Global and Distinct Targets of IRF-5 and IRF-7 during Innate Response to Viral Infection*

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The interferon regulatory factors (IRF) are transcriptional mediators of cellular response to viral invasion that play a critical role in the innate antiviral defense. Two of these factors, IRF-5 and IRF-7, play a critical role in the induction of interferon (IFNA) genes in infected cells; they are expressed constitutively in monocytes, B cells, and precursors of dendritic cells (pDC2) that are high producers of interferon α, and their expression can be further stimulated by type I interferon. The goal of the present study was to identify and analyze expression of cellular genes that are regulated by IRF-5 and IRF-7 during the innate response to viral infection. The transcription profiles of infected BJAB cells overexpressing IRF-5 or IRF-7 were determined by using oligonucleotide arrays with probe sets representing about 6800 human genes. This analysis shows that IRF-5 and IRF-7 activate a broad profile of heterologous genes encoding not only antiviral, inflammatory, and pro-apoptotic proteins but also proteins of other functional categories. The number of IRF-5- and IRF-7-modulated genes was significantly higher in infected than in uninfected cells, and the transcription signature was predominantly positive. Although IRF-5 and IRF-7 stimulated a large number of common genes, a distinct functional profile was associated with each of these IRFs. The noted difference was a broad antiviral and early inflammatory transcriptional profile in infected BJAB/IRF-5 cells, whereas the IRF-7-induced transcripts were enriched for the group of mitochondrial genes and genes affecting the DNA structure. Taken together, these data indicate that IRF-5 and IRF-7 act primarily as transcriptional activators and that IRF-5- and IRF-7-induced innate antiviral response results in a broad alteration of the transcriptional profile of cellular genes.

The rapid response to infection is essential for host defense. A regulatory network in which a set of transcription factors stimulates expression of the diverse genes encoding for early inflammatory proteins mediates this response. The functional diversity of these factors is dependent on their cell-specific expression, post-translational modifications, and interacting cross-talk.

Two families of transcription factors have been shown to play a critical role in the innate response to infection. The first is the NF-kB family of factors that are activated by viral infection as well as by binding of bacterial envelopes and double-stranded nucleic acids to Toll-like receptors (TLR). The second is the family of interferon (IFN) regulatory factors (IRF), particularly IRF-3, IRF-5, and IRF-7, that are activated by virus infection and/or ligands binding to TLR3, TLR4, and TLR7 (1). These factors play a critical role in the induction of antiviral proteins, type I IFNs, as well as some chemokines.

The IRF are transcription mediators of virus and IFN-induced signaling pathways and have been shown to play a critical role in antiviral defense, immune response, cell growth regulation, and apoptosis. To date, nine cellular IRF genes (IRF-1, IRF-2, IRF-3, IRF-4/Ip/i/CTS, IRF-5, IRF-6, IRF-7, ICSBP/IRF-8, and ISGF3γ/p48/IRF-9), as well as virus-encoded analogues of cellular IRF have been identified (2, 3). These factors can function as transcriptional activators (e.g. IRF-1, IRF-3, and IRF-9), repressors (e.g. IRF-8), or both (e.g. IRF-2, IRF-4, IRF-5, and IRF-7). They all share significant homology in the N-terminal 115 amino acids, which comprises the DNA-binding domain, characterized by five tryptophan repeats. Three of these repeats contact DNA with specific recognition of the GAA and AANNGAA sequences (4). However, the unique function of a particular IRF is accounted for by a cell type-specific expression, its intrinsic transcriptional potential, and an ability to interact with other members of the IRF family or other transcription factors and cofactors (5).

Three IRF (IRF-3, IRF-5, and IRF-7) function as direct transducers of virus-mediated signaling and play a crucial role in the expression of type I IFN genes and some chemokines, including RANTES (6–8). Although IRF-3 is constitutively expressed in all cell types (9), expression of IRF-7 and IRF-5 can be detected predominantly in cells of lymphoid origin and can be further

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The abbreviations used are: TLR, Toll-like receptors; IRF, interferon regulatory factors; IFN, interferon; RT, reverse transcriptase; GFP, green fluorescent protein; NDV, Newcastle disease virus; TNF, the tumor necrosis factor; TNFR, TNF receptor; RANTES, regulated on activation normal T cell expressed and secreted; MAP, mitogen-activated protein; PCBP, poly(C)-binding proteins; CBP, CREB-binding protein; OAS, 2′,5′-oligoadenylate synthetase; E2, ubiquitin carrier protein; Pip, double-stranded RNA; TTP, TATA box-binding protein; PKR, double-stranded RNA-activated protein kinase.

1 A. Schoenemeyer, B. J. Barnes, M. E. Manci, E. Latz, J. I. Ihoue, P. M. Pitha, K. A. Fitzgerald, and D. T. Golenbock, unpublished results.
stimulated by type I IFN (10, 11). In splenic B cells, monocytes, and particularly in precursor dendritic cells (pDC2) that are high producers of IFNα, both IRF-5 and IRF-7 are expressed constitutively (12). In uninfected cells, IRF-3 and IRF-7 reside predominantly in the cytoplasm, but upon virus-induced phosphorylation of the C-terminal serine residues, mediated by IKKε and TBK1 (13, 14), they translocate to the nucleus where they bind to the transcriptional co-activator p300/CBP and IRF-E elements in the IFNA and -B promoters (15). These two factors were identified as components of the transcriptional complex enhanceosome, binding to the promoters of IFNA1 and IFNB genes in infected cells (16, 17). Although IRF-5 is also activated by viral infection, it shows several distinct features from IRF-3 and IRF-7 (7, 18). The IRF-5 polypeptide contains two nuclear localization signals that are not present in IRF-3 or IRF-7, and low levels of nuclear IRF-5 can be detected in uninfected cells (7, 11). Although viral infection results in the further accumulation of nuclear IRF-5, activation of IRF-5 appears to be virus-specific, and thus, it can be phosphorylated in NDV-infected but not in Sendai virus-infected cells (7).

Although IRF-3 expression is sufficient for the induction of IFNB (19, 20), IRF-5 and IRF-7 have critical roles in the induction of IFNA genes in human cells (11, 21). Reconstitution of IRF-5 or IRF-7 expression in human cells, which under normal conditions express only the IFNB gene, results in the virus-mediated induction of IFNA genes (11, 21). Most interestingly, subtypes of IFNA genes induced by IRF-5 and IRF-7 are distinct. These results demonstrate both the essential and distinct roles of IRF-5 and IRF-7, which together ensure the transcriptional regulation of diverse IFNA genes during the antiviral response.

In addition to their role in innate immunity, IRF-5 and IRF-7 have distinct roles in the differentiation of lymphoid cells and apoptosis. IRF-7 was found to be a macrophage differentiation factor (22), and its expression is silenced by CpG methylation in some tumor cell lines (23). On the other hand, IRF-5 is transcriptionally activated by the tumor suppressor p53 (24); it induces p21WAF1/CIP1 and arrests cells in the G2/M phase of the cell cycle (25). Furthermore, overexpression of IRF-5 induces expression of several pro-apoptotic genes and apoptosis in a p53-independent manner. Most interestingly, a high percentage of primary lymphocytic malignancies shows a lack of IRF-5 expression (25). The chromosomal localizations of the IRF-5 and IRF-7 genes also suggest their growth regulatory functions. IRF-7 is localized on chromosome 11p15.5 (22), and IRF-5 is on chromosome 7q32 (11). Both of these regions contain a number of imprinted genes. These findings indicate that both of these IRF have a distinct role in the physiology of lymphoid cells and that deregulation of their inherent functions may lead to tumorigenesis.

Although both IRF-5 and IRF-7 can support virus-mediated induction of IFNA gene expression, the observation that IRF-5 and IRF-7 activate expression of different IFNA subtypes (11), and that their functions in uninfected cells appear distinct, suggests that IRF-5 and IRF-7 are not redundant in their roles. The goal of the present study was therefore to identify and analyze cellular genes involved with the antiviral and anti-inflammatory host response, expression of which is modulated by IRF-5 or IRF-7. The data reveal that overexpression of IRF-5 or IRF-7 in B cells modulates the expression of a large group of distinct and overlapping genes in both infected and uninfected cells. Both these factors induced a large group of genes involved in transcriptional activation and signaling. However, although the signature of IRF-5 in infected cells was a strong immune response and induction of adhesion genes, IRF-7 selectively up-regulated the expression of mitochondrial genes and genes involved in the DNA repair and structure.

MATERIALS AND METHODS

Cell Culture and Virus—Parental BJAB cells were grown in RPMI 1640 with 10% fetal calf serum. BJAB cells constitutively expressing IRF-5 or IRF-7 were generated by transfection of the N-terminal FLAG-tagged IRF-5, IRF-7, or empty vector expressing plasmid in which the respective cDNAs were under the control of the cytomegalovirus pIE1 promoter, as described previously (11, 25). Newcastle disease virus (NDV) was purchased from the ATCC (VR-699). Cells were infected with NDV at a multiplicity of infection of 2.0 for 6 h.

Preparation of cRNA and Gene Chip Hybridization—Total RNA was isolated from infected and uninfected BJAB vector control cells and BJAB/IRF-5 and BJAB/IRF-7 cells by the TriZol method and subsequently used to generate labeled cRNA probes. Preparation of cRNA, hybridization, and scanning of the HuGeneU95Av2 arrays were performed according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA). Briefly, 5 μg of the RNA was converted into double-stranded cDNA by reverse transcription using a cDNA synthesis kit (Superscript Choice, Invitrogen). After second strand synthesis, labeled cRNA was generated from the cDNA sample by an in vitro transcription reaction supplemented with biotin-11-CTP and biotin-16-UTP (Enzo, Farmingdale, NY). The labeled cRNA was purified by using RNasy spin columns (Qiagen, Valencia, CA). Fifteen micrograms of each cRNA sample was fragmented by mild alkaline treatment and then hybridized to human U95A oligonucleotide probe array complementary to 6800 genes (Affymetrix). The arrays were then washed with 6× SSPE and 0.5× SSPE with streptavidin-phycocyanin (Molecular Probes), washed again, and read by using a confocal microscope scanner with the 580-nm long pass filter (Amersham Biosciences; Affymetrix). GeneChip 3.0 software was used to perform data analysis. This software includes algorithms that determine whether a gene is absent or present and whether the expression level of a gene in an experimental sample is significantly increased or decreased relative to a control sample. To assess differences in gene expression, we selected genes that had a greater than 2-fold change and a p value of less than 0.01. The p values were calculated by using two-sample t test on log-transformed data. The microarray data are available on the following web site: www.ncbi.nlm.nih.gov/geo using the accession numbers GPL1332, GPL1333, GPL1334, and GPL1335.

PCR—Single-stranded cDNA was synthesized from total RNA by using Superscript II reverse transcriptase (Invitrogen) and random hexamers (Invitrogen) as primers. Preliminary experiments were performed to determine the conditions under which cDNAs were amplified in the linear region of the PCR curve. The reaction mixture was composed of 10 ng of cDNA template; 25 pmol of primers; 25 nmol each of dNTP; 0.2 units of Platinum TaqDNA polymerase (Invitrogen); 5 μl of 1× PCR buffer in a final volume of 25 μl. Primers used for IRF-5, IRF-7, and β-actin amplification have been described previously (11, 21). The nucleotide sequences of primers used for these studies are shown in Table I. PCR amplification conditions used are as follows: denaturation at 94 °C for 5 min, amplification during the various number of cycles composed of denaturation at 94 °C for 30 s, annealing at 52–68 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

SDS-PAGE and Western Blotting—Total proteins (25 μg) were dialyzed in Laemmli sample buffer, resolved by an SDS-PAGE in a 10% polyacrylamide gel, and transferred onto a 0.22-μm nitrocellulose membrane (Schleicher & Schuell). The membranes were incubated for 2 h in blocking buffer containing 5% milk in 10 mM Tris-HCl, pH 7.5; 2.5 mM EDTA, pH 8; 50 mM NaCl. Subsequent incubation was performed for 1 h with a M2 anti-FLAG monoclonal antibody (Sigma). The membranes were subsequently incubated for 1 h with a horseradish peroxidase-conjugated anti-mouse antibody (Amersham Biosciences), and immunodetection was realized by ECL reagents (Amersham Biosciences) followed by autoradiography on hyperfilm MP (Amersham Biosciences).

Subcellular Localization of GFP-IRF-5 or GFP-IRF-7 Proteins—BJAB cells (2 × 106) were transfected with GFP-IRF-5 (10 μg) (7) or GFP-IRF-7 (10 μg) (10) using the Amaxa nucleofector reagent and electroporation, as described by the manufacturer. Twelve hours post-transfection, cells were either left uninfected or infected with NDV for 3 or 6 h. Cells were then examined under a fluorescence microscope at ×40 magnification.

RESULTS

Characterization of BJAB Cells Overexpressing IRF-5 and IRF-7—In order to identify genes modulated by IRF-5 or IRF-7 in uninfected and virus-infected cells, we have utilized the previously generated BJAB/IRF-5 and BJAB/IRF-7 overexpressing
The signaling pathway is operative in these cells. IRF-5 or IRF-7. The results in Fig. 1 show that while in the uninfected transfected BJAB cells, GFP-IRF-7 was detected by immunoblot analysis in BJAB/IRF-7 cells but not in BJAB parental cells (data not shown). Nonetheless, a clear change in cytoplasmic expression of IRF-5 and IRF-7 proteins encoded by the ectopic plasmids was high. As shown in Fig. 2A, the relative levels of IRF-5 and IRF-7 transcripts in BJAB cells are very low, but they increase in IFNα-stimulated cells indicating that the type I IFN signaling pathway is operative in these cells.

We and others have shown that both IRF-3 and IRF-7 are activated by phosphorylation in infected cells which results in nuclear accumulation of these factors. The nuclear localization of IRF-5 and IRF-7 is essential for their transcriptional activity. Therefore, we examined whether NDV infection of BJAB cells induces nuclear translocation of the ectopic GFP-labeled IRF-5 or IRF-7. The results in Fig. 1B show that while in the uninfected transfected BJAB cells, GFP-IRF-7 resides primarily in the cytoplasm and not in the nucleus; as early as 3 h post-infection, GFP-IRF-7 localizes in the nucleus and can also be observed in the nucleus 6 h post-infection. It should be kept in mind that BJAB cells are nonadherent cells, and therefore a clear distinction between the nucleus and the cytoplasm is not as well defined as in adherent cells. Similar redistribution can also be seen for GFP-IRF-5, although as we have shown previously (11), low levels of IRF-5 are also present in the nucleus of uninfected cells (7). Nonetheless, a clear change in cytoplasmic to nuclear translocation is observed. These data indicate that NDV induces activation of IRF-5 and IRF-7 and their translocation to the nucleus of BJAB cells.

The second reason for selection of BJAB cells for this study was that neither IRF-5 nor IRF-7 proteins could be detected in unstimulated BJAB cells (data not shown), whereas the expression of IRF-5 and IRF-7 transcripts encoded by the ectopic plasmids was high. As shown in Fig. 2A, the relative levels of IRF-7 transcripts were significantly lower in BJAB cells (lanes 3 and 4) than in BJAB/IRF-7-expressing cells (lanes 2 and 4), and these levels were unaffected by virus infection (lanes 6 and 8). In addition, the IRF-7 protein was detected in lysates of BJAB/IRF-5 cells but not in BJAB parental cells (data not shown). Similarly, IRF-5 transcripts were detected only in lysates of uninfected (lanes 2 and 4) and infected (lanes 6 and 8) BJAB/IRF-5 cells and not in the infected (lanes 5 and 7) and uninfected (lanes 1 and 3) BJAB cells. In addition, the ectopic IRF-5 protein was also detected by immunoblot analysis in BJAB/IRF-5 cells. Thus, these cell lines allow us to analyze and compare global gene expression in the same cell type that

### Table 1

| Primers used for the RT-PCR analysis of selected genes up-regulated in the microarray analysis |
|-----------------------------------------------|
| IFI-16F | 5′-CTACCTTGGACCACTGCACATGTA-3′ |
| IFI-16R | 5′-GATTCTTGGACCACTGCACATGTA-3′ |
| FITI1F | 5′-TTCCTGGAGCGAGGCTGAG-3′ |
| FITI1R | 5′-GATCTTGGACCACTGCACATGTA-3′ |
| IFIT7F | 5′-CTACCTTGGAGACAGTGAAG-3′ |
| IFIT7R | 5′-AACATTTGAGACAGTGAAG-3′ |
| IP41F | 5′-AGAAGGAGGACTTTGGACTTGT-3′ |
| IP41R | 5′-TCAAGGAGGACTTTGGACTTGT-3′ |
| ISG-20F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| ISG-20R | 5′-TCAAGGAGGACTTTGGACTTGT-3′ |
| PAI-1F | 5′-TTGGAGGACTTTGGACTTGT-3′ |
| PAI-1R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| PKRF | 5′-TTGGAGGACTTTGGACTTGT-3′ |
| PKRR | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| OAS1F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| OAS1R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| OAS2F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| OAS2R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| CXCL11F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| CXCL11R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| CCL4F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| CCL4R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| TNFS10F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| TNFS10R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| UBE2E3F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| UBE2E3R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| USP9F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| USP9R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| UBE2G1F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| UBE2G1R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| UBE2D3F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| UBE2D3R | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| UBE2D1F | 5′-GTTGAGGACTTTGGACTTGT-3′ |
| UBE2D1R | 5′-GTTGAGGACTTTGGACTTGT-3′ |

**Fig. 1.** Kinetics of IRF-5 and IRF-7 induction/activation in IFN-treated or NDV-infected BJAB cells. A, endogenous IRF-5 and IRF-7 transcripts are up-regulated by IFNα in parental BJAB cells. BJAB cells were either left untreated or treated with IFNα-2a for 16 or 24 h at 500 units/ml. IRF-5, IRF-7, and β-actin transcripts were analyzed by RT-PCR from total RNA preparations. Lane 1, BJAB control cells; lane 2, BJAB treated with IFNα-2a for 16 h; lane 3, BJAB treated with IFNα-2a for 24 h, β-actin transcripts are shown as a control for RNA levels. B, the subcellular localization of GFP-IRF-5 or GFP-IRF-5 in uninfected control BJAB cells (0 h) or NDV-infected (6 h) BJAB cells is shown. BJAB cells were transiently transfected with either GFP-IRF-5 or GFP-IRF-5 by using the Amx-1 nucleofactor reagent and electroporation, as described under “Materials and Methods.”
differ from IRF-5 in infected cells (Table II), whereas only 15% of the genes were commonly up-regulated by IRF-5 and IRF-7 in uninfected cells. Data were generated from two independent experiments, in which two independent RNA isolations from the same cell preparations were hybridized to U95A Affymetrix oligonucleotide microarrays containing 6,800 human genes, and the data were analysis was GeneChip 3 software. The pattern of expression was remarkably similar between the two independent analyses, and genes modified by IRF-5 by 2-fold or greater, with a p value < 0.01, were selected for further analysis. IRF-7 modulated the expression of a large number of cellular genes (Table II). This number was significantly enhanced in cells following NDV infection compared with uninfected cells (693 and 232 genes, respectively). The large majority of regulated genes were positively influenced by IRF-7 (630 up-regulated genes), and only 63 genes were down-regulated. A large number (58%) of genes were commonly up-regulated by IRF-7 and IRF-5 in infected cells (Table II), whereas only 15% of the genes were commonly up-regulated by both of these factors in infected cells. The up-regulated genes were then grouped into functional categories.

**Gene Expression Profile of BJAB Cells Overexpressing IRF-7—**

By using microarray analysis, gene expression signatures unique to IRF-7 were determined by comparison of gene expression in infected and uninfected BJAB/vector and BJAB/IRF-7-expressing cells. Because IRF-7 was shown to play a critical role in the induction of an antiviral immune response, we have examined the effects of IRF-7 overexpression on the virus-stimulated gene profile in uninfected and NDV-infected cells. The up-regulated genes were then grouped into functional categories.

**TABLE II**

| Modification       | IRF5 | IRF7 | Common genes |
|--------------------|------|------|--------------|
| Up-regulated       | 568  | 630  | 371          |
| Infected           |      |      |              |
| Uninfected         | 88   | 216  | 33           |
| Down-regulated     | 89   | 63   | 26           |
| Infected           |      |      |              |
| Uninfected         | 67   | 16   | 3            |

**TABLE III**

| Category                      | IRF-7 Uninfected | IRF-7 Infected | IRF-5 Uninfected | IRF-5 Infected |
|-------------------------------|------------------|----------------|------------------|----------------|
| Adhesion molecules            | 0                | 0              | <5               | 7              |
| Apoptosis                     | <5               | 19             | 15               | 13             |
| Biosynthesis                  | <5               | 20             | <5               | 20             |
| Cell cycle                    | 10               | 33             | <5               | 33             |
| Cell structure                | 0                | 9              | <5               | 25             |
| Chaperons                     | <5               | 5              | <5               | 5              |
| Chromatin structure           | <5               | 17             | <5               | 9              |
| DNA repair/synthesis          | <5               | 20             | >5               | 5              |
| Immune response               | <5               | 16             | 5                | 29             |
| Membrane transport            | <5               | 18             | <5               | 20             |
| Metabolism                    | <5               | 19             | <5               | 15             |
| Trafficking                   | 8                | 33             | <5               | 22             |
| Protein degradation           | <5               | 25             | 5                | 24             |
| RNA processing                | 8                | 44             | <5               | 27             |
| Signal transduction           | 24               | 71             | 19               | 83             |
| Transcription factors         | 13               | 69             | 18               | 86             |
| Mitochondrial proteins        | <5               | 32             | <5               | 11             |
| Redox                         | <5               | 6              | <5               | 5              |
| Etc.                          | 16               | 147            | 6                | 23             |
| Adhesion molecules | | | |
|-------------------|---|---|---|
| CD22 antigen      | Hs.171763 | 3.8 | |
| Presenilin 1      | Hs.3260  | 3.8 | 2.9 |
| Apoptosis         | | | |
| Phorbol 12-myristate 13-acetate-induced protein 1 | Hs.96  | 9.3 | |
| BCL2-like 1       | Hs.305890 | 7.8 | |
| BAX inhibitor 1   | Hs.74637 | 7.0 | 3.0 |
| Program cell death | Hs.41639 | 5.7 | |
| Biosynthesis      | | | |
| Sterol-C5-desaturase | Hs.288031 | 7.0 | 2.2 |
| S-Adenosylmethionine decarboxylase 1 | Hs.262476 | 6.5 | 2.3 |
| 3-Hydroxy-3-methylglutaryl-CoA synthase 1 (soluble) | Hs.77910 | 6.2 | 3.8 |
| Methionine adenosyltransferase II, α | Hs.77502 | 5.0 | 3.5 |
| Cell cycle        | | | |
| Cyclin G1         | Hs.79101 | 15.1 | 12.4 |
| CDC2 (cell division cycle 2) | Hs.334562 | 7.6 | 2.2 |
| p21-activated kinase 2 | Hs.30692 | 6.7 | 4.5 |
| Cyclin C          | Hs.118442 | 5.5 | 2.8 |
| NIMA              | Hs.153704 | 4.7 | |
| Tsg101            | Hs.118910 | 3.6 | |
| Cell structure    | | | |
| Syndecan-binding protein (syntenin) | Hs.8180 | 7.2 | |
| Microfil-associated protein 1 | Hs.61418 | 4.5 | |
| Chaperones        | | | |
| Calreticulin      | Hs.16488 | 16.5 | |
| Heat shock 70-kDa protein 1B | Hs.274402 | 10.2 | 8.9 |
| DnaJ (Hsp40) homologue, subfamily A, member 1 | Hs.388392 | 5.9 | |
| Retinitis pigmentosa 2 | Hs.44766 | 4.7 | |
| Chromatin structure | | | |
| H2B histone family, member C | Hs.137594 | 5.1 | 3.4 |
| SWI/SNF-related, matrix-associated | Hs.9456 | 4.6 | |
| DNA synthesis/repair | | | |
| Topoisomerase I   | Hs.317    | 7.8 | 2.4 |
| Topoisomerase IIα | Hs.156346 | 4.4 | 3.2 |
| Replication factor C | Hs.171075 | 3.9 | 3.7 |
| Mutator gene MSH2 | Hs.44039 | 3.8 | |
| Immune/antiviral response | | | |
| Interferon β1     | Hs.93177 | 8.6 | |
| cig5, viperin      | Hs.17518 | 8.6 | |
| IRF-8              | Hs.14453 | 8.6 | 4.5 |
| Thymopoietin      | Hs.11355 | 6.3 | 5.5 |
| Phospholipid scramblase 1 | Hs.198282 | 5.7 | |
| CD 80(B7-1)       | Hs.838    | 3.2 | |
| Intracellular/organelle trafficking | | | |
| RAB2, member RAS oncogene family | Hs.78305 | 6.8 | |
| ADP-ribosylation factor 4 | Hs.75290 | 6.0 | |
| Insulin-induced gene 1 | Hs.238513 | 5.0 | 2.0 |
| Vesicle-associated membrane protein 1 | Hs.20021 | 5.0 | 4.2 |
| Sortin nexin 3    | Hs.12102 | 4.8 | |
| Secretory carrier membrane protein 1 | Hs.31218 | 4.6 | |
| Membrane transport | | | |
| ATPase, H+ -transporting | Hs.281866 | 15.8 | 3.3 |
| ATP synthetase    | Hs.281866 | 5.3 | 5.4 |
| Metabolism        | | | |
| Glutamate dehydrogenase-2 | Hs.272497 | 4.2 | 3.8 |
| Methionine adenosyltransferase II, α | Hs.77502 | 5.0 | 3.5 |
| Lipase A          | Hs.85226 | 4.2 | |
| Mitochondrial proteins | | | |
| ATP synthase, H+ -transporting, mitochondrial F1 complex, α | Hs.155101 | 5.4 | 5.3 |
| Translocase of mitochondrial membrane 17 | Hs.20716 | 4.1 | |
| Protein degradation | | | |
| Ubiquitin-conjugating enzyme E2D1 | Hs.129683 | 5.9 | 3.0 |
| Proteasome subunit α3 | Hs.346918 | 4.3 | |
| Ubiquitin-specific protease 9 | Hs.77578 | 3.9 | 2.8 |
| RNA processing    | | | |
| DEA/D/H box 3     | Hs.147918 | 8.2 | 2.9 |
| Poly(A) polymerase α | Hs.334648 | 5.6 | 4.4 |
| KH domain RNA-binding protein | Hs.119537 | 4.8 | |
| Poly(rC)-binding protein 1 | Hs.2853 | 3.8 | |
| PAI-1 mRNA-binding protein | Hs.356427 | 3.3 | 2.1 |
| Signal transduction | | | |
| Fibroblast growth factor receptor 4 | Hs.165950 | 9.7 | 3.3 |
| Rag D protein     | Hs.238679 | 6.5 | |
| v-RAF-1           | Hs.349650 | 6.2 | 2.2 |
| Transducer of ERBB2, 1 | Hs.178137 | 5.6 | |
| Protein kinase, cAMP-dependent, regulatory, type I, α | Hs.183037 | 5.5 | 2.7 |
The group of transcription factors that include general transcription factor IIb, MAX-interacting protein 1, two zinc finger proteins, basic transcription factor 3, and the MAX protein were highly stimulated by IRF-7 in infected cells. All of these factors were induced only in infected cells overexpressing IRF-7, and their role in the induction of the early inflammatory genes has yet to be established.

A group of genes associated with apoptosis such as program cell death gene and BAX inhibitor 1 gene were induced by IRF-7 in infected cells. Also, numerous chaperone-encoding genes were induced by IRF-7 in infected cells, where the HSP70 1B gene was induced by IRF-7 also in uninfected cells. IRF-7 also mediated an enhanced expression of genes, encoding RNA-binding proteins such as KH domain RNA-binding protein that functions as an RNA helicase, poly(I)-binding protein (PCBC), and PAI-1 mRNA-binding protein. Most interestingly, both in infected and uninfected cells, IRF-7 stimulated expression of genes involved in the modulation of DNA structure and DNA repair, such as topoisomerase I and II, and mutation gene MSH2.

Finally, genes encoding proteins of the ubiquitination pathway represented a large group of genes induced in infected IRF-7-expressing cells. These included several variants of ubiquitin-conjugating enzyme E2, ubiquitin-deconjugating enzymes, such as ubiquitin-specific protease 9 and proteasome subunits.

**Gene Expression Profile of BJAB Cells Overexpressing IRF-5**—In a similar manner, we have analyzed the genes targeted by IRF-5 by using the previously generated BJAB/IRF-5-overexpressing cell line (25). RNA isolated from infected and uninfected BJAB- and BJAB/IRF-5-expressing cells were analyzed by microarray as described for IRF-7 analysis. Two independent experiments were performed, and the pattern of gene expression between them was highly similar. Although IRF-5 modulated about the same number of genes in infected cells as IRF-7 (Table II), the number of genes regulated in uninfected cells was significantly lower (155 genes). Because the levels of ectopic IRF-5 and IRF-7 protein in the stable expressing BJAB cell lines were comparable (Fig. 2), it is unlikely that the fewer numbers of cellular genes modulated in uninfected BJAB/IRF-5 cells reflect a difference in the relative levels of IRF-5 and IRF-7 expressed in these cell lines.
| Table V: Selected genes up-regulated by IRF-5 |
|---------------------------------------------|
| **Unigene no.** | **Change infected** | **Change noninfected** |
| Adhesion molecules |  |
| Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) | Hs.512682 | 8.1 |
| Catenin α1 | Hs.178452 | 3.8 |
| CD 164 antigen | Hs.43910 | 3.5 |
| Basement membrane-induced gene | Hs.10649 | 3.5 |
| SDF-1 | Hs.6354 | 3.3 |
| Neural cell adhesion molecule 1 | Hs.167988 | 3.1 |
| Apoptosis |  |
| Phorbol 12-myristate 13-acetate-induced protein 1 | Hs.96 | 11.4 |
| Tumor necrosis factor (ligand) superfamily, member 10 | Hs.83429 | 7.7 |
| Kruppel-type zinc finger (C2H2) | Hs.142150 | 5.8 |
| TNF-interacting serine-threonine kinase | HS296327 | 4.5 |
| Caspase 3 | Hs.74552 | 4.4 |
| Bcl-2 antagonist | Hs.38213 | 4.3 |
| Programmed cell death 2 | Hs.41639 | 3.5 |
| Biosynthesis |  |
| GTP cyclohydrolase 1 (dopa-responsive dystonia) | Hs.86724 | 6.6 |
| Sterol-C5-desaturase (fungal ERG3, 5-desaturase)-like | Hs.288031 | 6.1 |
| 3-Hydroxy-3-methylglutaryl-CoA reductase | Hs.11899 | 5.5 |
| Argininosuccinate synthetase | Hs.160766 | 5.4 |
| Cell cycle |  |
| Cyclin G1 | Hs.79101 | 6.6 |
| CDC2 | Hs.334562 | 5.6 |
| Cyclin-dependent kinase inhibitor 1A | Hs.179665 | 4.8 |
| Cyclin A2 | Hs.86137 | 4.1 |
| p21-activated kinase | Hs.30692 | 3.5 |
| Chaperones |  |
| Heat shock 70-kDa protein 1B | Hs.274402 | 20.5 |
| Heat shock 70-kDa protein 1A | Hs.8997 | 9.5 |
| Protein-disulfide isomerase-related protein | Hs.182429 | 5.8 |
| Heat shock protein, 70 kDa | Hs.75452 | 5.2 |
| Retinitis pigmentosa 2 | Hs.44766 | 4.0 |
| Chromatin structure |  |
| SWI/SNF-related chromatin regulator | Hs.9456 | 4.2 |
| Telomeric repeat binding factor | Hs.194652 | 4.0 |
| Nucleosome assembly protein 1 | Hs.302649 | 3.7 |
| Retinoblastoma-binding protein 4 | Hs.16003 | 3.7 |
| Immune response |  |
| sig5, viperin | Hs.17518 | 50.3 |
| Interferon, β1, fibroblast | Hs.93177 | 26.0 |
| Small inducible cytokine subfamily B (Cys-X-Cys), member 11 | Hs.103982 | 24.1 |
| Phospholipid scramblase 1 | Hs.198228 | 13.0 |
| Small inducible cytokine A4 (homologous to mouse Mip-1b) | Hs.75703 | 12.6 |
| Interferon-induced protein with tetrasacopeptide repeats 1 | Hs.20315 | 9.1 |
| Small inducible cytokine A3 (homologous to mouse Mip-1a) | Hs.73817 | 8.3 |
| Interferon-induced protein 41 | Hs.38125 | 7.4 |
| Pre-B cell colony-enhancing factor | Hs.239138 | 6.5 |
| Protein kinase, interferon-inducible, double-stranded, RNA-dependent | Hs.274382 | 5.6 |
| OAS1 (40–46 kDa) | Hs.82396 | 5.3 |
| Interferon-induced, hepatitis C-associated microtubular aggregate protein (44 kDa) | Hs.82316 | 5.2 |
| Thymopoietin | Hs.11355 | 3.6 |
| Intragranular trafficking |  |
| Insulin-induced gene 1 | Hs.56205 | 5.7 |
| Vesicle-associated membrane protein 1 | Hs.20021 | 3.6 |
| Metabolism |  |
| Lipase A, lysosomal acid, cholesterol esterase (Wolman disease) | Hs.85226 | 6.4 |
| Glutamine synthetase | Hs.170171 | 4.3 |
| Methionine adenosyltransferase II, α | Hs.77837 | 3.8 |
| RNA processing/translation |  |
| Zinc finger protein 36, C3H type, homologue (mouse) | Hs.343586 | 5.7 |
| KH domain RNA-binding protein | Hs.15020 | 4.8 |
| DEAD box polypeptide 18 | Hs.100555 | 3.9 |
| Poly(A) polymerase α | Hs.334648 | 3.9 |
| PolyrC-binding protein 1 | Hs.28353 | 2.9 |
| Fragile X mental retardation | Hs.89764 | 3.4 |
| PAI-1 mRNA-binding protein | Hs.165998 | 3.0 |
| Protein degradation |  |
| Ubiquitin-conjugating enzyme E2D | Hs.118797 | 4.8 |
| Proteasome subunit β5 | Hs.180062 | 4.3 |
| Proteasome subunit α3 | Hs.346918 | 4.3 |
| Ubiquitin conjugation enzyme E2G1 | Hs.78563 | 3.5 |
| Ubiquitin-specific protease 9 | Hs.77778 | 3.5 |
| Signal transduction |  |
| Fibroblast growth factor receptor 4 | Hs.165950 | 8.4 |
18, poly(A) polymerase α, poly(C)-binding protein, and Fragile X mental retardation gene.

Comparison of Gene Expression Profiles between IRF-5- and IRF-7-overexpressing BJAB Cells Infected with NDV—Among the up-regulated genes in NDV-infected BJAB/IRF-5- and BJAB/IRF-7-expressing cells, about 60% were common to both IRF-5 and IRF-7 (Table II). In infected cells, IRF-5 and IRF-7 up-regulated 371 common genes, and 33 genes were up-regulated in uninfected cells. From the genes up-regulated by IRF-5 or IRF-7 in infected cells, 42 and 43 genes, respectively, function in cellular signaling and transcription (Table III). Remarkably, several transcription factors that play a role in the induction of an antiviral early inflammatory response and cell growth were induced by both IRF-7 and IRF-5. The stimulated genes included MAX and the MAX-interacting proteins, IRF-1, IRF-8 (ICSBP), STAT1, STAT3, and STAT5B (Table VI). Zinc finger gene 267 and basic transcription factor 3 that regulates the activity of RNAse polymerase II were also up-regulated both by IRF-5 and IRF-7. Many of these factors, such as the IRFs and STATs, are essential components of the transcription complexes formed on the promoters of type I IFN genes and ISG genes, and thus play a critical role in their inducible expression (27–30). Altogether, there were 14 genes, encoding proteins with immunoregulatory and antiviral functions, commonly up-regulated by greater than 2-fold in IRF-5- and IRF-7-expressing BJAB cells (Table VI). Unexpectedly, IRF-5 was a much stronger activator of the IFNB gene than IRF-7 (26- and 9-fold induction, respectively). Expression of IRF-5 was detected in primary B cells, and pDC2 cells that both induce higher levels of IFNα than IFNβ upon viral infection, and IFN-5 is activated by R848 that predominantly induces IFNα synthesis (31). On the other hand, IRF-7 is a component of the transcriptional complex enhanceosome assembled on the promoter of the IFNB gene (17).

Another large group of common genes induced by both IRF-5 and IRF-7 were the different members of the ubiquitin-conjugating enzymes UBE2 and subunits of proteasomes. Further-
intracellular trafficking genes (20 genes) that are involved in nuclear entry and exit, vesicular fusion, or docking (32). From these, insulin-induced gene 1 and vesicle-associated membrane protein 1 were stimulated in both IRF-5- and IRF-7-expressing BJAB cells. The expression of genes encoding RNA-binding proteins, such as putative helicase DEAE box 16, poly(C)-binding protein, and KH domain binding protein were also induced in both infected IRF-5- and IRF-7-expressing cells. These data demonstrate that overexpression of IRF-5 or IRF-7 in BJAB cells stimulates similar cellular programs resulting in the induction of overlapping genes.

Nonetheless, overexpression of IRF-5 and IRF-7 also induced a set of distinct, nonoverlapping genes belonging to different functional categories. Thus, IRF-7 induced more effectively mitochondrial and DNA repair genes. In the case of mitochondrial genes, IRF-7 stimulated the expression of 32 genes, whereas IRF-5 induced only 9 genes. Also, a larger number of DNA repair and structure-modulating genes was induced in IRF-7-expressing infected cells (20 genes) than in IRF-5-expressing cells (5 genes). Most interestingly, a number of these genes, such as topoisomerase I and II and replication factor C, were induced both in infected and uninfected IRF-7-expressing cells. We have shown previously (33) that changes in chromatin structure are sufficient to induce expression of the IRF-7 gene, and thus, an interesting question that remains to be answered is if any of these proteins could recognize viral DNA and initiate the innate immune response.

Two functional groups were induced more effectively by IRF-5 than by IRF-7. These are as follows: 1) genes involved in the immune responses; and 2) genes involved in adhesion. Thus, whereas IRF-5 induced 23 ISG genes in BJAB cells, only 14 ISG genes were stimulated by IRF-7 (Table VI). In particular, the antiviral protein viferin was induced much more effectively by IRF-5 (50-fold enhancement) than by IRF-7 (9-fold enhancement). Furthermore, IRF-5 overexpression induced more effectively expression of the IFNB gene. The expression of chemokine genes, including MIP-1α and -β and the subfamily of B chemokines, which are involved in the recruitment of macrophages and T cells to sites of infection or inflammation, was found to be increased only in infected IRF-5-expressing cells, whereas in BJAB/IRF-7-expressing cells, only CXCL11 was expressed (Fig. 6). The induction of chemokines CCL3, CCL4, and CCL5 by IRF-5 has been observed previously (7). In the group of adhesion molecules, IRF-5 stimulated seven genes encoding adhesion proteins in infected cells, whereas IRF-7 induced only two genes. Among the IRF-5-induced genes were carcinoembryonic antigen-related cell adhesion molecule 1, cadherin-associated protein cathepin α1, CD164 antigen, and SDF-1.

**Suppression of Gene Expression by IRF-5 and IRF-7**—Relatively few genes were down-regulated in IRF-5- and IRF-7-expressing cells (Table II). In uninfected BJAB/IRF-5 or BJAB/IRF-7 cells, only 67 and 16 genes, respectively, were down-regulated, and the level of suppression was only in the range of 2–4-fold reduction. From these, only 3 genes were down-regulated by both IRF-5 and IRF-7. In infected cells, 89 and 67 genes were down-regulated by IRF-5 and IRF-7, respectively (Table II). Both IRF-5 and IRF-7 down-regulated the expression of tetraspanine and CD151 that regulates adhesion (34, 35). In infected cells, IRF-7, but not IRF-5, down-regulated 10 genes that function in signal transduction pathways, including Ca-promoted Ras activator (4.5-fold decrease) and genes encoding POU domain transcription factor 1 and 2 (4-fold decrease). Considering that IRF-5 and IRF-7 each induced about 650 genes in infected cells, these results indicate that both of these factors function predominantly as transcriptional activators.

**Analysis of Selected Gene Expression by Semiquantitative RT-PCR Analysis**—To confirm the relative levels of gene expression from microarray analysis, we have selected several genes to compare their levels by semi-quantitative RT-PCR. The results of the microarray analysis have shown that IRF-7 is induced in BJAB/IRF-5-expressing cells after virus infection, but no IRF-5 transcripts were detected in IRF-7-expressing cells. Because the RNA for the microarray analysis was isolated from BJAB- and BJAB/IRF-overexpressing cells at 6 h post-infection, we next examined the kinetics of induction of these two factors in NDV-infected parental BJAB cells (Fig. 3A). Total RNA was isolated at 2, 4, 8, and 16 h post-infection; yet at this time point, IRF-5 and IFNA transcripts were undetectable. At 16 h post-infection, IRF-5, IRF-7, and IFNA transcripts were present in the cells, but the relative levels of IFNA transcripts were significantly decreased. Transient expression of the IFNB gene was observed previously (36). These data indicate that in infected BJAB cells, expression of IRF-7 and IFNA genes precedes induction of the IRF-5 and IFNA genes.

The high levels of IFNB gene expression in infected BJAB/IRF-5-expressing cells was unexpected because we had previously associated IRF-5 primarily with the induction of IFNA...
genes (11). However, stimulation of IFNA genes was not detected by the microarray analysis. To confirm the data from microarray analysis, we have compared the relative levels of IFNA and IFNB transcripts in BJAB, BJAB/IRF-5, and BJAB/IRF-7 cells at 6 h post-NDV infection to that in uninfected cells (Fig. 3B). Although no IFNA or IFNB transcripts could be detected in uninfected BJAB cells, IFNB transcripts were detected in these cells at 6 h post-infection. In BJAB/IRF-5 cells, IFNB transcripts were present in uninfected cells, whereas NDV infection profoundly enhanced the relative levels of these transcripts. Low levels of IFNA transcripts were also detected in infected BJAB/IRF-5, yet their levels were enhanced after NDV infection (Fig. 3B, lanes 3 and 4). These results correlate with our previous findings that IFR-5 overexpression in the human fibroblast cell line 2iTHG induces low levels of IFNA gene expression. Because the IRF-5 polypeptide contains two functional nuclear localization signals, low levels of nuclear IRF-5 can be also detected in uninfected cells (7). In contrast, in BJAB/IRF-5 and BJAB/IRF-7 cells IFNA and IFNB transcripts could be detected only after NDV infection (Fig. 3B, lanes 5 and 6). Finally, we have analyzed the levels of endogenous biologically active IFNs produced in BJAB, BJAB/IRF-5, or BJAB/IRF-7 cells at 6 h post-NDV infection. These results correlate with the RT-PCR analysis. In infected BJAB cells, only low levels of IFNB were induced, whereas in BJAB/IRF-5 and BJAB/IRF-7 cells, synthesis of both IFNb and IFNb could be detected. The levels of biologically active IFNb synthetized in BJAB/IRF-5 cells was about 4-fold higher than in BJAB/IRF-7 cells; the microarray analysis has shown about a 3-fold difference between the levels of IFNB transcripts in IRF-5- and IRF-7-expressing cells. Because both IRF-5 and IRF-7 play a role in the antiviral innate immune response, we next examined the induction of several ISGs that were shown to be up-regulated by microarray analysis (Fig. 4). The OAS genes play an important role in the antiviral effect of type I IFNs (29), and both OAS1 and OAS2 were found to be up-regulated in microarray analysis of RNA from infected cells (Table VI). The RT-PCR analysis (Fig. 4A) confirmed that OAS1 and OAS2 transcripts were indeed up-regulated in the infected BJAB/IRF-5 cells, whereas their up-regulation in BJAB/IRF-7 cells was significantly lower. Several other ISGs were then tested (Fig. 4B), and all of these genes have shown higher levels of expression in infected BJAB/IRF-5 cells than in the infected BJAB cells. Although the majority of tested ISGs was also stimulated in BJAB/IRF-7 cells, although to a lesser extent, no stimulation of PAI-1, IFIT4, or IFI-16 was detected in infected BJAB/IRF-7 cells. However, because BJAB/IRF-5 expressed higher levels of IFNb at 6 h post-infec-

tion than BJAB/IRF-7 cells, we could not exclude the possibility that the increased expression of some of the ISG genes was IFNb-mediated.

To determine whether the enhanced expression of the genes in infected BJAB/IRF-5 and BJAB/IRF-7 cells was IFNb-mediated, we examined and compared expression of some of these genes in BJAB and BJAB/IRF-5 cells treated with IFNb to that in NDV-infected U3A and U3A/IFR-5 expressing cells (Fig. 5). U3A cells have a defect in STAT-1 signaling and therefore are not sensitive to IFNA or -IFN (37). RT-PCR analysis has shown that ectopic IFNb is well expressed in U3A cells, whereas these cells do not express endogenous IRF-7 (Fig. 5A). The results have also shown that most of the IFNb-stimulated genes in BJAB cells are also induced by IFNb in the absence of type I IFN signaling (Fig. 5B, lanes 7 and 8), although some genes, such as ISG20, PKR, and UBE2E, can be induced only by IFNb in BJAB cells (Fig. 5B, lane 2) and not in NDV-infected U3A cells. However, these genes are constitutively expressed in BJAB/IRF-5 and BJAB/IRF-7 cells and NDV-infected U3A/IRF-5-expressing cells, indicating that IFNb and not IFNb is the inducer. Other genes such as UBE2D1 and USP9 are constitutively expressed both in interferon-sensitive and insensitive cells. From the selected genes, only IRF-7 was not expressed in infected or uninfected U3A/IRF-5, whereas it could be induced by IFNb in BJAB cells (Fig. 1). Most interestingly, the induction of TNFS10 was completely dependent on IRF-5 expression.

In the microarray analysis, we have detected significant enhancement of both CXCL11 and CCL4/MIP-1b transcripts (24- and 12-fold, respectively) in BJAB/IRF-5 cells, but no significant enhancement was seen in infected BJAB/IRF-7 cells. We have therefore analyzed expression of these two chemokine genes in IRF-5- and IRF-7-expressing BJAB cells by RT-PCR. Although the relative levels of both CXCL11 and CCL4 (MIP-1b) transcripts were enhanced in infected BJAB/IRF-5 cells and results were comparable with those obtained in the microarrays and our previous studies, stimulation of CXCL11 transcription was observed in infected BJAB/IRF-5 cells by RT-PCR analysis even though no significant enhancement of CXCL11 expression was observed in these cells by microarray analysis (Fig. 6A).

The microarray analysis has shown stimulation of a large number of genes encoding ubiquitin-conjugating enzymes (E2D, E, and G) and ubiquitin-deconjugating enzymes (Ube4a and USP9) in infected IRF-5- and IRF-7-expressing cells. Because induction of ubiquitin-conjugated and -deconjugated enzymes by viral infection was not observed previously, we have examined whether an enhanced expression of these genes can
DISCUSSION

The results presented here show that the transcription factors IRF-5 and IRF-7 activate a signature of IFN exposure as well as a transcription profile unrelated to IFN exposure. When the induced transcripts were grouped into functional categories, it became clear that whereas IRF-5 and IRF-7 stimulated a large number of common genes belonging to all categories, a distinct functional profile was associated with each of these IRFs. A somewhat unexpected finding was that these factors activated a large profile of heterologous genes encoding not only the inflammatory and pro-apoptotic proteins but also proteins of many functional categories. Altogether, the number of IRF-5 and IRF-7 modulated genes was significantly higher in infected than in uninfected cells. Furthermore, the IRF-5 and IRF-7 signatures were predominantly positive, as a much smaller group of transcripts was down-regulated by these two factors. Taken together, these data indicate that IRF-5 and IRF-7 act primarily as transcriptional activators rather than repressors and that the IRF-5- and IRF-7-activated innate antiviral response results in a broad perturbation in the transcriptional profile of cellular genes.

Previous studies have shown that both IRF-5 and IRF-7 are modified by phosphorylation in infected cells and are consequently transported to the nucleus, where they interact with the CBP/p300 acetyltransferases and are part of the transcription complex enhanceosome binding to promoters of IFNA and IFNB genes (6, 7, 17, 25). Thus, it does not come as a surprise that the IRF-5- and IRF-7-activated innate antiviral response results in a broad perturbation in the transcriptional profile of cellular genes.
inhibitor dependent kinases, as well as the cyclin-dependent kinase stimulated by IRF-5 and IRF-7, including cyclins and cyclin-NDV-infected BJAB/IRF-5 cells, indicating that IRF-5-targeted BCL-2 any distinct effect on the growth rate of BJAB or 2fTGH cells, fibrosarcoma line, 2fTGH, did not significantly modulate cell function (25). Of note, overexpression of IRF-5 in the human The growth regulatory function of IRF-5 may be specific to cells may be associated with the activation of cyclin G1 (25). Previously observed growth inhibitory activity of IRF-5 in BJAB ARF-p53 and pRB pathway (39), suggesting that the previous data suggest that a certain nuclear threshold of these IRFs increased the levels of IRF-5 and IRF-7 in the nucleus, these may be required for the transcriptional activation of the majority of induced genes. Another possibility is that transcriptional activation requires the activation of another factor, such as IRF-3, which is localized in the nucleus only after virus-induced phosphorylation by TBK-1 (40).

The established BJAB/IRF-5 cell line, although viable, has shown a progressive reduction in cell growth, upon in vitro propagation, due to the induction of cell cycle regulatory genes, such as p21, and the pro-apoptotic genes BAK, BAX, and caspase 8 (25). In contrast, the BJAB/IRF-7 cell line has grown efficiently on soft agar and did not show any significant changes in growth rate or cell cycle progression (25). For the Affymetrix microarray analysis, the early passages of BJAB/ IRF-5 cells were used, which at that point did not show any significant changes in cell cycle regulation, growth, or induction of apoptosis compared with the BJAB parental cells. Even though both IRF-5 and IRF-7 stimulated the expression of a number of pro-apoptotic genes, a larger number was up-regulated by IRF-5. Most interestingly, although in the later passages of BJAB/IRF-5 uninfected cells we have previously observed the expression of pro-apoptotic genes BAK1, caspase 8, BAX, DR5, and DAP kinase (25), the present analysis detected enhanced expression of a distinct set of apoptotic genes including the TNF superfamily, TNF receptor interacting serine-threonine kinase, BCL-2 antagonist, and caspase 3 only in NDV-infected BJAB/IRF-5 cells, indicating that IRF-5-targeted genes in infected and uninfected cells may be distinct.

In infected cells, several cell cycle regulatory genes were stimulated by IRF-5 and IRF-7, including cyclins and cyclin-dependent kinases, as well as the cyclin-dependent kinase inhibitor p21. From these, the cyclin G1 gene was enhanced most efficiently by both IRF-7 and IRF-5 in infected cells. The growth inhibitory function of cyclin G1 has been linked to the ARF-p53 and pRB pathway (39), suggesting that the previously observed growth inhibitory activity of IRF-5 in BJAB cells may be associated with the activation of cyclin G1 (25). The growth regulatory function of IRF-5 may be specific to lymphoid cells because a number of primary lymphoid cell malignancies show a lack of IRF-5 expression, suggesting that in these cells IRF-5 may have a growth-suppressing function (25). Of note, overexpression of IRF-5 in the human fibrosarcoma line, 2fTGH, did not significantly modulate cell growth (11). Whereas IRF-7 overexpression has not shown any distinct effect on the growth rate of BJAB or 2fTGH cells, expression of cyclin G1, cyclin-activated kinase 2 (CDK2), and cyclin C was also induced in uninfected BJAB/IRF-7-expressing cells. We have shown previously that overexpression of IRF-7 in monocytes induced differentiation to macrophages, indicating that the IRF-7-mediated growth or differentiation effects may be cell type-specific (22).

The largest functional group of genes modulated by IRF-5 and IRF-7 were the genes encoding the signal transduction and transcription-activating proteins. In the signaling category, both IRF-5 and IRF-7 induced several genes encoding the signaling receptors and adaptors such as FGFR4 and MYD88. MYD88 is an important adapter in the TLR7-mediated signaling pathway leading to the activation of IRF-5 and IRF-7. Both IRF factors also induced mitogen-activated protein kinase 9 (MAP) pathways. Although activation of the MAP pathway is generally not associated with the IFN-induced signaling pathway, the gene array analysis of IFN-stimulated genes also identified activation of MAP 2 and MAP 8 kinases (40). Among the transcription factors activated in infected IRF-5- or IRF-7-expressing cells (Table III) were basic transcription factor 3 and zinc finger proteins. IRF-5 induced expression of the STAT3 gene that is activated by IL-6 and IL-10; however, expression of these cytokines was not detected in this analysis. The array analysis has also identified up-regulation of several IRF genes that were also shown to be up-regulated in the array analysis of IFN-induced transcripts (40). Altogether, these data indicate a profound up-regulation of signaling and transcriptional machinery that may explain the unexpectedly broad transcriptional stimulation of the large number of cellular genes belonging to so many functional categories.

Because our study has been focused on genes induced during the antiviral immune response, the first question we asked was which of the IRF-5- and IRF-7-stimulated genes belong to this category. In addition to the cluster of antiviral and immunoregulatory genes, IRF-5 and IRF-7 induced two other gene clusters with known roles in the innate antiviral response. First, genes encoding cellular chaperones such as the various heat shock proteins. The induction of heat shock proteins has been associated with stress responses including viral infection, and it was shown that recognition of HSP70 by TLR1 activates the innate immune response (41–43). Of interest was also the induction of X-linked retinitis pigmentosa 2 gene; although the function of this protein has not yet been completely established, it was shown that it may function as a chaperone (44). These observations indicate that the activation of these genes by IRF-5 and IRF-7 in infected cell may be part of a stress response to viral infection.

The second cluster of genes included those encoding proteins that have a role in protein degradation, such as proteasome subunits and several ubiquitin-conjugating enzymes and ISG15. Proteasome subunits cleave peptides in an ATP-ubiquitin-dependent process in a nonlysosomal pathway. The essential function of immunoproteasome is the processing of class I major histocompatibility complex peptides (45). Ubiquitin-specific protease 9 is the ubiquitin-specific protease on the Y chromosome (46). Most interestingly, both IRF-5 and IRF-7 stimulated expression of several ubiquitin-conjugating enzyme E2 genes, as well as the ubiquitin-deconjugating enzyme, ubiquitin-specific protease 9. The analysis of the promoter region of the UBE2 gene (www.genomatix.de) has shown the presence of both an interferon-stimulated response element and an IRF-binding site. There are several indications that the IFN response may be associated with a modulation of the ubiquitin degradation pathway (47–49). Furthermore, IFN induces the ubiquitin-like protein ISG15 that is conjugated to other proteins (50). Both ubiquitin and ISG15 are using a common ubiquitin ligase E2 (51). The expression of Ube43 protease that removes ISG15 from target proteins is also induced by IFN (53); however, these transcripts were not detected in our RNA preparations. Although there is no evidence that conjugation of ISG15 targets proteins for degradation, it was shown that one of the ISG15-targeted proteins is STAT1. Thus, the ability of IRF-5 and IRF-7 to modulate the components of the ubiquitin pathway may be an important part of the antiviral inflammatory response to infection.

A third set of induced genes with a possible role in the antiviral response, induced preferentially in BJAB/IRF-5 cells, encoded chemokines that play a role in the leukocyte recruitment cascade to sites of inflammation (7). CC-chemokines have also been shown to modulate activity of NK cells and induce tumor rejection (54), and Epstein-Barr virus-induced CCL19 promotes IFN-γ-mediated antitumor response (55). IRF-5 also induced expression of genes modulating cell adhesion like cate-
nin α1, which is a key regulator of cadherin function and affects cell adhesion (56), and stromal cell-derived factor SDF-1 that is a lymphocyte chemoattractant (57).

Our second question was focused on the identification of genes that are direct targets of IRF-5 or IRF-7 in infected cells versus those that are stimulated by type I IFN. We have shown previously (6, 18, 21) in virus-infected cells that IRF-5 and IRF-7 stimulate expression of IFNA, RANTES, and a select group of chemokine genes. The gene array analysis has shown that in infected BJAB cells, IRF-5 induced a stronger antiviral transcription profile than IRF-7 and was a very strong inducer of the IFNB gene. IRF-5 was shown to have a critical role in the induction of IFNA genes (11), but its role in the induction of the IFNB gene has not been addressed. Although we have not detected enhanced levels of IFNA transcripts by the gene array analysis, the RT-PCR analysis has shown that IFNA genes are induced both in BJAB/IRF-5- and BJAB/IRF-7-infected cells.

The set of ISGs expressed in infected IRF-5-expressing BJAB cells only partially overlapped with the profile of highly induced IFNb genes identified by the Affymetrix arrays in IFNβ-treated HT1060 cells (58). Some genes, including those encoding PKR, 2’-5’-OAS, scramblase, and ISG15, were induced at high levels both by IFNβ and IRF-5, whereas we have not detected expression of genes encoding Staf 50, Mx, or Cox mRNA that were detected in IFN-treated cells (58). Also, the transcripts of G proteins, including RAN, NET1A, and GEM and potential modulators of the Ras G protein that were enhanced in IFN-treated cells (40), were not increased in IRF-5- or IRF-7-infected cells. Furthermore, as our data indicate, some of the genes such as ISG20, PKR, UBE2E, or IFI 35 were induced by IRF-5 in U3A cells that have a defect in the type I IFN-signaling pathway, and therefore their induction was independent of type I IFN. In contrast, the IRF-7 gene, transcription of which is stimulated by IFN but not by virus infection, was not induced in virus-infected U3A/IRF-5-expressing cells. However, because U3A cells are derived from the 2fTGH cells that do not express IFN-7, because of hypermethylated (23), the lack of expression of IRF-7 in these cells is not unexpected. These data indicate that the up-regulated gene transcripts detected by microarray analysis belong to the three following categories: 1) genes directly targeted by IRF-5 or IRF-7; 2) genes stimulated both by IFN and IRF-5 or IRF-7; and 3) genes induced only by type I IFN. Our future analysis of gene expression in infected cells expressing IRF-5 and/or IRF-7 has to take into consideration the complexity of the system.

There was also only a partial gene overlap between IRF-5, IRF-7, and dsRNA-induced pathways, because only a few common genes were induced both in infected BJAB/IRF-5 or BJAB/IRF-7 cells and dsRNA-induced cells (59). The common genes include IRF-1, interferon-induced proteins with tetratopeptide repeats 1 (IFIT1), small inducible cytokine A (SCYA4), and a transcription factor E74-like factor 3. Some of the IRF-5- or IRF-7-induced ISGs were also shown recently to be induced by small interfering RNA at high concentrations (60). Thus, the transcription profile of antiviral and inflammatory-related genes induced by IRF-5 and IRF-7 in infected cells is both overlapping and distinct from the transcription profile induced by IFNβ or dsRNA.

Recent studies have demonstrated that viral nucleic acids are recognized by TLR and that the distinct TLRs function as signaling receptors for single-stranded RNA, dsRNA, or bacterial or viral DNA (61). Binding of the dsRNA to TLR3 results in the activation of TBK-1 kinase and phosphorylation of IRF-3 and IRF-7 (40), whereas recognition of single-stranded RNA by TLR7 and TLR8 (62, 63) results in activation and phosphorylation of IRF-5 and IRF-7 but not IRF-3.2 Recently, it was shown that TLR9, which recognizes unmethylated CpG-rich DNA (64), can be also activated by HSV-2 DNA (65). Moreover, our recent unpublished results, as well as the results of others, indicate that virus or dsRNA (66, 67) can induce signaling in a TLR-independent manner. We have therefore searched for an induced expression of transcripts encoding proteins that are not components of the TLR pathway but could recognize viral nucleic acids and possibly activate the antiviral pathway in infected cells. Several transcripts of RNA-binding proteins were identified in infected IRF-5- and IRF-7-expressing cells. These include the putative ATP-dependent RNA helicase DEAE (Asp-Glu-Ala-Asp) box polypeptides, implicated in cellular processes that involve modulation of the RNA secondary structure (68) and pre-mRNA processing and splicing (69), poly(C)-binding proteins (PCBP), where PCBP2 interacts with the stem loop of the internal ribosome entry site of poliovirus RNA and functions as a translational co-activator (70), whereas other PCBP have diverse functions in post-transcriptional controls (71). The plasminogen activator inhibitor 1 (PAI-1) gene, encoding the chromodomain helicase DNA-binding protein 3 interacting protein, was also induced both in infected IRF-5- and IRF-7-expressing cells. This protein interacts with the C-terminal region of the human chromatin-remodeling factor CHD-3 and has been implicated as a risk factor for IgE-mediated asthma and allergic disease (72). Both IRF-5 and IRF-7 also induced transcripts of KH domain containing RNA-binding proteins. The KH domain is a conserved domain in many RNA-binding proteins including a protein encoded by the fragile X mental retardation gene product (FMRI). Mutation in KH domain impairs RNA binding (73).

It is noteworthy to consider, in this context, whether induction of the DNA and chromatin-modulating genes such as topoisomerase I and II or SWI/SNF, also has a role in the recognition of viral DNA, because we have shown previously (22) that changes in chromatin architecture were able to activate transcription of the IRF-7 gene. Although there is no evidence yet that any of these nucleic acid-binding proteins are recognized by viral nucleic acids in infected cells, or that they activate the antiviral pathways, the role of RNA helicase RIG-1 in the cellular antiviral response to viral infection was demonstrated recently (74).

Taken together, the results presented in this study show that IRF-5 and IRF-7 activate a heterogeneous transcriptional profile in the B cell line BJAB, and that the majority of regulated genes could be grouped into a large number of functional categories. As such, there is only partial overlap between IRF-5- and IRF-7-modulated genes and genes induced by IFNβ or dsRNA. Although IRF-5 and IRF-7 stimulated common sets of genes in all clusters, the profile of the IRF-5-induced genes was not a subgroup of genes induced by IRF-7. The noted differences were a strong transcriptional profile of the antiviral early inflammatory genes in infected IRF-5-expressing cells, whereas the transcripts induced specifically by IRF-7 were enriched in the group of mitochondrial genes and genes affecting the DNA structure. These data indicate that the antiviral response is accompanied by modulation of expression of a large number of cellular genes belonging to many functional categories. It still remains to be elucidated which of the IRF-5- and IRF-7-targeted genes, identified in this study, plays a role in a natural resistance to infection or in the pathogenesis of autoimmune disease.

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Global and Distinct Targets of IRF-5 and IRF-7 during Innate Response to Viral Infection
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