN responses indicate the usefulness of applying oligonucleotide arrays for determining global gene profiles in response to other cytokines and extracellular stimuli.

MATERIALS AND METHODS

Cell Culture and RNA Isolation. HT1080 cells were cultured using DMEM supplemented with fetal bovine serum (10%) and antibiotics. Cells (10^7) were plated on 15-cm tissue culture plates and cultured overnight before being treated with 1,000 international units (IU)/ml of each IFN, IFN-α (IFN-alpha2a, Roche, specific activity, 2.7×10^8 IU/mg), IFN-β (IFN-beta, Berlex, specific activity, 2×10^8 IU/mg), or IFN-γ (IFN-gamma, GIBCO/BRL, specific activity, 10^7 IU/mg). After treatment with the IFNs for 6 hr, total RNA was isolated (Trizol, GIBCO/BRL).

Preparation of cRNA. The methods for preparation of cRNA and subsequent steps leading to hybridization and scanning of the Hu6800 GeneChip Arrays were provided by the manufacturer (Affymetrix, Santa Clara, CA). Briefly, poly(A)$^+$ RNA was isolated from 100 μg total RNA of each sample with Oligotex (Qiagen) and converted into double-stranded cDNA using a cDNA synthesis kit (SuperScript Choice, GIBCO/BRL) with a special oligo(dT)$_{24}$ primer containing a T7 RNA polymerase promoter site added 3′ of the poly T tract (Genset). After second-strand synthesis, labeled cRNA was generated from the cDNA sample by an *in vitro* transcription (IVT) reaction (T7 MegaScript System, Ambion) supplemented with biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics). The labeled cRNA was purified by using RNeasy spin columns (Qiagen). Fifty micrograms of each cRNA sample was fragmented by mild alkaline treatment, at 94°C for 35 min in fragmentation buffer (40 mM Tris-acetate, pH 8.1/100 mM potassium acetate/30 mM magnesium acetate) and then used to prepare 1 ml of master hybridization mix [0.1 mg/ml of herring sperm DNA (Sigma)/1 M sodium chloride/10 mM Tris, pH 7.6/0.005% Triton X-100). A mixture of four control cRNAs for bacterial and phage genes was included in the mix (BioB, BioC, BioD, and cre, at 1.5, 5, 25, and 100 pM, respectively; referred to as "staggered spikes") to serve as

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Abbreviations: IFN, interferon; 2–5A, 2′-5′ oligoadenylate; PKR, double-stranded RNA-activated protein kinase; STAT, signal transducer and activator of transcription; IRF, IFN regulatory factor; VEGF, vascular endothelial growth factor; VRP, VEGF-related protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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tools for comparing hybridization efficiency between arrays and for relative quantitation of measured transcript levels. A biotinylated oligonucleotide, B2, also was added that hybridizes to unique features at the center and four corners of each chip to facilitate accurate orientation and mapping of the probe sets.

Oligonucleotide Array Hybridization and Scanning. Before hybridization, the cRNA samples were heated to 94°C for 5 min, equilibrated to 40°C for 5 min, and clarified by centrifugation (14,000 × *g*) at room temperature for 5 min. Aliquots of each sample (10 μg of cRNA in 200 μl of the master mix) were hybridized to Hu6800 GeneChip arrays at 40°C for 16 hr in a rotisserie oven set at 60 rpm. After this, the arrays were washed with 6× SSPE and 0.5× SSPE, stained with streptavidin-phycoerythrin (Molecular Probes), washed again, and read by using a confocal microscope scanner with the 560-nm long-pass filter (Molecular Dynamics; Affymetrix).

Data Analysis. Data analysis was performed by using GENECHIP 3.0 software. Initial absolute analysis for gene expression was performed without scaling while subsequent comparison analysis files were created by scaling the six probe sets for 5′, middle (M), and 3′ of actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in all data sets to a uniform value, 50,000, and normalizing to all genes.

RESULTS

To identify IFN type-specific changes in gene expression, we prepared biotin-labeled cRNA samples from untreated HT1080 cells and cells stimulated for 6 hr with IFN-α, -β, or -γ (1,000 IU/ml each). The Hu6800 GENECHIP array represents a set of four individual chips, A–D, each containing unique probe sets to more than 1,700 genes, collectively representing more than 6,800 human genes. For each gene, there are 20 oligonucleotide probes to different sequences in the mRNA and 20 corresponding single, mismatch control oligonucleotides (Fig. 1). Analysis of the HT1080 samples was performed by hybridizing aliquots of cRNA (10 μg each) simultaneously to the GENECHIP arrays, A–D. Thus, a total of 16 distinct array hybridizations were performed to complete the analysis of the four cRNA samples. After hybridization, staining, and scanning of the arrays, the data were analyzed using GENECHIP 3.0 software.

To assess variability in array hybridization and cDNA synthesis efficiency between samples, two types of hybridization controls were examined. Hybridization efficiency between arrays was assessed by comparing the signal intensities for the four control bacterial and phage gene cRNA "spikes," BioB, BioC, BioD, and cre, which had been added to the hybridization mixtures at known concentrations (1.5, 5, 25, and 100 pM, respectively). Among the 16 total arrays screened, BioB usually was undetectable, while BioC, BioD, and cre were detected as present in all data sets. This

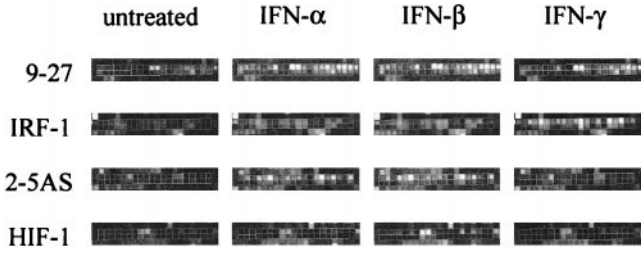

FIG. 1. Genes up-regulated by IFNs. The probe sets from hybridized oligonucleotide arrays corresponding to the indicated IFN-induced genes are represented. 9–27 is an example of a gene that is regulated by all IFNs. IRF-1 is preferentially induced by IFN-γ. 2–5A-Synthetase (1.8 kb mRNA; 2–5AS) is induced selectively by IFN-α and -β, but not IFN-γ. HIF-1α is selectively induced by IFN-β. Perfect-match oligonucleotides are tiled across the upper rows of each boxed probe set while corresponding single-base mismatch oligonucleotides are tiled across the lower rows.

established that, under these hybridization conditions, the lower detection limit was in the range of 1.5–5 pM. Each array also contains distinct probe sets corresponding to the 5′, M, and 3′ regions for both actin and GAPDH. The cumulative hybridization intensities for either of these gene transcripts were used to provide normalization between different RNA samples, as is commonly done in conventional mRNA detection methods. Furthermore, the efficiency of full-length cDNA synthesis for a given cRNA sample can be assessed by comparing the respective 3′ signal intensities to that of the upstream 5′ and middle regions. Since each sample was hybridized to four separate DNA arrays, it was essential to determine the consistency among arrays by calculating the average intensities for all of the actin and GAPDH probe sets among the data sets (Table 1). Importantly, we observed that the average hybridization signals for each of the six different actin and GAPDH probe sets differed by less than 20% among the four samples. To compare the efficiency of cDNA synthesis between samples, the ratios for the 5′ and middle intensities relative to the 3′ intensities were calculated for actin and GAPDH. Among the four samples, the intensities for 5′-GAPDH signals were 81–86% of the 3′-GAPDH intensities, while M-GAPDH signals were 101–110% of the 3′-GAPDH values; the 5′- and M-actin intensities relative to that of the 3′ values were 130–146% and 146–157%, respectively. The higher signal intensities for the M- and 5′-GAPDH regions as compared with the 3′ region are reflective of the intrinsic hybridization characteristics of these probes sets (C. Harrington, personal communication). Also, a high degree of similarity was observed in the overall patterns of 5′, M, and 3′ intensities between samples. Taken together, these data indicate that a high level of both efficiency and consistency was achieved in the cDNA and cRNA synthesis for these four samples.

We next determined which gene transcripts were detectable and their respective signal intensities. The total number of mRNAs detected as present among the four samples was similar: untreated, 2,207; IFN-α, 2,225; IFN-β, 2,403; and IFN-γ, 2,320. The intensities for gene transcripts detected as present represented a range spanning 3 orders of magnitude, from approximately 20 to the highest recorded value of 18,524 for metallothionein (data not shown). The majority of these mRNAs, approximately 85–90%, were detected with intensities of less than 1,000. The genes associated with intensities greater than 1,000 (≈300 genes) include many of the ribosomal genes and commonly known, high-abundance genes such as tubulin, elongation factor 1-α, and ubiquitin. It is interesting to note that among this set of highly abundant mRNAs, stimulation by any of the three IFNs did not cause dramatic changes in their expression levels, with any detectable changes being less than 2-fold (data not shown). The singular exception was β2 microglobulin, a known ISG (12), with a basal expression level of 1,111 that was increased 1.7-, 2.1-, and 1.3-fold, respectively, by IFN-α, -β, and -γ (Table 2).

To identify IFN-regulated genes, pairwise comparisons were generated between the data sets from each of the IFN-treated samples and the untreated HT1080 cell data set. More than

Table 1. Average intensity values for actin and GAPDH

Sample	5′	M	3′
	Actin		
1 (Untreated)	5,662 (1.46)	6,089 (1.57)	3,875 (1)
2 (IFN-α)	7,195 (1.30)	8,073 (1.46)	5,532 (1)
3 (IFN-β)	7,311 (1.33)	8,167 (1.48)	5,484 (1)
4 (IFN-γ)	7,860 (1.49)	8,254 (1.56)	5,281 (1)
	GAPDH		
1 (Untreated)	6,314 (0.82)	8,493 (1.10)	7,707 (1)
2 (IFN-α)	8,107 (0.86)	10,230 (1.09)	9,417 (1)
3 (IFN-β)	6,949 (0.81)	8,635 (1.01)	8,542 (1)
4 (IFN-γ)	8,101 (0.85)	10,319 (1.09)	9,441 (1)

The average intensity values for the probe sets indicated were calculated from the respective values obtained by hybridization of each sample to the four arrays that comprise a Hu6800 GENECHIP set. The ratios of the 5′ and middle intensities relative to 3′ intensities are indicated in parentheses.

Table 2. Genes up-regulated by IFNs

GenBank accession no.	Known ISG	Gene description	Untreated, intensity		IFN-α Intensity	IFN-α Fold increase	IFN-β Intensity	IFN-β Fold increase	IFN-γ Intensity	IFN-γ Fold increase
M24594	+	ISG-56K	16	*	588	>29.4	2,492	>124.6	15	0
X02875	+	2-5A synthetase (1.8 kb RNA)	18	*	584	>29.2	505	>25.2	24	0
X57522	+	RING4	29	*	800	28	602	21	1,407	49.2
J04164	+	9-27	62	*	1,431	23.2	1,349	21.9	504	8.2
M33882	+	MxA	19	*	426	>21.3	625	>31.3	25	0
M13755	+	IFN-induced 17/15-kDa protein	148		3,054	20.7	2,409	16.3	268	1.8
U22970	+	6-16	−12	*	405	>20.2	433	>21.7	−7	0
U52513	+	RIG-G	19	*	358	>17.9	583	>29.2	63	>3.1
M62800		52-kD SS-A/Ro autoantigen	−34	*	204	>10.2	131	>6.6	126	>6.3
M876503	+	p48/ISGF3γ	50	*	499	10.1	478	9.6	392	7.9
U72882	+	IFP35	55		460	8.4	395	7.2	258	4.7
Z14982	+	LMP7	47	*	389	8.3	342	7.3	535	11.4
AF008445		Phospholipid scramblase	72		594	8.2	723	10	208	2.9
X02874	+	2-5A synthetase (1.6 kb RNA)	33	*	262	8	217	6.6	43	0
L22342	+	IFN-induced nuclear phosphoprotein	4	*	156	>7.8	199	>10.0	119	>6.0
M30818	+	MxB	3	*	114	>5.7	254	>12.7	36	0
M97936	+	STAT1 (84 kDa)	−31	*	111	>5.6	224	>11.2	40	>2.0
U43142		VEGF-C/VRP	37	*	198	5.3	150	0	264	7.1
M87434	+	2-5A synthetase (71 kDa)	10	*	104	>5.2	181	>9.1	25	0
Z35491		RAP46/Bag-1	−2	*	99	>5.0	134	>6.7	86	>4.3
X74262		RbAp48	24	*	119	5	175	7.3	100	4.2
D49824		Human HLA-B null allele	79		395	5	527	6.7	360	4.6
M63838	+	IFI 16	8	*	98	>4.9	254	>12.7	67	>3.3
M97935	+	STAT1 (91 kDa)	52		258	4.9	446	8.5	155	3
D50919		KIAA0129	33		155	4.7	198	6	76	0
M92642		Alpha-1 type XVI collagen	43	*	179	4.2	153	0	190	4.4
M55542	+	GBP-2	12	*	82	>4.1	175	>8.7	147	>7.3
X14454	+	IRF-1	22	*	88	4	117	5.3	682	31.1
M79463	+	PML-2	70		280	4	279	4	250	3.6
X82200	+	Staf50 mRNA	46		177	3.9	274	6	55	0
X66401	+	LMP2	108		388	3.6	250	2.3	581	5.4
D28137		BST-2	22	*	86	3.9	100	4.5	32	1.4
X90846		Mixed lineage kinase 2	17	*	71	>3.6	95	>4.8	95	>4.8
X04602		Interleukin BSF-2	21	*	76	3.6	65	3.1	65	3
U59321		DEAD-box protein p72	−37	*	70	>3.5	32	>1.6	−32	0
U04285		Lysosomal acid lipase (LIPA)	17	*	69	>3.5	81	>4.0	31	0
M79462	+	PML-1	35	*	121	3.5	134	0	124	3.6
M24470		Glucose-6-P dehydrogenase	18	*	68	>3.4	71	0	45	0
U89606		Pyridoxal kinase	28	*	89	3.2	168	6	93	0
U83463		Scaffold protein Pbp1	13	*	64	>3.2	64	0	37	>1.9
J04080	+	Complement component C1r	11	*	62	>3.1	65	>3.3	103	>5.2
U32849		Hou	18	*	59	>3.0	92	>4.6	49	>2.4
M20022	+	MHC Class I	151		445	3	536	3.6	912	6.1
X58536	+	MHC Class I	120		337	2.8	363	3	276	2.3
D28915		Hepatitis C-associated p44	27	*	74	2.8	128	4.8	26	0
L40387		TRIP14	117	*	318	2.7	416	3.5	110	0
M22877		Somatic cytochrome c	93		253	2.7	333	3.6	163	1.7
U09825		Acid finger protein	76	*	200	2.6	214	2.8	153	2
X15949	+	IRF-2	5	*	50	>2.5	77	>3.9	42	0
U41515		DSS1	67		168	2.5	201	3	117	1.8
X83492	+	Fas/Apo-1	6	*	48	>2.4	51	0	52	>2.6
L14778		PPP3CA	7	*	47	>2.4	76	−3.8	26	>1.3
X61123		BTG1	80		192	2.4	158	2	183	2.3
J04611		Lupus p70 (Ku) autoantigen	524		1,250	2.4	1,253	2.4	1,025	2
X75755		PR264	150		364	2.4	342	2.3	326	2.2
D89052		Proton-ATPase-like protein	408		912	2.2	720	1.8	1,005	2.5
U84573		PLOD2	58		28	2.1	162	2.8	23	2.5
U10439	+	dsRNA adenosine deaminase	152		324	2.1	450	3	304	2
Z47087		Pol II elongation factor-like protein	134		263	2	201	1.5	238	1.8
M94556		Mitochondrial SSB	309		542	1.8	619	2	581	1.9
D32129	+	HLA class-I (HLA-A26) heavy chain	252		449	1.8	461	1.8	518	2.1
J00105	+	β-2 microglobulin	1,019		1,700	1.7	2,150	2.1	1,296	1.3
L25081		GTPase (rhoC)	432		696	1.6	652	1.5	912	2.1
U20998		SRP9	105		157	1.5	277	2.6	137	1.3
X95648		eIF-2B α subunit	90		138	1.5	167	1.9	196	2.2
U07802		Tis11d	10	*	28	>1.4	39	>2.0	30	>1.5
U49837		LIM protein MLP	−16	*	26	>1.3	50	>2.5	24	>1.2
X16707		Fra-1	18	*	44	0	214	>10.7	254	>12.7
M21388		Unproductively rearranged IgM	−7		180	0	193	>9.7	251	>12.5
D38293		Clathrin-like protein	28	*	27	0	246	8.9	15	0
Y09836		3′ UTR of unknown protein	11	*	−19	0	150	>7.5	35	0
U22431		Hypoxia-inducible factor-1	29	*	33	0	204	7.1	50	0
M35663	+	Pkr	39	*	68	0	271	7	33	0
X89416		Protein phosphatase 5	49		103	0	305	6.2	160	3.3
U88047		DRX	−40	*	77	0	107	>5.4	106	0
Z50194		PQ-rich protein	46		103	0	245	5.3	210	4.5
U41766		MDC9	25	*	30	0	135	5.3	19	0
U79291		Clone 23721	16	*	−2	0	97	>4.9	20	0
U58046		p167	21		17	0	100	4.7	14	0
D79999		KIAA0177	32	*	21	0	140	4.4	43	0
U41387		Gu protein	53		56	0	226	4.3	57	0

IFN-regulated genes identified by GENECHIP array analysis were divided into three subgroups. The first subgroup was organized in decreasing order on the basis of fold increase in response to IFN-α, while the second and third were ordered similarly by decreasing fold increase in response to IFN-β and IFN-γ, respectively. A plus sign in the "Known ISG" column indicates that previous studies had identified the given gene as being regulated by IFNs. An asterisk beside an intensity value indicates that the given gene transcript was determined by the GENECHIP software as nondetectable in that particular sample. A "greater than" sign (>) beside a fold increase value for a given gene indicates that this likely represents an underestimation since expression of the gene was determined as nondetectable in the untreated sample, and, consequently, the GENECHIP software used a minimal value of 20 as a divisor to calculate the fold change for the IFN-treated sample. MHC, major histocompatibility complex; dsRNA, double-stranded RNA.

Table 2. Continued

GenBank accession no.	Known ISG	Gene description	Untreated, intensity		IFN-α		IFN-β		IFN-γ	
					Intensity	Fold increase	Intensity	Fold increase	Intensity	Fold increase
X63563		RNA polymerase II, 140 kDa	56		105	1.9	230	4.1	88	0
X70649		Clone 1042 of DEAD box family	49	*	81	0	199	4.1	50	0
D29640		KIAA0051	26		32	0	103	4	37	0
Z29064		AF-1p	23	*	54	2.4	88	3.9	42	1.8
D79986		KIAA0164	4	*	30	0	76	>3.8	19	0
U44378		DPC4	15	*	34	>1.7	77	>3.8	16	0
J03473		Poly (ADP-ribose) polymerase	61		130	0	224	3.7	146	2.4
D90070		PMA-responsive gene (APR)	4	*	28	>1.4	69	>3.5	42	>2.1
D63875		KIAA0155	14		13	0	70	>3.5	26	0
M85164		SAP-1	11	*	21	0	67	>3.4	32	>1.6
U34605	+	ISG-58K	8	*	15	0	66	>3.3	20	0
D14043		MGC-24	55		94	1.7	182	3.3	33	0
J04102		Ets-2	20	*	14	0	67	3.3	34	0
D16481		Mitochondrial 3-ketoacyl-CoA thiolase	45	*	86	0	147	3.2	103	2.3
Z69915		Clone ICRFp507L1876	23	*	28	0	73	3.2	28	0
X98260		M-phase phosphoprotein, mpp11	−9	*	7	0	61	>3.1	−6	0
M14660	+	ISG-54K	−62	*	−5	>1.0	61	>3.1	−4	>1.0
M82882		Human cis-acting sequence	2	*	21	0	63	>3.1	9	0
U05040		FUSE binding protein	60	*	84	0	189	3.1	63	0
M28249		Collagen receptor α-2 subunit	31	*	28	0	97	3.1	22	0
X74039		uPA receptor	22	*	52	0	68	3.1	49	0
M83216		Caldesmon	3	*	9	0	62	>3.1	5	0
AB001106		Glia maturation factor	6	*	14	0	61	>3.0	21	0
U19252		Putative transmembrane protein	11	*	24	0	59	>3.0	30	0
M63896		TEF1	15	*	22	0	59	>3.0	9	0
X54326		Glutaminyl-tRNA synthetase	49	*	39	0	150	3	44	0
J03909	+	IP-30	4	*	44	>2.2	35	>1.8	239	>12.0
U82987		Bcl-2 binding component 3 (bbc3)	−27	*	46	>2.3	8	0	102	>5.1
M83667		NF-IL6-beta	24		30	0	59	2.5	108	4.5
M24283	+	ICAM-1	−8	*	−4	0	−15	0	86	>4.3
U63824		RTEF-1	40	*	87	2.2	64	0	162	4
M21533	+	MHC Class I	239		437	1.8	517	2.2	938	3.9
X96752		L-3-hydroxyacyl-CoA dehydrogenase	17	*	32	0	50	>2.5	77	>3.8
U31628		IL15RA	36	*	87	0	111	0	135	3.8
X85237		Splicing factor SF3a120	52	*	49	0	101	0	192	3.7
X98172		MACH-1	23	*	53	2.3	65	2.8	74	3.2
X71874		MECL-1	184		312	1.7	292	1.6	563	3.1
X84213	+	BAK	57		78	0	82	0	190	3.3
U18009		Human 17q21 clone LF113	30	*	64	0	52	0	94	3.1
U65011		PRAME	37	*	72	0	67	0	111	3
L07633	+	IFN-γ-inducible gene, I-5111	250		478	1.9	411	1.6	714	2.9

1,000 genes were identified whose mRNA expression levels changed after IFN stimulation. The majority of these (>75%) represented genes for which the degree of change was less than 2-fold. The number of genes whose expression changed more than 10-, 4-, or 2-fold was 10, 26, and 94 for IFN-α; 14, 56, and 268 for IFN-β; and 4, 25, and 129 for IFN-γ, respectively.

To select the genes most likely to represent bona fide IFN-regulated genes, we empirically tested different criteria to make further selections from the list of more than 1,000 differentially expressed genes. By using known ISGs as "guideposts," we determined reasonable but conservative delineations for fold-change values. After applying a given criterion to yield a shortened list of genes, its effectiveness was assessed by the number of known ISGs remaining and, in particular, how many remained near the bottom of the list. Three criteria ultimately were applied to generate a list of 122 genes (Table 2): (*i*) gene transcripts with at least 4-fold increased expression by any IFN type, (*ii*) gene transcripts with at least 3-fold increase by any IFN type and detected as absent in untreated HT1080 cells, and (*iii*) gene transcripts with at least 2-fold increase in response to all three types of IFNs. These genes were then ordered in three subgroups by first listing genes on the basis of highest fold change in response to IFN-α, followed by responses to IFN-β and, finally, to IFN-γ. A useful aspect of the GENECHIP software allows the special identification of genes whose expression was nondetectable in the untreated or baseline sample, but detectable in the IFN-treated sample. In these cases, the fold change is calculated by dividing the intensity value for the gene from the IFN-treated data set by a minimum value of 20 (which approximates the lower limit of detection), and, thus, the calculated fold change most likely represents a underestimation of the true fold change. The genes falling into this category are identified in Table 2 with a "greater than" symbol (>) preceding the fold-change value.

Among the 122 listed genes in Table 2, at least 40 genes represent known ISGs as indicated in previous published studies while the remaining 82 represent novel IFN-regulated genes. The characteristic induction profiles for these genes allow for them to be tentatively categorized as (*i*) genes induced by all three types of IFNs, (*ii*) genes induced by IFN-α or IFN-β, (*iii*) genes preferentially induced by IFN-β, and (*iv*) genes preferentially induced by IFN-γ; 9–27, 2–5A synthetase, hypoxia inducible factor (HIF)-1α, and IRF-1 represent examples for each of categories, respectively (Fig. 1 and Table 2). There were no convincing examples of genes preferentially induced at 6 hr by IFN-α but not by IFN-β. While there are examples of genes listed in Table 2 with significant fold increases in response to IFN-α, but with 0 representing the fold increase by IFN-β, the respective IFN-induced intensity values are, in fact, similar. As examples, the respective IFN-α- and IFN-β-treated intensities for α-1 type XVI collagen are 220 and 184, and for PML-1 are 84 and 88, despite a 0-fold increase by IFN-β displayed for both genes. When analyzing two data sets, the GENECHIP software uses a decision matrix that includes other parameters in addition to the net change in intensity values to decide whether a given gene is differentially expressed. Consequently, once a decision is made that there was no significant difference in expression, "0" is automatically entered as the fold-change value for that gene regardless of the actual ratio between the two intensity values. Therefore, in the overall analysis, it is important to consider actual intensity values in addition to the fold change. Furthermore, intensity values of less than 50 with increases of no greater than about 100 are less reliable than higher intensity values and should be regarded cautiously as possible indications of gene induction. Similar interpretations apply to down-regulated genes

with intensity values of less than about 100 before IFN treatments. In this regard, a group of genes exhibited decreased mRNA levels after IFN treatment. By applying two criteria, any genes with at least a 3-fold decrease by any IFN and genes with at least a 2-fold decrease by all 3 IFNs, 21 genes were identified as IRGs (Table 3).

DISCUSSION

We have used oligonucleotide arrays to study the changes in mRNA expression after stimulation of the human HT1080 cell line with different IFNs. The effectiveness of this approach was evident by the ability to successfully identify and quantify the mRNA levels for many known ISGs. Previous Northern blot analyses of HT1080 cells showed that while IFN-α induced 6–16 mRNA levels by more than 20-fold, there was no detectable induction by IFN-γ; also, 9–27 mRNA levels were induced by both IFN-α and IFN-γ, although the level of 9–27 induction by IFN-α was 3-fold higher than by IFN-γ (5). These characteristics were replicated with remarkable similarity by the oligonucleotide arrays: 6–16 was absent in untreated cells and induced at least 20- and 21-fold by IFN-α or IFN-β, respectively, but not by IFN-γ; 9–27 was induced 23- and 22-fold by IFN-α or IFN-β and 8-fold by IFN-γ (Fig. 1 and Table 2). The consistency between our data and previous studies regarding IFN-specific inducibility further extends to several other genes (Table 2). ISGs that are preferentially induced by IFN-α but not IFN-γ include the 2′-5′ oligoadenylate synthetase family of genes, MxA and MxB genes, and the gene family comprising ISG54, ISG56, ISG58, and RIG-G. The differential induction of 2–5A synthetase mRNA by IFN-α and IFN-γ was confirmed in the RNA samples by Northern blot analysis before preparing cRNA for this analysis (data not shown). ISGs preferentially induced by IFN-γ include IRF-1, IP-30, and CIITA (data not shown). ISGs that are responsive to all IFNs tested include major histocompatibility complex Class I genes STAT1 and GBP. It is noteworthy that although the mRNA expression profiles for many of the above genes were established in cell lines with differing backgrounds, including HeLa, Daudi, and NB4 cells, their IFN-specific induction characteristics were similar in HT1080 cells.

Using known ISGs as guideposts, we determined that it was reasonable to attribute greater significance toward genes whose expression levels changed from nondetectable in untreated cells to detectable in IFN-treated cells. Thus, the three criteria we applied to select the 122 IFN-regulated genes described in Table 2 represent conservative cutoffs. Known ISGs also were among the hundreds of genes that were detected originally as differentially expressed but excluded by our cutoff criteria, and, therefore, by inference, other novel ISGs were also likely represented. It is likely that the mRNA levels for certain ISGs simply do not change by more than 3-fold at the 6-hr time point we used to prepare cRNA. Consequently, further analysis of candidates from the comprehensive list of differentially expressed genes may yet reveal additional genuine ISGs (the comprehensive data set is available at our web site, www.lri.ccf.org/ri/pi/williams.html).

We anticipated that identification of genes with previously known biological properties may facilitate reasonable inferences as to their potential roles as mediators of known IFN activities or indicate new areas of IFN action. Given the role of apoptosis as an antiviral mechanism, we were intrigued to note the identification of apoptosis regulators as novel ISGs. Phospholipid scramblase was among the most strongly inducible of novel ISGs identified, with expression levels increased 8- and 10-fold by IFN-α and IFN-β, respectively, and a more modest 3-fold increase by IFN-γ (Table 2). This enzyme has an important role in the apoptosis program by flipping phosphatidylserine from the inner to the outer leaf of the cell membrane (13). Exposed phosphatidylserine serves as a surface marker for recruited phagocytes to recognize and eliminate apoptotic bodies. Therefore, increased levels of phospholipid scramblase in response to IFN may serve as a mechanism that enables more efficient removal of virus-infected cells undergoing apoptosis. The proapoptotic gene for HIF-1α also was identified as a novel ISG (see below) (14). We also have identified two antiapoptotic IFN-regulated genes. Expression of RAP46, the human homologue for the mouse gene, BAG-1 (15, 16), was consistently induced about 3-fold by all IFNs (Table 2). In addition, Bcl-2 expression was modestly increased by IFN-α or IFN-β (data not shown). In the context of a viral infection, induction of antiapoptotic genes by IFNs may serve to protect cells from immediate virus-mediated cytopathic effects, thus allowing time for directed cellular antiviral activities to combat the infection. The notion that IFNs can also regulate antiapoptotic genes provides a satisfying balance to the characterization to date of ISGs having primarily proapoptotic functions, a list that includes PKR (17), 2–5A-dependent ribonuclease (RNaseL) (18, 19), IRF-1 (20, 21), and STAT1 (22, 23).

Crosstalk between the IFN system and other signaling pathways are suggested by novel ISGs such as vascular endothelial growth factor (VEGF)-related protein (VRP), which was induced by all three IFNs. VRP, also known as VEGF-C, is the unique ligand for the endothelial cell-specific tyrosine kinase receptor, Flt4 (24). Stimulation with VRP promotes proliferation in lung and bone marrow endothelial cells and leads to activation of downstream signaling events involving JNK and RAFTK. IFN-α has been studied both as an antitumor agent and as an angiogenic inhibitor (2), including the treatment of hemangiomas

Table 3. Genes down-regulated by IFNs

GenBank accession no.	Gene description	Untreated Intensity	IFN-α Intensity	IFN-α Fold decrease	IFN-β Intensity	IFN-β Fold decrease	IFN-γ Intensity	IFN-γ Fold decrease
L77701	COX17	107	17	>5.3	60	0	28	3.8
D86971	KIAA0217	74	14	>3.7	63	0	54	1.4
U07000	BCR	77	24	3.2	30	0	21	3.7
M16706	Histone H4	62	8	>3.1	1	>3.1	−16	>3.1
U60062	FEZ1-T	60	22	2.8	30	0	18	>3.0
U62962	Int-6	237	121	2	473	(2)	158	1.5
Z78289	Clone 1D2	182	101	0	24	7.6	151	0
X03562	Insulin-like growth factor 2	129	92	0	0	>6.4	115	0
U79294	Clone 23748	366	140	2.6	74	5	165	0
X78817	*H. sapiens* partial C1 mRNA	206	145	0	46	4.5	175	0
U76010	Zinc transporter ZnT-3	469	289	0	118	4	296	0
U62437	Nicotinic acetylcholine receptor	78	−11	0	−28	>3.9	20	0
U61232	Tubulin-folding cofactor E	69	51	0	10	>3.5	51	0
U30894	*N*-sulphoglucosamine sulphohydrolase	71	39	0	16	>3.5	56	0
U82311	Unknown protein	231	187	1.2	66	3.5	200	0
X17025	Homolog of IPP isomerase	109	55	0	147	0	8	>5.5
U78798	TRAF6	117	44	0	41	2.8	28	4.1
Z31695	Inositol polyphosphate 5-phosphatase	79	37	0	50	0	17	>3.9
M15841	U2 RNA-associated B antigen	129	104	0	121	0	38	3.4
X97748	PTX3	90	63	0	107	0	26	3.5
L33243	Polycystic kidney disease 1 protein	60	33	0	38	0	4	>3.0

(25). The role of VRP in tumor formation or angiogenesis currently is unclear, but may be worthwhile investigating in tumor models or clinical situations where IFNs is used as a therapeutic agent. Genes that were not known previously to be selectively regulated by IFN-γ include the IL-15α receptor and NF-IL6β. Interactions between these cytokine pathways would be consistent with their mutual involvement in proinflammatory responses and immunomodulatory activities.

Many studies have established that different IFN-α subtypes and IFN-β can manifest distinct biological responses despite binding a common Type I IFN receptor. Previously, βR1 was the only known ISG that is selectively induced by IFN-β, but not IFN-α or IFN-γ (26). Our data suggest more than 20 potential candidate genes whose mRNA levels may be preferentially upregulated by IFN-β but not by IFN-α, although further studies are required to confirm these tentative conclusions. These include a gene encoding a clathrin-like protein, PKR (discussed below), HIF-1α, and the *fos* family member, Fra-1. HIF-1α is an important transcription factor that regulates apoptosis and expression of genes such as VEGF, p53, and p21 in response to hypoxia (14). Two additional candidate IFN-β-specific genes are ISG54 and ISG58, and, interestingly, the two other members of this gene family, ISG56 and RIG-G, both exhibited higher levels of inducibility in response to IFN-β as compared with IFN-α (Table 2). Genes that also appear to be preferentially induced by IFN-β over IFN-α include IFI16, STAT1, and GBP-2. Although probe sets for βR1 are present on the oligonucleotide arrays used, no basal or inducible expression was detectable among any of our samples, possibly because βR1 mRNA levels usually peak later than the 6-hr time point used in this study (R. Ransohoff, personal communication).

Unexpectedly, PKR was characterized by the oligo array data as inducible by IFN-β, but not IFN-α. However, as evident from Table 2, the intensity values for PKR did increase modestly from 42 in the untreated sample to 83 in the IFN-α-treated sample, but these numbers are too small to make firm conclusions. Previous studies involving Northern blot and nuclear run-on analyses determined that PKR gene transcription in Daudi cells reaches maximal levels 2 hr after IFN-α stimulation and that PKR mRNA levels are significantly elevated by 4 hr (27). While further analyses are needed to determine the kinetics of PKR gene transcription in HT1080 cells, these results suggest the intriguing possibilities that not only may PKR be preferentially induced by IFN-β, cellular background may also contribute distinct characteristics to the profile of PKR mRNA induction by distinct IFN subtypes. Furthermore, the concept of differential responsiveness by certain IFNs in a cell type-specific manner also may be generalized to other ISGs.

While we were further surprised that so many genes appeared to be preferentially induced by IFN-β, it should be emphasized that data were obtained only after treatments for 6 hr. Therefore, confirmation that particular genes are preferentially induced by a particular type of IFN requires further studies involving different times points. Nevertheless, stimulation by either IFN-α or IFN-γ resulted in approximately 100 genes with differences greater than 2-fold and 25 genes with greater than a 4-fold change. IFN-β stimulation, on the other hand, resulted in the identification of twice as many IFN-induced transcripts, 268 genes with greater than 2-fold increases, and 56 genes with greater than a 4-fold change. Since the molar amounts of IFN-α and -β protein used in our experiments were similar and the respective intensity values for the majority of ISGs induced by both IFN-α and IFN-β also were generally similar, this suggests that IFN-β may regulate a wider range of ISGs, at least in fibrosarcoma cells. In addition, it also suggests that ISGs that are inducible by IFN-α or IFN-β may be distinguished into three categories, ISGs that are similarly regulated by IFN-α/β, ISGs that are selectively regulated by IFN-β, and ISGs that are differentially regulated by IFN-α and IFN-β but still inducible by both. We did not detect ISGs that are preferentially induced by IFN-α but not IFN-β (using strict criteria, see *Results*). It will be important to determine whether certain IFN-α subtype proteins can also differentially regulate ISG induction and whether these expression profiles are cell type-specific. Lastly, we have identified IRGs with decreased expression after IFN treatment that include COX17, ZnT-3, clone 23748, clone 1D2, C1, and insulin-like growth factor 2 (IGF-2) (Table 3).

The identification of novel IFN-regulated genes in our study may lead to improved therapeutic uses for IFNs in different ways. Such genes may represent better markers to monitor the effectiveness of IFN therapies, as 2–5A synthetase and β2-microglobulin have been used in previous studies (18). The new pathways of IFN action suggested by our findings could also lead to identifying novel therapeutic agents and strategies. For example, the antitumor properties of IFNs may be improved by inhibitors of VRP/VEGF-C if, in fact, IFN-induced VRP/VEGF-C expression augments tumor angiogenesis. Lastly, the identification of novel IFN-regulated pathways may indicate diseases for which IFN therapy had not been previously considered. The realization of simultaneous monitoring for thousands of genes at the mRNA level, by oligonucleotide arrays or other methods, now shifts the challenge in biological research toward achieving more efficient utilization of this information as it relates to understanding gene function.

This study was enabled by access to GENECHIP arrays and expert support provided by Dr. Chris Harrington through the Affymetrix Academic User Center, funded by National Institutes of Health Grant PO1 HG0132. We also thank Dr. Ernest C. Borden (Cleveland) for helpful discussions. This work was supported by additional grants from the National Institutes of Health: AI34039 (B.R.G.W.), CA44059 (R.H.S.), and CA62220 (B.R.G.W. and R.H.S.).

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