Cell-type and Donor-specific Transcriptional Responses to Interferon-α

USE OF CUSTOMIZED GENE ARRAYS*

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A sensitive, specific, reproducible, robust, and cost-effective customized cDNA array system based on established nylon membrane technology has been developed for convenient multisample expression profiling for several hundred genes of choice. The genes represented are easily adjusted (depending on the availability of corresponding cDNAs) and the method is accordingly readily applicable to a wide variety of systems. Here we have focused on the expression profiles for interferon-α2a, the most widely used interferon for the treatment of viral hepatitis and malignancies, in primary cells (peripheral blood mononuclear cells, T cells, and dendritic cells) and cell lines (Kit255, HT1080, HepG2, and HuH7). Of 150 genes studied, only six were consistently induced in all cell types and donors, whereas 74 genes were induced in at least one cell type. IRF-7 was identified as the only gene exclusively induced in the hematopoietic cells. No gene was exclusively induced in the nonhematopoietic cell lines. In T cells 12, and in dendritic cells, 25 genes were induced in all donors whereas 45 and 42 genes, respectively, were induced in at least one donor. The data suggest that signaling through IFN-α2 can be substantially modulated to yield significant cell-type and donor-specific qualitative and quantitative differences in gene expression in response to this cytokine under highly standardized conditions.

Type I (predominantly α and β) and type II (γ) IFNs† play a central role in mediating antiviral, antiproliferative, and immunomodulatory responses. The pathways that are involved in IFN-induced gene expression include specific type I and II receptors, JAKs and STATs (1). Upon ligand binding, STATs form homo- or heterodimers through phosphotyrosine-SH2 interactions following activation by JAKs. Whereas STAT dimers bind to γ-activated sequence elements, both STAT1–2 heterodimers and STAT1 homodimers bind to p48 (ISGF-3/γ/IRF-9) resulting in a trimer that binds to interferon-stimulated regulatory elements in promoters of responsive genes (2).

To date the gene expression profile induced by IFN-α2 has been studied predominantly in fibrosarcoma and melanoma cell lines (3, 4). Little is known about the transcriptional profiles for other cell lines and nontransformed cells or of donor-specific differences. The definition of cell-type and donor-specific quantitative and qualitative differences is, however, central to a full understanding of the biology of the IFNs and their mechanisms of action.

Approaches through expression profiling are also of potential clinical importance. IFN-α2 is widely used in the treatment of diseases including chronic viral hepatitis B and C and several malignancies (5, 6). Only a minority of patients, however, respond to this therapy (7). The definition of gene expression profiles that correspond to “response” or “nonresponse” should ultimately result in further optimization of IFN treatment. Genes that are abnormally expressed in “nonresponders” to IFN-α2 may define novel pharmacological targets and provide further insight into the pathophysiology of the underlying disease.

To address these questions it is critical to use read-out systems that cover expression from a large number of genes. Technological advances have made possible the simultaneous detection of thousands of gene transcripts using small tissue or cell samples. These technologies include DNA chips (high density oligoarrays (8, 9) or microarrays (10)), differential display (11), differential cDNA arrays (12–14), serial analysis of gene expression (15), and expressed sequence tag data base comparison (16). These methods have been used to analyze gene expression in colon, breast, ovarian, and renal cell carcinomas, multiple sclerosis lesions, leukemic cells, and to monitor gene expression in resting, activated, and anergic lymphocytes (17–25). Although large scale array techniques are particularly useful to give a broad view of gene expression changes between samples and to discover “novel” genes that are induced by a particular cytokine or drug, they are, in general, costly, labor intensive, and unsuitable for the assay of multiple samples necessary for the detailed analysis of cytokine responses. Appropriate, customized, nylon membrane-based filter arrays, however, are attractive for precisely such analyses.

Here, we describe a customized cDNA array system that is specific, sensitive, robust, reproducible, convenient to use, and cost-effective. Using this technology we have defined significant quantitative and qualitative differences in the response of cells of hematopoietic and nonhematopoietic origin to IFN-α2a

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† The on-line version of this article (available at http://www.jbc.org) contains additional tables of original data.

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§ The abbreviations used are: IFN, interferon; IRF, interferon regulatory factor; JAK, Janus kinase; STAT, signal transducer and activator of transcription; DC, dendritic cell; PBMC, peripheral blood mononuclear cells; TC, peripheral blood T cells; rIL, recombinant interleukin; LB, Luria-Bertani; RPA, RNase protection assay; SNP, single nucleotide polymorphism; ISGF, interferon-stimulated gene factor.
Under highly standardized conditions. Substantial quantitative and qualitative donor-specific differences for T cells and DC were observed in response to this cytokine.

EXPERIMENTAL PROCEDURES

Cell Culture—Human HT1080 (fibrosarcoma), HepG2 (hepatoma), HuH7 (hepatoma), and Ki255 (T cell leukemia) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 2 mM t-glutamine, penicillin, and streptomycin at 37 °C in a humidified atmosphere containing 10% CO2. Recombinant human IFN-α2a was provided by Roche Molecular Biochemicals. The human IL-2-dependent T cell line Ki255 (26) was maintained in RPMI 1640 supplemented with heat-inactivated fetal calf serum and 20 ng/ml rIL-2 (Proleukin, Chiron, Emeryville, CA). Prior to treatment of cells with IFN-α2, Ki255 cells were washed and then cultured for 48 h in the absence of rIL-2. PBMC were isolated from buffy coat by density centrifugation on Lymphoprep (Nycomed, Norway). To obtain TC, PBMC were activated with phytohemagglutinin (Murex, UK) and maintained in RPMI 1640 supplemented with 10% inactivated fetal calf serum and human rIL-2 (20 ng/ml) for 1 week. Prior to treatment of cells with IFN-α2, TC were washed and then cultured for 48 h in the absence of rIL-2. To generate DC, monocytes were isolated from PBMC by magnetic cell sorting using anti-CD14 conjugated magnetic microbeads (Miltenyi Biotec, Cologne, Germany) and cultured for 6 days in RPMI 1640 supplemented with 10% inactivated fetal calf serum, 50 ng/ml granulocyte-macrophage colony-stimulating factor, and 50 ng/ml IL-4 (both from R&D Systems). All experiments were performed under stringent endotoxin-free conditions.

RNA Extraction—Total RNA was isolated from cells using Trizol (Invitrogen) according to the instructions of the manufacturer. RNA quantity and quality was analyzed by spectrophotometry and additional visualization by agarose gel electrophoresis.

Selection and Propagation of IMAGE Clones—Genes of interest were selected from the UniGene database (www.ncbi.nlm.nih.gov/UniGene/Hs.Home.html). 5’ IMAGE clones with 0.5–0.8 kb length were chosen and ordered from the Human Genome Mapping Project, Hinxton, UK (www.hgmp.mrc.ac.uk). Bacteria were streaked out onto 1.5% LB agar plates containing 75 μg/ml ampicillin and cultured overnight at 37 °C. Single clones were picked, transferred to 96-well plates with 200 μl of LB medium containing 75 μg/ml ampicillin and 10% glycerol, and grown overnight at 37 °C in an incubator without shaking. A 1/10 dilution of individual clones was set up in 96-well plates by adding 10 μl of bacterial culture to 90 μl of sterile ddH2O. Throughout the duration of the experiments, the number of genes present on the filters was continuously adjusted from 150 to 231 genes (Fig. 1A), reflecting the flexibility of the method. The data presented here are, however, restricted to the initial 150 genes (Table I).

Expansion of DNA from IMAGE Clones by PCR.—30-μl aliquots from the 10-fold diluted bacterial cultures were transferred into PCR strips on ice. The cDNA inserts were amplified in the presence of 50 mM KCl, 10 mM Tris, pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphates, and 50 units of reverse transcriptase (Superscript II, Invitrogen), dATP, dTTP, dGTP (0.5 mM each), and dCTP (2 μM, Amersham Biosciences) in the presence of 30 μl of [α-32P]dCTP (PerkinElmer Life Sciences, catalog number NE6013H). T23ACG anchored primers (1 μg), 1 μg of DNA, RNAse inhibitor (40 units Stratagene, Amsterdam, Netherlands, catalog number 300–151) in a total volume of 30 μl. After reverse transcription, residual RNA was hydrolyzed by alkaline treatment (15 μl of 1 M NaOH) at 70 °C for 20 min followed by neutralization with 15 μl of 1 M HCl. To remove unincorporated nucleotides the 32P-labeled cDNA was purified using Sephadex columns (ProbeQuanti G-50, Amersham Biosciences, catalog number 27-5330-01). Before hybridization to the arrays, the labeled cDNA was mixed with 50 μl of COT1-DNA (Invitrogen, catalog number 15279-011) and 10 μl of poly(dA) DNA (Amersham Biosciences, catalog number 27-7386-02) in 4× SSC, 0.1% SDS, denatured at 95 °C for 5 min, and hybridized for 1 h to minimize nonspecific binding to repetitive sequences and the poly(A) tail. After denaturation, the cDNA was added directly to medium sized hybridization bottles (260 × 40 mm, Amersham Biosciences, catalog number RPN2516) containing the membrane arrays prehybridized in 20 ml of CHURCH buffer for 30 min in a rotary hybridization oven. Hybridization with the 32P-labeled cDNA was for 16 h at 65 °C. After hybridization the hybridization buffer was discarded and replaced by 150 ml of washing buffer: the membranes were washed once in 2× SSC, 0.1% SDS, twice in 0.2× SSC, 0.1% SDS, and once in 0.1× SSC, 0.1% SDS for 20 min each at 65 °C. The membranes were transferred to a sheet of 3MM paper, immediately wrapped in Saran wrap, exposed to intensifying screens for 48 h, and scanned with a PhosphorImager at 200-micron resolution (Storm 820, Amersham Biosciences). Images were subsequently analyzed with ImageQuant (Amersham Biosciences) and converted into a table of signal intensities. Further data analysis was performed using Excel (Microsoft). For normalization between samples data were corrected for glyceraldehyde-3-phosphate dehydrogenase present in 18 copies on each filter. A detailed laboratory protocol for the cDNA array method described here is available on request from the Schlaak (joerg.schlaak@uni-essen.de) or Kerr (ian.kerr@cancer.org.uk) labs.

RNase Protection Assay—RPAs were carried out as described previously (27). Briefly, probes were synthesized from SP6/T7 transcription vectors and labeled with [32P]UTP to a specific activity of 2–5 × 108 cpm/μg of input DNA. Aliquots equivalent to 1–3 × 106 cpn of each probe and 13 μg of total RNA were used per assay. The integrity and concentration of radioactive bands were quantified using a PhosphorImager (Storm, Amersham Biosciences). Bands of interest were quantitated and corrected for background. Data are expressed as -fold induction compared with unstimulated samples.

Statistical Methods—Statistical analysis was performed using the two-sample Wilcoxon test.

RESULTS

Here we have developed a customized cDNA array methodology for ISGs based on nylon filter technology. At present this system permits the analysis of between 288 (triplicate spot) and 864 genes (single spot) for up to 12 samples per day (Fig. 1A). Throughout the duration of the experiments it was constantly extended from 150 to 231 genes. The analysis presented here, however, is restricted to the initial 150 genes (Table I). A substantial spectrum of known ISGs can be assayed with this macroarray (“macro”array: spot size >300 μm; “micro”array: spot size <300 μm) and it allows the convenient investigation of complex experimental settings including, for example, extensive kinetic and dose-response curves. Moreover the processing, analysis, storage, and recovery of the data is significantly easier and quicker compared with that for microarrays, because only genes of interest are investigated. Accordingly, the analysis of the data for 12 arrays takes ~60–90 min using standard Microsoft Excel software. Currently, each cDNA is
Fig. 1. Macroarray experiment with 12 different samples. A, digitized image of an experiment with 12 samples. Each nylon filter was spotted with DNA amplified from IMAGE clones representing 231 different genes including 18 copies of glyceraldehyde-3-phosphate dehydrogenase as normalization control. RNA was isolated from HT1080 cells, reverse transcribed, and hybridized to the membranes. All filters were exposed in one PhosphorImager screen for 48 h and subsequently scanned at 200-micron resolution. The original data for these 12 experiments are provided as Supplementary Materials. B, higher resolution of one subarray shown in A. Each gene was spotted in triplicate. The triplicate values for the spots shown here are provided as Supplementary Materials.
Genes of interest were selected from the UniGene database. These genes comprise known ISGs and genes of intrinsic interest that might or might not be induced by IFNs in different cell systems. They include genes involved in cell proliferation, immune responses, and the responses to a variety of cytokines. 5. IMAGE clones with 0.5–0.8 kb length were chosen and obtained from the Human Genome Mapping Project.

| Gene Accession No. | Gene Accession No. | Gene Accession No. | Gene Accession No. |
|--------------------|--------------------|--------------------|--------------------|
| 2–5A synthetase X02875 | Hou U32849 | NKC-4 M59807 |
| 5' Nucleotidase X55740 | HPAST protein AF001434 | p48/ISGF-3/yrF-9 M57503 |
| 60 S Ribosomal protein L11 U43522 | Hypoxia-induced factor-1 U22431 | Phospholipid scramblase 1 AF098642 |
| 6–16 U22970 | ICM-1 M42483 | PIAS x–β AF077954 |
| 72-kDa type IV collagenase J03210 | ICSB 1 M91196 | Pin-1 M16750 |
| 9–27 J04815 | IFI-16 M65833 | PKR U56648 |
| α-1-Antiplateletase K01396 | IFI-41 L23242 | Placental calcium-binding protein M80563 |
| Auto Ag SS-A/Ro NM003141 | IFN-αR1 J03171 | PLOD2 U84573 |
| BST2 D28137 | IFN-αR2 L42243 | PML-1 M79462 |
| BTG1 X61123 | IFN-γ M23833 | PFF3CA L14778 |
| Calcyclin J05763 | IFN-γR1 J03143 | PRAME U65011 |
| CASP protein AJ006470 | IFN-γR2 U05875 | Prolyl-4-hydroxylation α M24486 |
| Caspase 8 X96172 | IFP-35 U72882 | Proton-ATPase-like protein D890052 |
| Caspase-1 M87507 | IFP-53 X62570 | Putative serine/threonine kinase G383310 |
| Cathecol α-methyltransferase M58525 | IL-1α M28963 | Pyridoxal kinase U89606 |
| Cathepsin D M11233 | IL-10R U00672 | RAP46Bag-1 L35491 |
| CCR1 L90290 | IL-10R β Z17227 | RbAp48 X74262 |
| CCR5 U54994 | IL-12R β-2 U41498 | Reticuloctin D42073 |
| C1TA X74301 | IL-12R β-2α L14072 | RING12 X62741 |
| c-jun J04111 | IL-12R β-2α U14407 | RING4 U57522 |
| c-myc L00558 | IL-12Rβ-1 D16957 | RSG104 U73522 |
| Collagen α1 (I) Z74615 | IL-18-binding protein AB019504 | Smad1 U59423 |
| Collagen α2 (I) J05464 | IL-2Rα K05312 | Smad2 M50796 |
| Collagen, type XVI, α1 M92864 | IL-8 M28130 | Smad3 U47686 |
| Compl. compound C1r J04080 | Ind-2,3-Dioxigenase M43455 | Smad5 U73522 |
| COX1 L77701 | iNOS2A U20141 | Smad6 U59914 |
| CTRL-1 X71877 | Int-6 U62962 | Smad7 D89052 |
| CXCR4 AF005658 | Integrin β7 M62880 | Smooth muscle α-actin J00073 |
| DEAD box binding protein 1 AF077951 | IL-6 M92642 | Smooth muscle cell actin X10242 |
| DEAD-box protein p72 U59321 | IP-10 U4602 | SOCS1 N91935 |
| Destrin S65738 | IP-30 J03909 | SOCS3 AB004904 |
| DR-α J00194 | IRF-1 L05072 | SOCS4 AB006968 |
| EZF-1 U47877 | IRF-2 X15849 | SOCS4 M50796 |
| Elastase 2 M50379 | IRF-7 U73036 | SOCS5 NM003498 |
| ER M76184 | ISG-15 U31628 | STAT1 M97395 |
| F-actin binding protein U56637 | ISG-56K M29383 | STAT2 M97394 |
| Farnesyl pyrophosphate J03262 | KIAA0129 D28137 | STAT4 L78440 |
| FAS/Apo-1 M67454 | KIAA0336 L14778 | STAT5A L41142 |
| FK506-binding protein 6 AF038847 | LIPA U04285 | STAT5B U47866 |
| Folate receptor X62753 | LMP-1 L68901 | STAT6 U16031 |
| Galectin-1 J04456 | LMP-7 Z14982 | Succinyl-CoA ligase AF058953 |
| γ-Actin X04098 | L-selectin M52280 | TGF-βR1 L11695 |
| γ-Synegrin NM004274 | MEN1 U93237 | TGF-βR2 D56863 |
| GATA 3 X58072 | Mig U72755 | TGF-βR3 L07594 |
| GBP-1 M55542 | Mixed lineage kinase 2 X90846 | TRAIL U37518 |
| GBP-2 M55543 | MMP-1 M13509 | Transferrin M12350 |
| Granzyme B M17016 | MxA M33882 | Transhyretin D60986 |
| HCV-associated p44 D28970 | MxB M39818 | TRIP14 L40387 |
| HLA II Ag DP1 M38664 | Neural cell adhesion molecule M74387 | TTF2 AF03771 |
| HLA-E X56841 | NF-IL-6 X52560 | VEGF-C U43142 |

The high flexibility of the spotting procedure also permits the generation of filters with only one spot per gene that are particularly useful for scanning higher numbers of target genes with lower sensitivity.

To enhance the performance of the system it is critical to block nonspecific hybridization through repetitive sequences or the poly(A) tail using COT1-DNA and poly(dA) (Fig. 2). This is particularly useful for genes only marginally (1.5–2.5-fold) induced: differentials for highly induced genes are still detectable in the absence of prehybridization with COT1-DNA and poly(dA). The system offers a high degree of reproducibility as indicated by its low inter- and intra-assay variation (Fig. 3, A and B, Supplementary Materials for Table II, Table IV, experimental TC V/a-d). To achieve this it is essential to use strict endotoxin-free culture conditions because lipopolysaccharide can induce IFN-β and the expression of ISGs (28). For RNA extraction, to avoid artifacts induced by prolonged trypsinization and centrifugation, adherent cells were lysed directly on the tissue culture plates and suspension cells directly after spinning down without washing.

All of the IMAGE clones used were sequence verified. Comparability of the macroarray data for known genes with data obtained by alternative accepted RNase protection methodology was established by data from experiments carried out as an integral part of our ongoing program. An example of the data from one such experiment (involving an analysis of the responses obtained through the endogenous type I and II IFN receptors and a receptor chimera 2EgAβ (29), reveals, for the ISG-56k, IRF-1, and 9–27 ISGs, a good correlation (r = 0.89–

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2 J. F. Schlaak, unpublished data.
0.99) between the data from the two approaches (Fig. 4). Similarly good correlations have been obtained in a number of further experiments comparing the results by the two methods for the above and additional ISGs including IP-10, GBP-1, 6-16, MxA and 2-5OAS (for example, Fig. 7, Table V).

The sensitivity of the method has also been assessed. As a rule of thumb, in most micro- and macroarray systems a 2-fold change in the expression level is regarded as being significant. Statistical analysis showed that the macroarrays are capable of detecting smaller differences, after stimulation with very low concentrations of IFN-α (e.g. 10 IU/ml, Fig. 5), changes in gene expression of 30% or less are detectable with a high degree of significance (p value < 0.05) by this method. This permits the analysis of dose-response curves for poorly induced (<2-fold, Fig. 6) genes considered marginally significant by other methods. Using more replicates of the spotted DNA this high sensitivity may be enhanced even further. The physiological relevance of these relatively small changes, however, still have to be determined for the individual genes. Accordingly, we have retained 2-fold as the threshold level for significant inducibility for comparative expression profiling (Tables II–IV), which is, in addition, associated with a very high degree of statistical significance (p = 0.001 and less, Fig. 5).

Cell Type-specific Responses to IFN-α2a—To date little is known about the cell-type specificity for the expression profiles induced by the α-IFNs. The most substantial current data were obtained for human fibrosarcoma cell lines (3). Only limited information is available for nontransformed or hematopoietic cells. Accordingly, here expression profiles were compared for HT1080, HepG2, HuH7, and Kit255 cell lines and the nontransformed PBMC, T cells, and DC. Stimulation was with 1000 units/ml rhIFN-α2a for 6 (T cells) or 8 h (remainder); conditions known from further extensive experiments to yield maximal responses for each of the cell types represented. 150 genes (Table I) were represented on the arrays. Examples of the data from all seven cell types (Table II) and for different donors for the DC and T cells (Tables III and IV) are presented.

3 C. M. U. Hilkens, J. F. Schlaak, and I. M. Kerr, manuscript in preparation.
Pim-1, and SOCS2 were up-regulated in PBMC and T cells but not in DC, which might indicate that under these conditions these genes are predominantly induced in the T cell population. These genes were not, however, induced in all 5 T cell donors (Table IV). Of the genes represented none was exclusively induced in nonhematopoietic cells although RING4 and phospholipid scramblase 1 showed only low induction in 4 of 5 of the T cell and 2 of the 3 DC samples (Tables III and IV). In contrast to the donor variability observed for DC and T cells (Tables III and IV and below), for the cell lines similar results to those presented in Table II were obtained in additional experiments (see Supplementary Materials).

To establish further the validity of the comparative macroarrays and the cell type-specific differences between the T and DC cells for IP-10 in particular, a number of RPAs were also carried out on the T and DC cell RNAs for a spectrum of ISGs (e.g. Fig. 7, Table V). The data confirm the major differences in the induction of IP10 (strongly induced in DC but not T cells) and the similar patterns of induction observed for the ISGs by the two types of assay. Lower -fold inductions are frequently observed using macro- or microarray versus alternative assays. This is particularly obvious here for IP10 in DCs (Table V, a). In this instance the higher -fold induction by RPA (60–349-fold) versus macroarray (15.6–20.6-fold) reflects, in part at least, the very low background values obtained for IP10 in the RPAs (Fig. 7, lanes 1–4). For the 2-5AOS and 6-16 genes the generally lower -fold induction values by macroarray versus RPA almost certainly reflect, in part, similar differences in background inductions, but likely also donor variability (see below) and possibly the relative hybridization efficiencies of the particular image clones ‘‘chosen’’ for the macroarrays. Despite these differences in the -fold inductions observed by the RPAs and macroarrays for some of the ISGs, in general the agreement between the two types of assay is good with those ISGs registering as substantially or minimally induced by one assay registering similarly by the other. Overall, for the induced genes, the data appear consistent with limited cell-type specificity superimposed upon fairly wide inducibility for most genes, but with considerable quantitative variation between cell types and, see below, between the same cell type from individual donors.

### Table II

| Gene       | acc. no. | PBMC 1 DC 1 | TC 1 | K2555 HT1080 | HepG2 | HuH7 |
|------------|----------|-------------|------|--------------|-------|------|
| ISG-56k    | M24954   | 81.3        | 35.4 | 61.8         | 20.9  | 75.4 |
| ISG-15     | M13757   | 77.3        | 45.6 | 38.5         | 12.9  | 23.1 |
| IP-10      | X02530   | 81.4        | 15.6 | 1.9          | 1.3   | 1.1  |
| NR4A1      | M33982   | 95.3        | 26.6 | 5.6          | 4.6   | 5.5  |
| IGV-1       | D289115  | 32.2        | 4.3  | 2.7          | 7.1   | 19.3 |
| IFN-1      | M82638   | 7.5         | 5.9  | 15.4         | 2.2   | 1.7  |
| Mx1        | M30618   | 14.9        | 2.5  | 2.6          | 2.9   | 6.8  |
| GBP-1      | M66542   | 14.7        | 4.8  | 2.5          | 4.0   | 3.1  |
| YF271      | AF170311 | 14.7        | 7.6  | 1.2          | 0.7   | 1.2  |
| PRAME      | U56011   | 14.6        | 5.8  | 6.9          | 1.8   | 0.8  |
| STAT1      | M79054   | 13.5        | 2.1  | 2.3          | 10.1  | 1.5  |
| 9-27       | J04164   | 11.8        | 6.3  | 9.1          | 2.7   | 12.1 |
| 2-5AOS     | X02875   | 19.7        | 6.9  | 2.9          | 5.8   | 3.1  |
| IFN-3      | U73492   | 9.9         | 5.3  | 5.5          | 3.3   | 0.9  |
| IRF-7      | U73026   | 8.0         | 3.2  | 3.6          | 1.5   | 1.5  |
| Hou        | U52949   | 7.6         | 2.3  | 3.1          | 1.9   | 1.4  |
| PKR        | U60484   | 7.5         | 3.1  | 1.8          | 1.3   | 1.6  |
| E6-14      | U25970   | 7.3         | 4.0  | 17.3         | 3.9   | 21.2 |
| Phos. scramblase 1 | AF009642 | 6.7        | 2.6  | 4.1          | 2.8   | 2.7  |
| GBP-2      | M55543   | 6.4         | 3.1  | 1.4          | 3.1   | 0.8  |
| IL-15Ra    | D28137   | 6.1         | 2.9  | 3.8          | 4.1   | 1.7  |
| RAP46      | Z35401   | 6.0         | 3.5  | 1.3          | 1.6   | 1.7  |
| IF-31      | J22433   | 5.4         | 3.5  | 4.5          | 2.4   | 1.3  |
| RING4      | X67522   | 5.0         | 2.1  | 2.7          | 2.3   | 3.3  |
| YEGF-C     | U43142   | 4.9         | 1.9  | 3.2          | 1.6   | 1.3  |
| CCN1       | L36220   | 3.9         | 1.3  | 1.8          | 1.9   | 1.4  |
| Gasparase-1 | M67655   | 3.8         | 3.2  | 2.1          | 1.1   | 1.3  |
| IFN-1      | M67503   | 3.6         | 1.1  | 2.1          | 2.2   | 2.3  |
| IL-4       | G6582    | 3.5         | 1.5  | 2.9          | 1.3   | 1.5  |
| EGF-1      | U47677   | 3.5         | 0.9  | 2.1          | 0.9   | 0.9  |
| Pim-2      | M17659   | 3.5         | 0.9  | 2.8          | 0.7   | 1.0  |
| LMP-2      | X84041   | 3.4         | 3.2  | 2.3          | 2.9   | 3.4  |
| RING12     | X62741   | 3.3         | 2.4  | 1.0          | 2.5   | 1.9  |
| HIV-1      | U24351   | 3.2         | 0.6  | 1.7          | 0.8   | 0.5  |
| SOCS2      | AF009906 | 3.1         | 1.0  | 2.2          | 0.9   | 1.5  | 1.1  |
Donor-type Specific Responses in DC and T Cells

Little is known about donor-specific differences in the transcriptional response to IFN-α/H9251. To address this question DC and T cells from a number of donors were isolated, cultured, and treated with IFN-α/H9251 under identical conditions.

Dendritic Cells

In three independent experiments with DC derived from 3 different healthy individuals (DC I-III, Table III), a total of 42 genes was found to be induced in at least one donor, whereas 30 (71%) genes were up-regulated in at least 2 donors and 25 (60%) in all 3 donors (Table III). 11 (26%) ISGs were found with apparent qualitative rather than quantitative differences as defined by inductions of >2-fold and ≤1.3-fold each for at least one donor: TTF2, collagen type XVI, PRAME, phospholipid scramblase 1, Mig, IP-10, RAP46, HLA-E, p48/ISGF-3/IRF-9, iNOS2A, and granzyme B. 5 genes were exclusively induced in donor DC I, 7 genes were found to be up-regulated only in donor DC III, whereas no gene was exclusively induced in donor DC II. The larger among these differences, e.g. those for TTF2, collagen type XVI, and PRAME, at least, are unlikely to reflect simple quantitative differences because, overall, a surprising degree of quantitative consistency was observed between donors for the majority of the induced genes (for example, IP-10, MxB, IRF-7, IFP-35, and 2-5OAS).

T Cells

In five independent experiments with T cells from 5 different healthy donors (TC I-V, Table IV), a total of 45 genes was found to be induced (>2-fold) by IFN-α/H9251 for at least one donor, whereas 35 (78%) of these were up-regulated in at least 2 donors, 22 (49%) in at least 3 donors, 14 (31%) in at least 4 donors, and 10 (22%) in all 5 donors (Table IV). For donor TC V, data were obtained in quadruplicate. For this donor, the median of the 4 replicates was used to define the inducibility of the individual genes. 28 (62%) ISGs were found with apparent qualitative rather than quantitative differences, i.e. substan-
Cells were stimulated with 1000 units/ml IFN-α2a for 6 h. Data are shown as fold change compared to a matched control. Shaded areas denote inductions >2-fold for donor V T C V/H11349. Additional experiments revealed similar correlations between replicates, indicating consistency of the observed effects across different cell cultures.

| gene       | acc. no. | T C I | T C II | T C III | T C IV | T C V | T C VI | T C VII | T C VIII |
|------------|----------|------|--------|---------|--------|-------|--------|---------|----------|
| IFN-β      |          | 0.7  | 0.9    | 0.7     | 0.9    | 0.9   | 0.9    | 0.9     | 0.9      |
| ISG-56k    |          | 10   | 13     | 12      | 12     | 12    | 12     | 12      | 12       |
| MxA        |          | 3.8  | 3.8    | 3.8     | 3.8    | 3.8   | 3.8    | 3.8     | 3.8      |
| IFN-α1     |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| IFN-α2     |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT1      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT2      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT3      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT4      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT5      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT6      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT7      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT8      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT9      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT10     |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| TGF-β      |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| IFNAR2     |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| IFNAR1     |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| JAK1       |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| JAK2       |          | 0.7  | 0.7    | 0.7     | 0.7    | 0.7   | 0.7    | 0.7     | 0.7      |
| STAT5     |          | 0.7  | 0.7    | 0.7     | 0.7     | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| STAT6     |          | 0.7  | 0.7    | 0.7     | 0.7     | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| STAT7     |          | 0.7  | 0.7    | 0.7     | 0.7     | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| STAT8     |          | 0.7  | 0.7    | 0.7     | 0.7     | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| STAT9     |          | 0.7  | 0.7    | 0.7     | 0.7     | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| STAT10    |          | 0.7  | 0.7    | 0.7     | 0.7     | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |

**DISCUSSION**

A highly sensitive, specific and reproducible customized cDNA array system has been established. It is suitable for the routine assay of multiple samples including, for example, those necessary for the dose response and kinetic analyses required for detailed comparisons of the effect of a given cytokine in different cell types (as here) or under different conditions or in response to the inhibition of a particular signal transduction pathway. These “custom” arrays are clearly designed to study a set of known genes, not to discover “novel” genes involved in particular signaling pathways or biological conditions. Cell lines offer the advantage of virtually unlimited availability of RNA. Access to larger amounts of RNA from possibly more biologically relevant nontransformed cells is, however, often limited. An advantage of the macroarrays is the relatively small amount of total RNA required (5–10 μg) per sample. Accordingly, “macros” are suitable for the assay of sufficient samples for significant analyses of readily (PBMC) or less readily available (DC) nontransformed cells. Systematic information on the transcriptional response of such cells to IFN-α2a has been generated here. The modular design of this technology allows easy adjustment to address alternative questions across species borders provided the genes of interest are known and cDNAs, ideally in the form of IMAGE clones, are available. Based on the methodology described here, macroarrays for expression profiling in response to human IFN-γ, for type I and II murine IFNs, and for woodchuck ISGs have also been established and the technology is being used, for example, to study in vivo responses in PBMC from patients treated with IFN-α2a. In addition to signaling through ISGF-3 (STATs 1, 2, and 4/8/ISGF-3/IRF-9) type I IFNs variably activate additional STATs. STAT3 is activated in a variety of cell types (3.1) and plays a role in the transcriptional response of such cells to IFN-α2a directly activates (in addition to STATs 1, -2, and -3) STAT1 (IRF-9) type I IFNs variably activate additional STATs. STAT3 is activated in a variety of cell types (3.1) and plays a role in the transcriptional response of such cells to IFN-α2a directly activates (in addition to STATs 1, -2, and -3) STAT1 (IRF-9).
yet to be identified, together with differential STAT activation might a priori lead to the differential induction of subsets of genes in different cell types. Indeed an initial objective was to determine whether such subsets are in fact differentially induced. If so, this could, in turn, through shared promoter elements, shed light on the involvement of additional known or novel signaling pathways in the type I IFN response. Although there is little evidence here for discrete subsets of genes highly induced uniquely in one or only a few related cell types, some degree of cell-type specificity was observed. Relatively few genes appeared to be induced in all cell types (Table II). More particularly the induction of IRF-7 appeared to be specific to primary PMBC, DC, and T cells of the immune system, whereas numerous genes were substantially induced in one or more of the primary PMBC, DC, or T cells but not the cell lines. In addition, IFI-16, IP-10, and PRAME provide striking examples of substantial cell type and donor differentials in DC and T cells. Because the ISGs analyzed were mainly selected based on data obtained from experiments with fibrosarcoma cells, further differentials in the induction of, yet unknown, ISGs can be anticipated. Accordingly, to build up a comprehensive customized set of genes of interest.

IRF-7 has previously been reported to be predominantly expressed in cells of hematopoietic origin (39). Of the genes analyzed IRF-7 was the only one consistently induced in PMBC, DC, and T cells but not in nonhematopoietic cells. IRF-7 is induced by a variety of agents including viruses, lipopolysaccharide, and type I IFNs. It regulates the production of the α-IFNs, IFN-β, and the chemokines RANTES and IP-10 in virus-infected cells (40–42). It has also been identified as a key regulator for monocyte differentiation to macrophages (43). The lack of expression of IRF-7 in the fibrosarcoma cell line 2TGH correlates with hypermethylation of the CpG island in the human IRF-7 promoter (44). Such hypermethylation may represent one mechanism whereby differential cell type-specific responses to IFNs are generated.

Little is known about donor-specific responses to IFNs and their molecular background. It is, however, well established that both the clinical side effects of IFN-α therapy and its efficacy vary largely within groups of patients treated for chronic viral hepatitis or malignancies (45–50). This is, therefore, large donor-dependent variation in the clinical response to this cytokine. The data here support this in that substantial donor-specific IFN responses were observed. It is important to emphasize that differences similar to those observed between the expression profiles for different donors (Tables III and IV) were not observed for the remarkably reproducible profiles obtained on different occasions for the cell lines (e.g. Fig. 2). Overall the data are consistent with complex modulation ranging from possible specificity for particular lineages or cell-type (IRF-7) to universal high induction (ISG-56k and ISG-15), with the majority of genes showing substantial quantitative variation between cell types and donors indicative of the likely modulatory effects of differential activation of STATs and additional pathways. Superimposed on this one can reasonably expect some degree of quantitative cell type and donor variation with IFN dose and kinetics.

SNP in the promoter and coding regions of ISGs may also affect their transcription and function thus contributing to donor-specific differentials. SNPs in IRF-1 in several human liver cancer cell lines result in different antiproliferative effects of type I IFNs (51) and have been associated with juvenile idiopathic arthritis (52) and childhood atopic asthma (53), whereas SNPs in IRF-2 were found to be associated with atopic dermatitis (54). Although donor-specific responses to IFNs were not analyzed in these studies, accepting the secondary requirement for IRFs in the induction of many ISGs, one can assume that such SNPs can lead to altered function and result in differential responses to the IFNs. In principle, SNPs in promoter or coding regions of JAKs or STATs could affect their transcription and function thus contributing to downstream signaling events. Relevant data are not yet available for STAT1 and -2 but SNPs in the first exon of the STAT6 gene are associated with the development of allergic diseases (55).

Here, nylon membrane-based cDNA array technology has been further developed and optimized to provide a sensitive, robust, and convenient method for the analysis of expression profiles for a substantial “customized” set of genes of interest. The method has been used to study, in a number of both primary cells and cell lines, cell-type and donor-specific responses to IFN-α2, the most widely used IFN for the treatment of viral hepatitis and malignancies. The data have revealed that signaling in response to IFN-α2 is substantially modulated leading to significant cell-type and donor-specific qualitative and quantitative differences in the response to this cytokine. Further analysis of the in vivo responses to IFN-α2 and definition of the modulatory factors responsible for the heterogenous transcriptional responses to this agent will lead to further insight into the biology of IFN-α signaling and ultimately to further improvements in IFN therapy.
