Two Discrete Promoters Regulate the Alternatively Spliced Human Interferon Regulatory Factor-5 Isoforms

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Interferon regulatory factor-5 (IRF-5) is a mediator of virus-induced immune activation and type I interferon (IFN) gene regulation. In human primary plasmacytoid dendritic cells (PDC), IRF-5 is transcribed into four distinct alternatively spliced isoforms (V1, V2, V3, and V4), whereas in human primary blood mononuclear cells two additional new isoforms (V5 and V6) were identified. The IRF-5 V1, V2, and V3 transcripts have different noncoding first exons and distinct insertion/deletion patterns in exon 6. Here we showed that V1 and V3 have distinct transcription start sites and are regulated by two discrete promoters. The V1 promoter (P-V1) is constitutively active, contains an IFN-E consensus-binding site, and is further stimulated in virus-infected cells by IRF family members. In contrast, endogenous V3 transcripts were up-regulated by type I IFNs, and the V3 promoter (P-V3) contains an IFN-stimulated responsive element-binding site that confers responsiveness to IFN through binding of the ISGF3 complex. In addition to V5 and V6, we have identified three more alternatively spliced IRF-5 isoforms (V7, V8, and V9); V3 and V6 were expressed in peripheral blood mononuclear cells from healthy donors and in immortalized B and T cell malignancies, whereas expression of V7, V8, and V9 transcripts were detected only in human cancers. The results of this study demonstrated the existence of multiple IRF-5 spliced isoforms with distinct cell type-specific expression, cellular localization, differential regulation, and dissimilar functions in virus-mediated type I IFN gene induction.

Virus infection results in the activation of a defined set of cellular genes involved in host antiviral defense. Transcription factors of the interferon (IFN) regulatory factor (IRF) family have been identified as having important roles in innate immunity by participating in both the immediate-early transcriptional response to infection and the secondary response to cytokines (1–4). While playing an important role in the antiviral immune response, IRFs also participate in cell growth regulation and apoptosis (5, 6). IRF-5 is one of the most recently characterized members of the IRF family (2, 7–14). It encodes a 60–63-kDa polypeptide that was originally identified as a regulator of type I IFN gene expression (7, 9). However, recent studies have indicated that it plays a role in many aspects of host defense (11), including induction of multiple cytokines and chemokines involved in the recruitment of T lymphocytes (8). Subsequently, it has been shown that IRF-5 itself is regulated by type I IFN (8, 10), indicating an important regulatory pathway for the controlled induction of multiple immunomodulatory genes (11).

The role of IRF family members in the induction of cell arrest and cell death has been well established (5, 10, 12, 15–17). Although IRF-5 participates in the virus-induced signaling cascade, its expression is also modulated by p53 (12), and it has a role in the apoptotic signaling pathway that is p53-independent (10). Thus, IRF-5 modulates the expression of a number of factors involved in cell cycle regulation and apoptosis, independent of viral infection (10). The ability of IRF-5 to induce expression of proteins involved in the regulation of cell growth, apoptosis, and immunomodulation represents an important defense mechanism against extracellular stress, including viral infection. How significant is the overlapping cross-talk between IFN and apoptotic signaling pathways is currently unknown, yet it has been suggested recently that type I IFN can also induce p53 (6). Altogether, the current data suggest an important role for IRF-5 in both IFN- and p53-mediated signaling pathways.

To date, a large number of IRF-5 sequences have been deposited to GenBank™. Most interesting, the first complete coding sequence of human IRF-5, isolated from lymphocytes and deposited in 1996 to GenBank™ (accession number U51127), was not functionally characterized. Subsequently, we had cloned two new isoforms of IRF-5, here termed variants 3 and 4.®
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V3; GenBank™ accession number AY504946) and 4 (V4; GenBank™ accession number AY504947) from a dendritic cell library (7), and we have characterized the identical polypeptide they encode (2, 7–11). In the past 2 years, two additional isoforms, termed variant 1 (V1; isoform a; NM_002200; U51127) and variant 2 (V2; isoform b; NM_032643), have been deposited to GenBank™. These two cDNAs encode proteins distinct from the isoforms 3 and 4 that we have identified, and their functions have yet to be characterized. Sequence analyses of these four isoforms reveal that they differ in their first exon and in the pattern of deletion(s) in exon 6. Alternative splicing of pre-mRNA has been described for other IRF family members, including IRF-1, -3, and -7, in which each isoform may be differentially expressed depending on the cell or tissue type (18–22). However, differential isoform expression and regulation of these IRFs at the promoter levels have not been addressed yet, even though the promoters of each have been isolated and partially characterized (23–25).

The aim of the present study was to examine the regulated transcription of the human IRF-5 gene. The transcription analyses demonstrate that two distinct promoter regions differentially regulate exon 1 of V1-associated transcripts and exon 1 of V3 transcripts, where the P-V1 promoter responds to viral infection and the P-V3 promoter responds to IFN stimulation. However, the transcription pattern of the IRF-5 gene is more complex, and we have identified nine distinct alternatively spliced IRF-5 mRNAs (V1–V9). Here we describe the alternative splice patterns of the new IRF-5 isoforms, their cell type–specific expression, localization, inducibility, and function in virus-mediated type I IFN gene induction.

MATERIALS AND METHODS

Cell Culture and Reagents

Human HeLa, 293T, A549, and Madin-Darby bovine kidney cells were obtained from the American Type Tissue Collection (ATCC). Human fibroblasts (2fTGH) were from G. Stark (Cleveland Clinic), and primary human fibroblasts were from G. Hayward (Johns Hopkins University). These adherent cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Daudi, Namalwa, and BJAB B cell lymphomas, THP-1 acute monocytic leukemia, U937 monocytic lymphoma, and BC-1 primary effusion lymphomas (PEL) were from ATCC and cultivated in RPMI with 10% fetal bovine serum. Peripheral blood mononuclear cells (PBMC), plasmacytoid dendritic cells (PDC), monocytes (MO), natural killer (NK), and B and T cells were isolated and purified as described previously (26–28) from fresh heparinized peripheral blood obtained with informed consent from healthy volunteers. PDC (Lin−, CD123−, CD11c+, and human leukocyte antigen-DR+ cells) were isolated by using BDCA-4 microbeads, MO by using CD14 beads, NK by using CD16 beads, T cells by using CD3 beads, and B cells by using CD19 beads (Miltenyi Biotec). Cell purities were all greater than 95%, as determined by flow cytometry. The human studies were approved by the Institutional Review Board of the New Jersey Medical School. The experiments demonstrate that two distinct promoter regions differentially regulate exon 1 of V1-associated transcripts and exon 1 of V3 transcripts, where the P-V1 promoter responds to viral infection and the P-V3 promoter responds to IFN stimulation. However, the transcription pattern of the IRF-5 gene is more complex, and we have identified nine distinct alternatively spliced IRF-5 mRNAs (V1–V9). Here we describe the alternative splice patterns of the new IRF-5 isoforms, their cell type–specific expression, localization, inducibility, and function in virus-mediated type I IFN gene induction.

Reverse Transcription (RT)-PCR and IRF-5 Isoform Expression Analysis

Total RNA was isolated using the Qiagen RNeasy mini kit, and 1 µg was reverse-transcribed to cDNA using oligo(dT) primers. From this mixture of cDNAs, IRF-5, IRF-7, IFNA, IFNB, and β-actin cDNA were amplified by PCR as described (7, 29). The IRF-5 primers (7) (primers 19 and 20, Table 1) used to detect all known IRF-5 isoforms bind to a region in the carboxyl terminus spanning exons 7–9. For screening of IRF-5 isoform expression associated with a specific exon 1, sense primers that were specific for each first exon and a common antisense primer that results in amplification through exon 1 were used (Fig. 1B). IRF-5 V1 cDNA was amplified with PIRF5V1-F77 (specific to Ex1V1), V2 cDNA with PIRF5V1-F13 (specific to Ex1V2), or V3 cDNA with PIRF5V3-F25 (specific to Ex1V3) and PIRF5-R7 (exon 4 specific primers) (primers 21–24, Table 1). Optimal PCRs conditions were: 1 cycle at 94 °C (2 min); 25 cycles at 94 °C (1 min), 63.9 °C (1 min), and 72 °C (1 min 30 s); and 1 cycle at 72 °C (5 min). For the determination of exon 6 patterning in various cell types, primers that amplify exon 5 (primer 25) through exon 7 (primer 26) were used. Optimal PCR conditions were as follows: 1 cycle at 94 °C (4 min); 40 cycles at 94 °C (1 min), 63.9 °C (1 min), and 72 °C (1 min 30 s); and 1 cycle at 72 °C (5 min).

In order to distinguish between V1- and V4-specific expression and to determine expression of new IRF-5 variants associated with their specific deletion/insertion pattern(s) in exons 5–7, exons 1–7 were amplified with each specific exon 1 sense primer (primers 21–23, Table 1 and Fig. 1B) and a common antisense primer (primer 26, Table 1 and Fig. 1B). Optimal PCR conditions were identical to the exon 1–4 PCR amplification, except 37 cycles were used. PCR products were either electrophoresed on a 1.5% agarose gel and/or cloned to the pCR®2.1-TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and sequenced.

Expression Plasmids, SuperScript One-step RT-PCR, and Cloning of Full-length IRF-5 Isoforms

The IRF-1, IRF-3, IRF-5 (V3 and V4), and IRF-7 expression vectors were described (7, 30–32). Full-length IRF-5 V2 was subcloned from the pOTB7 plasmid (Open Biosystems) to pCMV-tag2b (Stratagene Inc.) at EcoRI and XhoI restriction sites. For the cloning of new IRF-5 isoforms, total RNA was isolated from PBMC or THP-1 cells. Superscript one-step PCR was performed using a Pfu proofreading polymerase with sense primers specific to each first exon (primers 21–23, Table 1) and a common antisense primer that results in amplification through exon 9 (primer 27, Table 1). Briefly, cDNA was synthesized at 45 °C (30 min)
and 94 °C (2 min). PCR cycling conditions were as follows: 35 cycles 94 °C (30 s), 60 °C (30 s), and 72 °C (1 min 30 s). PCR products were electrophoresed on a 1% agarose gel, purified, and then cloned directly to the pCR 4-Blunt-TOPO vector (Invitrogen). Nucleotide sequences (V5, accession number AY693665; V7, accession number AY693668; and V9, accession number AY693669) were deposited to GenBankTM.

Oligonucleotide Pull-down and Immunoblot Assays

Biotinylated antisense oligonucleotides were annealed with the corresponding sense oligonucleotides (primers 28–31, Table I). Briefly, 4 μg of biotinylated DNA was incubated with 100 μg of DynaBeads M-280 streptavidin (Dynal Inc.) for 16 h in 200 μl of TEN buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 0.1 M NaCl), and unbound DNA was removed by extensive washing. Cell extracts were prepared as described previously (29) from control untreated BJAB cells or cells stimulated with 500 units/ml IFN-α for 16 h. Extracts (300 μg of protein) were incubated with bound DNA (7, 24) and washed, and the bound proteins were identified by immunoblotting. Rabbit polyclonal antibodies against ISGF3-γp48, STAT1, and STAT2 were purchased from Santa Cruz Biotechnologies. Signals were visualized using the ECL detection reagents (Amersham Biosciences).

Treatments, Transient Transfections, Infections, and Reporter Assays

Purified PBMC, PDC, MO, NK, and B and T cells were stimulated with either IFN-α2b (10,000 IU), HSV-1 (multiplicity of infection of 1), or CpG DNA (40 μg/ml) for 6 h at 37 °C with 5% CO2. BJAB and Daudi were stimulated with IFN-α2 (1000 units/ml) for 16 h; Daudi and THP-1 were infected with NDV (40 plaque-forming units) for 16 h; THP-1 was stimulated with VSV (multiplicity of infection of 2) or R848 (10 μM) for 16 h. 2TGH cells were transiently transfected with gfp-IRF-5 variant expression plasmids using the Superfect transfection reagent (Qiagen) for 30 h and then examined by fluorescent microscopy using a Nikon TE-200 and DXM12000F at ×40 magnification.

Dual Luciferase Assay—293T, 2TGH, or HeLa cells were transfected in 60-mm dishes using SuperFect. 2 μg of the reporter plasmid DNA and 0.1 μg of pRL-CMV (internal control; Promega) were used for each transfection. In co-transfection experiments a 1:1 ratio of the reporter and expression plasmid (2 μg each) was used. The final concentration of transfected DNA was kept constant in all co-transfection assays. Cells were harvested 24 h post-transfection unless additional treatments were performed. For further treatments, cells were split 12–16 h post-transfection into 6-well plates and incubated for another 8 h, after which medium was changed either with Dulbecco’s modified Eagle’s medium containing recombinant IFN-α2 (500 units/ml), or NDV (50 μM/mL). Treatments/infections were incubated an additional 16 h, and cells were then harvested and lysed with 1× Passive Lysis Buffer (Promega). Protein concentrations were determined using the Bio-Rad Protein Assay according to manufacturer’s instructions. Luciferase assays were carried out using the Dual Luciferase Assay kit according to the manufacturer’s specifications (Promega). Experiments were repeated at least three times in duplicate.

SAP Assay—2×105 2TGH or 2TGH/IRF-5 V3 (7) stable expressing cells were transfected with a constant amount of DNA (2 μg/6-well plate) by using the Superfect transfection reagent (Qiagen). Equal amounts of the indicated SAP reporter plasmids and IRF5-expressing plasmids were co-transfected with the β-galactosidase expression plasmid (50 ng). Transfected cells were split 16 h later, incubated for an additional 6 h, and either left uninfected or infected with NDV for another 16 h. The SAP was determined as described (29, 33). Each experiment was repeated at least three times; β-galactosidase expression levels were used to normalize the difference in transfection efficiency.

IFN Cytotoxic Effect Assay—2TGH cells (0.5 × 105 cells/well of a 6-well plate) were transiently transfected with 50 ng of each FLAG-tagged IRF-5 full-length variant expression vector. 16 h later, cells were split in duplicate to a 24-well plate, incubated for 8 h, and either left uninfected or infected with NDV for an additional 16 h. The levels of biologically active type I IFN or IFN-α were determined in the cell culture supernatants by the viral cytotoxic effect assay (34). VSV was used as the challenging virus, and the cytotoxic effect was determined in human fibroblasts (type I IFN) or Madin-Darby bovine kidney cells (IFN-α).
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RESULTS

Structural Analysis of Isoform-specific Exons of the Human IRF-5 Gene—Although cDNA sequences for human IRF-5 isoform 1 (V1) and 2 (V2) have been deposited to GenBank™, the proteins encoded by the cDNAs have not been functionally characterized. We had identified previously two new mRNA isoforms (V3 and V4), and we functionally characterized the identical polypeptide encoded by the two cDNAs (2, 7–11). Here we examine the transcriptional regulation of these four isoforms (Fig. 1).

Sequence alignments between the four IRF-5 isoforms indicate two major differences: (1) the utilization of distinct alternative first exons in V1 (Ex1V1), V2 (Ex1V2), and V3 (Ex1V3), where V1 and V4 share the same first exon; and (2) the distinct pattern of deletion(s) in exon 6 of V1, V2, and V3, where V3 and V4 share the identical deletion pattern (Fig. 1B). Considering these differences in cDNAs, we first determined the organization of introns and exons in the human IRF-5 gene (Fig. 1A). Searches for homologous sequences using BLAST software mapped the V1, V2, and V3 exon sequences to the recently described human chromosome 7 (35). This mapping is consistent with our previous chromosomal assignment of the IRF-5 gene (7). Based on the cDNAs of IRF-5 deposited to GenBank™, there are at least three first exons (Ex1V1, Ex1V2, and Ex1V3) and eight common exons thereafter (Fig. 1, A and B). Sequence alignments between the four IRF-5 isoforms are shown in boldface (pr#) and correspond to the primer list in Table 1. C, characterization of the 5'-UTR of the IRF-5 mRNA. 5'-RACE was performed using mRNA isolated from either unstimulated or IFN-stimulated BJAB cells. Three 5'-RACE experiments were performed, one for each first exon of IRF-5; the two successful experiments are shown. Electrophoretic analysis of Ex1V1 5'-RACE products from unstimulated BJAB cells shows a single band, resulting from nested PCR amplification of the three original bands. The identification of a nested PCR product from IRF-5 Ex1V3 following 5'-RACE was possible in IFNα2-stimulated BJAB cells. D, sequencing of 5'-RACE products demonstrated that the V1 and V3 transcription start sites match the consensus sequence for transcription initiation. The A residue in each site has been assigned as position +1 of the transcripts.

Characterization of the 5'-UTR of IRF-5 mRNA—to investigate the transcriptional mechanism(s) underlying the regulation of V1, V2, V3, and V4 transcript expression, we identified and characterized the IRF-5 gene promoter sequences. To this effect, we first determined the transcription start site(s) of the IRF-5 gene by 5'-RACE. Because BJAB cells contain a functional/inducible IRF-5 promoter, as demonstrated by the stimulation of IRF-5 gene expression after IFN treatment (8, 10), we used RNA extracted from BJAB cells for the 5'-RACE experiments. Based on the IRF-5 DNA sequences deposited to GenBank™, we performed three 5'-RACE experiments, one for each of the known first exons (Fig. 1A). As shown in Fig. 1C, when unstimulated BJAB cells were subjected to 5'-RACE using an antisense primer specific for Ex1V1, three distinct products were visualized. Following nested PCR, one distinct band was detected (Fig. 1C, left panel), indicating the presence of a transcription start site for IRF-5 isoforms containing Ex1V1 in unstimulated BJAB cells. When we used the same approach to identify the 5'-ends of IRF-5 V2 and V3 tran-
scripts, we were unable to amplify either of the two 5'-UTRs in unstimulated BJAB cells. This is likely because of the very low levels of general transcription initiation ATG codon of human IRF-5. By using the TRANSFAC data base (www.gene-regulation.com), we have identified a TATA box upstream of Ex1V1 and Ex1V3 but not upstream of Ex1V2 or exon 2. This analysis, combined with the 5'-RACE data, suggests that the IRF-5 gene contains only two promoters, as defined by the presence of the TATA boxes and transcription start sites upstream of Ex1V1 and Ex1V3. However, it is also possible that the Ex1V2 and exon 2 5'-flanking regions may be functioning TATA-less promoters. It has been shown previously that mRNA transcription initiating from multiple sites, as 5'-RACE demonstrates, is usually driven by TATA-less promoters, and thus, we cannot exclude the possibility that additional transcripts initiating from these two 5'-flanking regions will be identified.

In order to test whether all of these 5'-flanking regions (Fig. 1A, P-V1, P-V2, P-V3, and P-Ex2) function as constitutive or inducible promoters, DNA fragments corresponding to P-V1, P-V2, P-V3, and P-Ex2 (Fig. 1A) were amplified from BJAB genomic DNA and cloned into the promoter-less luciferase reporter pGL3-Basic. The putative promoter constructs were named according to the exon they flank. These four putative promoter constructs were transiently transfected to HeLa, 293T, or 2TGH cells and assayed for luciferase activity. Of the four constructs, only the P-V1 and P-V3 promoters displayed basal activity, suggesting that only these two 5' regions of the IRF-5 genome can function as constitutive promoters in the cell types examined (data not shown). Because the P-V1 promoter contains an IRF-E consensus site and the P-V3 contains an ISRE site, we have focused on the role of these two sites in the regulation of IRF-5 gene expression in virus-infected cells.

**Virus-mediated Activation of the IRF-5 Isoform 1 Promoter (P-V1)**—We first investigated the function of the IRF-E site, a known recognition site for members of the IRF family. Reporter constructs containing the entire wild type 0.64-kb P-V1 region or this region in which the IRF-E site was mutated were both inserted upstream of the luciferase gene in the pGL3-Basic vector (Fig. 2, A and B). The activities of these constructs were analyzed in transient transfection assays in
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293T, HeLa, and 2fTGH cell lines (Fig. 2, C and D, depict data from HeLa cells, however all cell lines gave similar results). Transfection of the full-length wild type P-V1 promoter resulted in significant luciferase reporter activity that was ~100 times higher than the activity of the promoterless pGL3-Basic control vector (Fig. 2C), indicating that in the cell types examined, the P-V1 promoter is constitutively active. Infection with NDV enhanced promoter activity by 3-fold. When the P-V1 promoter construct was co-transfected with pRL-CMV, and cells were treated with IFNs for 16 h. In all transfection assays, the levels of reporter firefly luciferase activity were normalized to a constant level of pRL-CMV Renilla luciferase activity that served as an internal control. The results are presented as a percentage of the luciferase activity of the P-V3 construct in untreated cells that is considered as 100%. The error bars represent the S.D. of three independent experiments repeated in duplicate. D, analyses of the binding profile of proteins to the ISRE consensus site and the IRF-5 P-V3 promoter ISRE site. Biotinylated oligodeoxynucleotides containing consensus ISRE and IRF-5 ISRE sites were incubated with extracts of IFN-α-stimulated and unstimulated BJAB cells. The bound proteins were eluted from the beads and analyzed by immunoblot (IB) with antibodies against ISGF-3p48, STAT-1, and STAT-2. The signals were visualized by the ECL kit.

Interferon-mediated Activation of the IRF-5 Isoform 3 Promoter (P-V3); Regulation by IFN—We have observed previously that transcription of the IRF-5 gene is stimulated by human IFN-α2 (8, 10) (Fig. 1C). Because 5′-RACE from the IRF-5 Ex1V3 was only successful in BJAB cells stimulated with IFN-α2 and the P-V3 promoter contains an ISRE site (Fig. 3A), suggesting its role in the IFNα2-mediated induction of IRF-5 V3 transcription (Fig. 1C), we examined whether the ISRE site was active. Reporter plasmids containing the entire 1.2-kb P-V3 region or its analogue with a mutated ISRE site (P-V3-IRFEM) were analyzed for reporter activity by transient transfection to 293T, HeLa, or 2fTGH cells (Fig. 3, B and C). Transfection of the full-length wild type putative P-V3 promoter resulted in significant luciferase reporter activity that was ~100 times higher than when the promoterless vector pGL3-Basic control was transfected alone (Fig. 3C), indicating that the P-V3 promoter is constitutively active in these cells. Treatment of the P-V3-transfected cells with IFN-α2 for 16 h resulted in about a 3-fold induction above the basal level activity (Fig. 3C), whereas IFN-γ treatment led to only a 2-fold increase. Mutation of the ISRE element (Fig. 3, A–C, P-V3-IRFEM) abolished the response to IFN-α2 without affecting the base-line activity of the P-V3 reporter (Fig. 3C). These results indicate that the 5′-flanking region of IRF-5 V3 can function as a promoter and that the ISRE site in this promoter confers the IFN-α2-mediated stimulation of the IRF-5 gene.

The induction of IFN-stimulated gene (ISG) promoters by IFN-α is generally mediated by binding of the ISGF3 complex, which consists of p48 (IRF-9), STAT1, and STAT2 proteins, to...
an ISRE consensus site (37). We have therefore used the oligonucleotide pull-down assay to examine whether the ISGF3 complex binds to the ISRE present in the IRF-5 P-V3 promoter. Because this ISRE differs slightly from the consensus ISRE sequence (Fig. 3B), we analyzed and compared the binding of the ISGF3 complex from whole cell extracts of unstimulated and IFN-α2-stimulated BJAB cells to the biotin-labeled P-V3 ISRE and the consensus ISRE oligonucleotides immobilized on magnetic beads. The bound proteins separated on SDS gels were identified by immunoblotting. Results show that both the P-V3 ISRE and the consensual ISRE bound p48, STAT1, and STAT2 from the extracts of IFN-treated cells, whereas only low levels of STAT2 bound in uninfected cells (Fig. 3D). These data demonstrate that the P-V3 ISRE is functional and that the ISGF3 complex contributes to the IFNα2-mediated activation of the IRF-5 P-V3 promoter.

**IRF-5 Isoforms Are Differentially Expressed and Regulated in Purified Primary Cell Subpopulations from Healthy Donors**—By Northern blot analysis and RT-PCR, we have shown previously that the constitutive expression of IRF-5 transcripts occurs primarily in normal lymphoid tissue, peripheral blood lymphocytes, and dendritic cells; yet these transcripts were not detected in a variety of immortalized B and T cell leukemias (7) or primary hematological malignancies (10). At the time of these studies, however, the presence or functional role of multiple IRF-5 isoforms was not known. To date, only the functional roles for IRF-5 V3/V4 have been elucidated (2, 7–11).

Recently, it was shown that IRF-5 transcripts were constitutively expressed at high levels in PDC (26, 38), and the high levels of IRF-5 expression may contribute to the high levels of IFN-α induced in these cells after stimulation with virus. IRF-5 V3 and V4 cDNAs were initially cloned from a dendritic cell library (7). Here we extend our previous findings to characterize further the constitutive and inducible expression of IRF-5 isoforms in purified PBMC, PDC, MO, NK, and T and B cells from healthy donors and in human immortalized cancer cell lines.

Primer sets that specifically recognize exon 1 of each isoform (Fig. 1B, Ex1V1, Ex1V2, and Ex1V3) and a common region in exon 4 of IRF-5 were optimized using cloned IRF-5 V1/4, V2, and V3 cDNAs. As shown in Fig. 4A, PCR amplification using the exon 1-specific sense primers was isoform-specific. Because we have detected previously high levels of general IRF-5 transcripts in PBMC and PDC (26), we first examined the levels of constitutive and inducible exon 1-specific IRF-5 isoform expression in similar purified cell populations. The isolated cell populations were greater than 95% pure as determined by flow cytometry. Cells (PBMC, PDC, MO, NK, and T and B cells) were either left unstimulated or stimulated with IFN-α, HSV-1, or CpG as described under "Materials and Methods." The effect of CpG DNA on IRF-5 gene transcription was examined because the stimulation of PDC with oligodeoxyribonucleotides containing unmethylated CpG motifs has been shown to produce high levels of IFNα (38–40). Results in Fig. 4B show...
that the Ex1V1 transcripts were detected in all types of unstimulated cells except T cells; the highest levels were detected in unstimulated MO and B cells (top panel). Although treatment with IFN-α or CpG had either no effect (PDC and B cells) or decreased (PBMC, MO, NK, and T cells) IRF-5 Ex1V1 transcript levels, stimulation with HSV-1 enhanced the levels of Ex1V1-associated transcripts in PBMC, NK, and T cells. Unstimulated and stimulated (with HSV or IFN-α) B cells expressed the highest levels of IRF-5 Ex1V1-associated transcripts that were unaffected by these inducers; expression was nearly identical in all three samples even at lower cycle numbers where amplification was not saturated but was in the linear range (data not shown). In comparison, transcripts associated with Ex1V2 were either very low (MO, NK, and B cells) or nearly undetectable (PBMC, PDC, and T cells) at 37 amplification cycles (Fig. 4B, middle panel). However, transcripts were detected in all cell types examined by increasing the amount of amplified cDNA and by using a higher number of amplification cycles compared with that required for the amplification of Ex1V1 or Ex1V3 transcripts. These results suggest that IRF-5 V2 transcripts, although present, were expressed at significantly lower levels than V1 or V3 transcripts (Fig. 4B). Furthermore, even at the higher number of amplification cycles, levels of the Ex1V2 transcripts were unaffected by stimulation with HSV-1, IFN-α, or CpG (data not shown). Although Ex1V3 transcripts were detected in unstimulated PDC, MO, and B cells, stimulation with IFN-α significantly up-regulated the levels of Ex1V3 transcripts in all cell types examined (Fig. 4B, lower panel). The origin of the multiple amplification bands for Ex1V3 PCR in the IFN-α-stimulated PBMC, PDC, and MO, and all B cell samples except for the CpG-stimulated B cells is presently unclear. However, these data may suggest the presence of multiple Ex1V3-associated IRF-5 isoforms. Although stimulation with HSV-1 did not affect Ex1V3 transcripts in most of the cells, transcript levels were dramatically decreased in MO and increased in NK, T, and B cells. CpG stimulation had either no effect or slightly decreased Ex1V3 transcript levels in all cell types except NK cells, where levels were up-regulated compared with unstimulated cells. However, stimulation with CpG did induce high levels of IFNA transcripts in PBMC and PDC but not in B cells and IRF-7 transcripts, and although dramatically up-regulated in PBMC, induction was less dramatic in PDC, and levels were down-regulated by CpG in B cells (Fig. 4B, right panel).

We next examined IRF-5 isoform expression in human immortalized cancer cell lines, Daudi, BC-3 and BCBL-1, THP-1, U937, and Namalwa. For these studies, Daudi cells were either left unstimulated, infected with NDV, or stimulated with IFN-α2. As shown in Fig. 4C, Ex1V1-associated transcript levels were up-regulated after infection with NDV, whereas V2 and V3 transcript levels were nearly undetectable in either the uninfected or infected samples. In comparison, IRF-5 V3 transcript levels were specifically induced after treatment with IFN-α2. Constitutive isoform expression was then examined in the two PEL cell lines: where Ex1V1-associated transcripts were expressed at high levels in BCBL-1 and not BC-3 cells, and both cell lines expressed low levels of constitutive IRF-5 V3. On the other hand, THP-1 cells expressed high constitutive levels of both Ex1V1 and Ex1V3 transcripts and low levels of Ex1V2 transcripts. Upon further stimulation with either NDV or VSV, only Ex1V1-associated transcript levels were up-regulated (Fig. 4C). With the recent finding that IRF-5 is critical for TLR7/8-mediated induction of type I IFNs in THP-1 cells, we examined the inducible expression of IRF-5 isoforms by R848 (41). We found that although THP-1 cells indeed expressed the

| IRF-5, first exon | No. of distinct new isoform clones | Total no. of isoform clones | Percent occurrence of alternative splicing from each distinct first exon |
|------------------|----------------------------------|-----------------------------|--------------------------|
| Ex1V1            | 100                              | 125                         | 80                       |
| Ex1V2            | 3                                | 60                          | 5                        |
| Ex1V3            | 4                                | 74                          | 5.4                      |

Ex1V1-, V2-, and V3-associated transcripts constitutively, albeit at distinct levels, only the IRF-5 V3 transcripts were up-regulated by R848. Furthermore, the examination of constitutive IRF-5 expression in U937 and Namalwa revealed the overlapping presence of Ex1V1-associated transcripts, and low levels of V3 transcripts were only detected in U937 cells. Levels of human β-actin transcripts are shown as a control for RNA levels.

Taken together, results from the 5’-RACE experiments, reporter assays and RT-PCR indicate that only the P-V3 promoter and its Ex1V3-associated transcripts are stimulated by IFN treatment (Fig. 3C and Fig. 4, B and C). Furthermore, these data support the finding that IRF-5 Ex1V1-associated transcript expression is regulated by the virus-mediated activation of P-V1 (Fig. 4, B and C). The differences in the levels of constitutive and inducible IRF-5 isoform expression in purified immune cell subpopulations from healthy donors and in tumor cell lines of different origin suggest that expression of the IRF-5 isoforms is cell type-specific.

**Isolation and Cloning of Five New Alternatively Spliced IRF-5 Isoforms**—Whereas our previous screening method for IRF-5 isoform expression yielded valuable information with regard to expression and induction of exon 1-associated transcripts, we were unable to distinguish between variants 1 and 4 because they both utilize the same exon 1 (Ex1V1). Moreover, the previous screening method did not allow for the identification of potential new alternatively spliced isoforms. In order to distinguish between V1 and V4 transcripts, we optimized primer sets that amplified from exon 1 to exon 7 that includes the region where the different deletion patterns occur (Fig. 1B). PCR fragments amplified with each of the three primer sets (primers 21–23 and 26, Table I) from THP-1, PDC, PBMC, Namalwa, U937, and/or BCBL1 were purified, cloned, and sequenced. At least 75 positive clones from each ligation reaction were isolated and sequenced. From the sequences of these clones, we identified nine new IRF-5 isoforms, most of which were alternatively spliced from Ex1V1 (Table II). Five of these new IRF-5 isoforms were re-cloned in order to obtain full-length IRF-5 cDNAs (Fig. 5A). Expression of the IRF-5 isoforms 5 and 6 was detected in PBMC and THP-1 cells, whereas expression of IRF-5 isoforms 7–9 was identified only in THP-1 cells. In addition, we were able to clone the full-length IRF-5 isoform 1 from THP-1 cells. All of the new IRF-5 isoforms share Ex1V1 except for V9 that contains Ex1V2. IRF-5 V5 contains the full genomic sequence of IRF-5 that does not show any deletions in exon 6 (Fig. 5A). IRF-5 V6 is a spliced isoform from Ex1V1 with a coding region identical to V2. IRF-5 V7 is lacking the majority of the DNA binding domain and thus may function as an endogenous dominant negative (DN) mutant of full-length IRF-5, it has the same deletion pattern as V2. IRF-5 V8 has a large single deletion that spans most of exons 6 and 7. For IRF-5 V9, although we obtained the full-length mRNA, alternative splicing led to the introduction of an early termination
Regulation of IRF-5 Spliced Isoforms

Figure 5: Identification of new alternatively spliced IRF-5 isoforms. A, multiple alignments of five new full-length IRF-5 isoforms amplified from human primary PBMC and/or human cancer cells. The five isoforms are here referred to as variant 5 (V5), variant 6 (V6), variant 7 (V7), variant 8 (V8), and variant 9 (V9). The exon 1 of each new isoform is shown, as are the deletion(s) in exon 5–7, and the deletion of exon 2 in V7. B, expression of each FLAG-tagged IRF-5 isoform in 2fTGH was determined by immunoblot analysis with M2 anti-FLAG antibodies (Sigma). 20 μg of whole cell lysates were run on 10% SDS-PAGE. Levels of actin are shown as a control for loading and protein integrity. C, subcellular localization of gfp-IRF-5 fusion proteins in uninfected cells.

codon, and the protein is therefore missing its carboxyl terminus. By using both RT-PCR and sequencing methods, the levels of the IRF-5 isoforms appear to be distinctly expressed. Puriﬁed PDC expressed only variant 7 (V7), variant 8 (V8), and variant 9 (V9). The exon 1 of each new isoform is shown, as are the deletion(s) in exon 5–7, and the deletion of exon 2 in V7. B, expression of each FLAG-tagged IRF-5 isoform in 2fTGH was determined by immunoblot analysis with M2 anti-FLAG antibodies (Sigma). 20 μg of whole cell lysates were run on 10% SDS-PAGE. Levels of actin are shown as a control for loading and protein integrity. C, subcellular localization of gfp-IRF-5 fusion proteins in uninfected cells.
V3/4 polypeptide was used as a positive control; this reporter was also activated by V6 and V9 polypeptides and to a lesser degree by V5, V7, and V8 (Fig. 6B). In contrast, IFNB reporter gene activity was only stimulated by V3/4, V6, and to a lower extent by V7, after NDV infection (Fig. 6C). To evaluate further the role of IRF-5 isoforms in mediating type I IFN gene responses, we measured the synthesis of endogenous biologically active type I IFN in NDV-infected 2fTGH cells transiently expressing each isoform. Infection of 2fTGH cells with NDV resulted in only low levels of endogenous type I IFN (Fig. 7A). In contrast, ectopic expression of FLAG-tagged IRF-5 isoforms (V2, V3, V4, and V6) conferred on these cells the ability to induce type I IFN upon NDV infection, albeit low levels by V2 (Fig. 7A). Most importantly, only IRF-5 V3 and V4 induced measurable levels of synthesized IFN-α assayed in Madin-Darby bovine kidney cells, indicating that the low levels of type I IFN induced by V2 and V6 are most likely IFN-β. We also measured the endogenous IFNA and IFNB transcript levels in these cells by semiquantitative RT-PCR. Fig. 7B shows the relative intensity of IFNA and IFNB transcripts induced by each isoform after normalization with β-actin levels. Again, measurable levels of IFNA transcripts could only be detected in NDV-infected 2TGH cells expressing the IRF-5 V3/V4 polypeptide. Altogether, these data indicate that whereas IRF-5 V6 is an efficient inducer of IFNB in infected cells, IRF-5 V3 and V4 are the primary inducers of type I IFN (Figs. 5–7).

It has been shown previously the presence of alternatively spliced isoforms of IRF-1, IRF-3, and IRF-7 that occur at the level of RNA splicing (18–19, 21, 42). In the case of IRF-3, an endogenous isoform lacking part of its DNA binding domain was isolated, termed IRF-3a (21–22), and shown to act as a DN of IRF-3-mediated IFNB gene induction. As such, we examined whether the IRF-5 V7 would act in a similar manner when co-expressed with the IRF-5 V3 or V4 and the IFNB promoter reporter. Data shown in Fig. 7C revealed the IRF-5 V7, which is lacking its DNA binding domain, potently inhibited the virus-induced activation of the IFNB promoter reporter by IRF-5 V3 in a dose-dependent manner. A noted difference, in the absence of endogenous IRF-5 (2TGH cells; Fig. 6, B and C), the V7 has low transactivation ability indicating that it may form functional heterodimers with IRF-3. The mechanism of this stimulation has yet to be determined.

**DISCUSSION**

The family of IRFs was originally identified as regulators and/or mediators of type I and II IFN signaling. IRF-3, IRF-5, and IRF-7 are central mediators controlling the expression of human type I IFNα (2). Whereas IRF-3 is constitutively expressed in all cell types, expression of IRF-5 and IRF-7 has been primarily detected in lymphoid cells and can be further...
stimulated by type I IFNs (2). It was shown that expression of IRF-3 is sufficient to support induction of IFNB, whereas IRF-5 or IRF-7 were sufficient for stimulation of IFNA gene expression in virus-infected cells (7, 29). Consistent with these observations, PDCs express high constitutive levels of IRF-3, IRF-5, and IRF-7 and are thus uniquely preprogrammed to respond rapidly to a range of viral pathogens with high levels of IFN α/β production (26). IRF-5, like IRF-3 and IRF-7, requires phosphorylation of carboxyl-terminal serines for the nuclear localization and transactivation (8). Most interesting is the fact that unlike IRF-3 or IRF-7, IRF-5 is activated in a virus-specific manner (7), and not by double-stranded RNA, suggesting that IRF-5 may be activated by distinct signaling pathways leading to virus-mediated type I IFN gene expression (8). With the recent finding that IRF-5 exists as multiple isoforms that are expressed in a cell type-specific manner, and the fact that at least two alternative promoters were found to differentially regulate expression of these isoforms, it seems likely that they would have distinct functions. Data from Lin et al. (13), examining for the first time the dissimilar polypeptides encoded by V1 and V2 cDNAs, support this hypothesis. Since it had been shown recently that the IKK-related kinases TBK1 and IKKe phosphorylate and activate IRF-3 and IRF-7 leading to the production of type I IFNs (43–44), they examined the in vitro phosphorylation and activation of IRF-5 V1 and V2 by TBK1 and IKKe and showed that although these two isoforms were indeed phosphorylated, phosphorylation did not lead to nuclear localization or activation (13). Whereas Lin et al. (13) did not examine localization/activation of these two isoforms by NDV, or their ability to induce type I IFNs after NDV infection, they concluded that IRF-5 might simply not be an activator of IFN gene expression. Important to note, Lin et al. (13) only examined the ability of Sendai virus to induce IRF-5-mediated transactivation of IFN promoter reporters even though it has been shown previously that Sendai virus is not an activator of IRF-5 (7, 41).

Additional data from our lab and others indicate that the IRF-5 V3/4 and V5 polypeptides are targets of TBK1 and/or IKKe by in vitro phosphorylation (14, 41). In the case of V5, it was shown that phosphorylation by TBK1 or IKKe led to IRF-5 nuclear translocation (14). Moreover, a critical role for the IRF-5 V3/4 polypeptide in TLR7/8-dependent type I IFN response was shown (41). In these studies, R848 stimulated the transactivation of both IRF-5 and IRF-7 but not IRF-3 and induced nuclear translocation of IRF-5 and IRF-7. The TLR7- and TLR8-induced activation of IRF-5 V3/4 was inhibited in a dose-dependent manner by the TBK1K38A and IKKeK38A kinase-inactive mutants, suggesting that phosphorylation of IRF-5 V3/4 by TBK1 and IKKe is functional. In support of these findings, a recent paper from Takaoka et al. (45) has shown an important role for IRF-5 in TLR signaling downstream of the TLR-MYD88 signaling pathway using spleen-derived dendritic cells and macrophages from wild type and IRF-5 /−/− mice. Of particular interest, whereas the induction of interleukin-6, interleukin-12, and tumor necrosis factor-α in these cells was shown to be dependent on IRF-5 expression after stimulation with CpG ODN (TLR9), polyI:C (TLR3), lipopolysaccharide (TLR4), flagellin (TLR5), or poly(U) (TLR7/8), only CpG-induced IFN-α was shown to be independent of IRF-5 (45). Whether the induction of murine IFN-α by the other TLR ligands or virus is dependent on IRF-5 expression was not examined and thus remains a very interesting and highly relevant question.

The presence of multiple IRF-5 isoforms suggests that their functions may not be redundant; instead, these isoforms may have distinct expression, regulation, and/or function, as has been shown for the IRF-3 isoforms (21, 22). Alternative splicing is a mechanism that allows for individual genes to express multiple mRNAs that encode proteins with diverse and even antagonistic function. This process generates segments of mRNA variability that can insert or remove amino acids, shift
reading frame, or introduce a termination codon. It also affects gene expression by removing or inserting elements controlling translation, mRNA stability, and/or localization. To this effect, we demonstrate that the IRF-5 alternatively spliced isoforms are expressed in a cell type-specific manner and are differentially regulated by at least two alternative promoters. In addition, the subcellular localization of IRF-5 isoforms was dependent on alternative splicing events because the isoforms gave distinct localization patterns, which likely lead to alternate functions (Figs. 5D, 6, and 7). For example, analysis of the IRF-5 V7 (IRF-5 DN) function indicated that in the absence of endogenous or ectopically expressed IRF-5, i.e. 2TGH cells (Fig. 6), V7 has a low level of transactivation ability. However, in cells that express IRF-5 V3, the IRF-5 V7 acts as a DN mutant and inhibits IRF-5 V3-mediated IFN induction. Most interestingly, because the IRF-5 V7 mRNA contains the non-coding exon 1 of V1 (Ex1V1), which is associated with P-V1, expression of this alternatively spliced isoform in virus-infected cells may provide an alternate mechanism for the virus to shut down type I IFN gene expression mediated by IRF-5 V3/4. We are currently examining this possibility. Altogether, these results imply that transcription of the IRF-5 isoforms is under multiple levels of control and that the alternatively spliced mRNAs encode for IRF-5 isoforms with potentially unique biological functions. A preliminary examination of the expression profile of mouse IRF-5 isoforms by gel electrophoresis of amplified exons 2–7 in RNA from peripheral blood lymphocytes of wild type C57Bl/6 mice or from the A20 murine B cell lymphoma cell line revealed a similar phenomenon of multiple banding patterns, indicating that murine IRF-5 may also exist as multiple isoforms (data not shown). A more detailed analysis is currently underway.

We have previously shown that the expression of IRF-5 is stimulated by IFN (8, 10), and results from the present study demonstrate that this induction is isoform-specific (Figs. 1C, 3C, and 4). As summarized in Fig. 8, only the Ex1V3-specific transcripts were up-regulated by IFN-α stimulation or treatment with the TLR7/8 ligand R848. Although stimulation with either of these two inducers had no significant effect on Ex1-associated transcripts, stimulation with HSV-1 primarily up-regulated the Ex1V1-associated transcripts. In addition, NDV-infected Daudi or THP-1 cells specifically up-regulated Ex1V1 transcripts and not Ex1V2 or Ex1V3 (Fig. 4C). Infection of IRF-5 expressing cells with virus or treatment with R848 leads to the phosphorylation and translocation of IRF-5 to the nucleus (7–8, 41). All of these data correlate well with that obtained by promoter reporter assays (Fig. 8). However, in contrast to the data reported by Takaoka et al. (45), we were unable to detect up-regulation of the human IRF-5 isoforms by CpG stimulation. This may reflect differences in the amount and time of CpG ODN stimulation where Takaoka et al. (45) examined IRF-5 transcript levels after only 2 h of treatment at 1 μM, and here we examined levels after 6 h at 40 μg/ml. Although they observed an up-regulation of murine IRF-5 transcripts by CpG, they found that IRF-5 was not a mediator of CpG-induced IFN-α (45). TLR7 and TLR9 signaling are both MyD88-dependent, and are potent inducers of IFN, yet the ability of either R848 or CpG to up-regulate IRF-5 transcripts levels may not be a prerequisite for its involvement in either signaling pathway since unstimulated PBMC and PDC already express IRF-5. Both R848 and CpG are capable of inducing IRF-5 nuclear translocation (41, 45). A distinct difference between these two signaling pathways could be in the mechanism of IRF-5 induction, activation, and/or the diversification of transcription factors activated downstream of MyD88/TRAF6/IRF-5-dependent signaling.

A further characterization of IRF-5 isoform expression in purified PBMC and PDC from healthy donors and immortalized tumor cell lines (THP-1, Namalwa, U937, and BCBL-1) led to the identification and cloning of five new IRF-5 isoforms, V5–V9 (Fig. 5A). These data revealed that human PDCs express only IRF-5 isoforms 1–4, whereas PBMC express, in addition, two new isoforms, V5 and V6. Moreover, IRF-5 V7, V8, and V9 were found to be expressed only in hematological malignancies. In comparing the function of these nine IRF-5 isoforms with regard to virus-mediated type I IFN gene expression, we found that the PDC-expressing isoforms did transactivate IFNA and IFNB gene promoters to varying degrees, but IRF-5 V3/4 was the most potent transcriptional activator of virus-induced IFNA and IFNB promoters (Fig. 6A). The IRF-5 V1, V6, and V9 also transactivated the IFNA1 reporter, whereas V2, V6, and V7 transactivated the IFNB reporter. Moreover, the synthesis of endogenous biologically active IFN-α in NDV-infected 2TGH cells transiently transfected with each individual IRF-5 cDNA was only observed in cells transfected with either IRF-5 V3 or V4 cDNA, whereas IRF-5 V2 and V6 induced lower but detectable levels of IFN-β.

Finally, we and others have shown recently that IRF-5 is a component of the p53 pathway where IRF-5 transcripts were up-regulated by p53 overexpression or activation of p53 by DNA-damaging agents (10, 12), yet IRF-5 expression can also be up-regulated by DNA-damaging agents in the absence of p53. Most interesting, stress induced by electroporation of B cells or treatment of colon cancer cells with the DNA-damaging agent Irinotecan (CPT-11) specifically induced the IRF-5 V3

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transcripts. The factors that are involved in the stimulation of IRF-5 expression in response to stress have yet to be identified. Although the molecular mechanism of IRF-5 induction or activation in uninfected cells is not known, we have identified a number of IRF-5-specific target genes in uninfected cells, such as the cell-cycle regulator p21, pro-apoptotic genes Bax, Bak, and caspase 8 (10). These genes are regulated specifically by the V3- and V4-encoded polypeptides. Currently, it is not clear whether these genes would also be regulated by the polypeptides encoded by V1 or V2 cDNAs. The absence of IRF-5 general transcripts in a number of primary leukemias and lymphomas (10) suggests that IRF-5 may act as tumor suppressor gene in which its function is deleted in certain cancers. Because expression of the IRF-5 V7, V8, and V9 mRNA transcripts has only been observed to date in cancer cell lines, another potential role for the IRF-5 DN (V7) may be to inhibit the cell cycle regulatory, growth inhibitory, and/or pro-apoptotic effects of IRF-5 V3/4. Whether V8 and V9 also have distinct functions specific to cancer cell growth is currently unknown. Altogether, these data suggest that further analysis of the molecular mechanism(s) regulating expression, induction, and biologic function of the IRF-5 isoforms is likely to reveal potential new biomarkers for cancer detection and new immunomodulatory strategies for inflammation-associated disorders.

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