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Genes transcriptionally modulated by interferon α2a correlate with the cytokine activity

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A B S T R A C T

Background and Objectives. Interferon α2a (IFNα2a) mediates important antiviral, antiproliferative and immunomodulatory responses and is employed in the treatment of human diseases, including chronic myelogenous leukemia. Here, we report the IFNα2a-dependent expression profiles of three malignant cell lines derived from liver, lymphocytes and muscle.

Design and Methods. The experiments were performed in the presence of cycloheximide, thus our results exclusively reflect direct transcriptional modulation. The short exposure time i.e. 5 hours evidences only the early events, excluding the effects of complex phenotypic changes on the expression.

Results. Our findings indicate that IFNα2a rapidly up-regulates the expression of STAT1, STAT2 and ISGF3G genes. This activity should result in the amplification of the cellular response to the cytokine. Moreover, IFNα2a directly modulates the expression of: (i) important transcriptional factors, e.g. IRF1 and IRF7 which control pivotal cellular events, and (ii) enzymes involved in the IFNα2a-dependent antiviral and apoptotic response. Interestingly, we showed that the cytokine induces transcriptional expression of Sjögren's syndrome antigen A1, a protein involved in several autoimmune diseases.

Interpretation and Conclusions. The observed changes induced by IFNα2a could be related to the development of autoimmune syndromes observed during IFNα2a treatment. A number of genes transcriptionally regulated by the cytokine have been identified for the first time; these might represent additional effectors of IFNα2a activity.

Key words: K562; RH-30, HepG2, interferon α2a, expression profile.

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Interferon-α2a (IFNα2a) plays a central role in mediating a large number of pivotal physiological events, in particular antiproliferative, antiviral and immunomodulatory responses. Moreover, the cytokine is employed in the treatment of chronic myelogenous leukemia, as well as of other diseases including hairy cell leukemia, non-Hodgkin's lymphoma, AIDS-related Kaposi's sarcoma and chronic, viral hepatitis B and C.[1] Furthermore, IFNα2a, alone or in association with additional drugs, has been proposed for the therapy of several other malignancies, including melanoma and rhabdomyosarcoma.[2,3] Thus, the knowledge of the molecular events following cytokine receptor engagement is particularly important in order to understand the basis of IFNα2a activities.

IFNα2a binds to its specific receptors and initiates a signaling cascade involving the JAK family of tyrosine kinases and the STAT family of transcription factors.[4] After receptor engagement, STAT form homo- or heterodimers through phosphotyrosine SH2 interactions, following activation by JAK. In particular, STAT1 (STAT1α or β) and STAT2 heterodimers or homodimers bind to p48 (ISGF-3γ/IRF-9) resulting in a complex (ISGF3) that recognizes regulatory elements in the promoter region of responsive genes (IFNα2a-stimulated genes or ISG).[5]

The identification of genes whose expression is modulated by IFNα2a represents a fundamental key to unraveling the basis of the cytokine's effects. This knowledge could also lead to: i) identification of new targets for therapy, bypassing the negative side effects of IFNα2a treatment, and ii) understanding the basis of the different responses to cytokine-based therapy. To date, gene expression profiles induced by IFNα2a are

available only in fibrosarcoma[6] melanoma,[7] and cutaneous T-cell lymphoma cell lines.[8] A small customized gene array, capable of analyzing the transcription of 155 genes, has been used to study the expression profile of cell lines derived from fibrosarcoma, hepatoma and T-cell leukemia.[9] Finally, additional knowledge can be gathered from studies,[10-13] which are not strictly gene expression profile studies using microarrays, but which do contribute important information about the activity of IFN on gene transcription.

Nevertheless, a large, contemporaneous analysis of the transcriptional profile of a number of cell lines is still lacking. Furthermore, to the best of our knowledge, the available data do not allow the genes regulated directly (*in cis*) by IFNα2a to be distinguished from those which represent indirect (*in trans*) target genes. Indeed, the majority of the reported profiles were obtained after days of treatment and represent the result of complex phenotypic changes and not solely of direct cytokine activity. On the other hand, the knowledge of early responses to the molecule is of pivotal importance in order to construct a precise scheme of the IFNα2a-dependent cascade of events.

In the present study, we selected cell models and experimental approaches to gather information only on the initial transcriptional events following the addition of IFNα2a. Our findings provide an interesting picture of the early molecular effects of the cytokine and allow the identification of new genes transcriptionally controlled by this immunomodulatory molecule.

Design and Methods

Cell cultures and treatment

HepG2, K562 and RH-30 cells were obtained and cultured as previously described.[14] In all the experiments, IFNα2a was added at a concentration of 1,000 units/mL to the medium of the cells along with cycloheximide (36 μM final concentration).[15] Control cell cultures contained only the protein synthesis inhibitor. After 5 hours, the cells were collected, centrifuged, washed with phosphate-buffered saline (PBS), centrifuged again and immediately employed for the RNA extraction.

cDNA microarray analysis

Microarray probing and analysis was performed by IncyteGenomics (Incyte Pharmaceuticals, Inc., Palo Alto, CA). Briefly, 1 μg of poly $(A)^+$ RNA, isolated from treated and untreated cell lines,[15,16] was reverse transcribed to generate Cy3 and Cy5 fluorescent-labeled cDNA probes. cDNA probes were competitively hybridized to a human UniGEM-V cDNA microarray containing 7075 immobilized cDNA fragments (4107 for known genes and 2968 for EST). Microarrays were scanned in both Cy3 and Cy5 channels with an Axon GenePix scanner (Foster City, CA, USA). P1 and P2 signals are the intensity reading obtained by the scanner for Cy3 and Cy5 channels. The balanced differential expression was calculated using the ratio between the P1 signal (intensity reading for probe 1) and the balanced P2 signal (intensity reading for probe 2 adjusted by using the balanced coefficient).

Incyte GEMtools software was used for image analysis. A gridding and region detection algorithm determined the elements. The area surrounding each element image was used to calculate a local background and was subtracted from the total element signal. Background subtracted element signals were used to calculate the Cy3:Cy5 ratio. The average of the resulting total Cy3 and Cy5 signal gave a ratio that was used to balance or normalize the signals.

Quantitative reverse transcriptase (qRT) assay

All reverse transcriptase reactions were performed using SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen, San Diego, CA, USA) with oligo-dT priming according to the manufacturer's intructions. The qRT assay was performed employing the iCycler iQ RealTime PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and using cDNA from 250 ng of total RNA for each reaction. Each gene's expression was assessed in a separate polymerase chain reaction (PCR). The PCR mixture consisted of 1 μM each primer, 1 unit of AmpliTaq Gold polymerase (Applied Biosystems, Branchburg, NJ, USA), 200 μM of each deoxynucleotide triphosphate, 4.5 mM $MgCl_2$, and 10 × AmpliTaq Buffer in a final volume of 25 μL. Samples were amplified with a precycling hold at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min (annealing at 58°C only for the aldo-keto reductase family 1 gene), and extension at 72°C for 1 min. Each assay was repeated at least twice to verify the results, and the mean copy number was used for analyses. The standard deviation between assays was not statistically significant (<5%) for any of the genes studied. Standard RealTime PCR curves were established for quantifying mRNA copy number by using nine known copy numbers of serially diluted (10^0 to 10^8 copies) plasmids containing the specific cDNA. Each cDNA was synthesized by reverse transcriptase-PCR and extracted from 2% agarose gels using the QIAquick gel extraction method (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The cDNA was ligated into pCR II-TOPO cloning vector (Invitrogen), the cDNA clones were transfected into *Escherichia coli* DH5-α cells, and cultures were expanded. Plasmids containing the target gene were purified and quantified for use in the qRT setup. To confirm that the inserted PCR product size was correct, plasmids were digested with spe-

cific restriction enzymes, and cDNA clone PCR products were then run on gel electrophoresis. The amplification plot of the PCR reaction was used to determine the threshold cycle. This value represented the PCR cycle at which an increase in reporter fluorescence above the line of the optimal value was first detected. The initial copy number of the target mRNA was calculated by a plot of CT against the input target quantity.

Finally, in order to analyze the results obtained from the qRT assays, the software *Gene Expression Relative Quantization* (update January 2004, BioRad), was employed.

Primers for the quantitative RT PCR were selected by using Oligo Primer Analysis Software, version 6.0 (National Biomedical Systems, Plymouth, MN, USA). In order to avoid possible amplification of contaminating genomic DNA, primers were designed so that each PCR product covered at least one intron.

The primers sequences used were as follows.

Pre-B-cell leukemia transcription factor 3: 5'-TGGATACCCTCCGTCATGTT-3' (forward), 5'-TATCCGAGTGCACACTTCCT-3' (reverse); *Aldo-keto reductase family 1, member C4*: 5'-GAGCAGCGGATCAGAGAGAA-3' (forward), 5'-CATCACCATCCACACACAGG-3' (reverse); *STAT1*: 5'-CCGTTTTCATGACCTCCTGT-3' (forward), 5'-AGCTGATCCAA-GCAAGCATT-3' (reverse); *STAT2*: 5'-GAACACTGAGCCAATG-GAAA-3' (forward), 5'-GAGGAGTA-GGAAGGGCAAGA-3' (reverse); *Sjögren's syndrome antigen A1*: 5'-AGATCACTGGGGGAGAAAGA-3' (forward), 5'-CTGGCACATGGCACACAC-3' (reverse); *ISGF3G*: 5'-CATCAAAGCGACAGCACAGT-3' (forward), 5'-CACTAGGAT-GCCCCTCTCAA-3' (reverse); *GAPDH*: 5'-GAGCCAAAAGGGTCATCATCTC-3' (forward); 5'-CTTCCACGATACCAAAGTTGTC-3' (reverse).

Analysis of promoter regions

The analysis of the promoter region of Sjögren's syndrome antigen A1, Mal, solute carrier family 35 (CMP-sialic acid transporter) and glucosaminyl (N-acetyl) transferase 2, I-branching enzyme was carried out by MatInspector[17] and Alibaba software.

Results

To examine the effect of IFNα2a on global patterns of genome transcription, we employed an mRNA differential screen of 7075 unique clones using a gene expression microarray (UniGEM-V) based on competitive hybridization expression profiling. The analysis was performed on three cell lines representing important target tissues of the cytokine: we employed K562 cells and HepG2 cells, established from a chronic leukemia in blast crisis and from a liver carcinoma, respectively, since IFNα2a is employed as first line drug in the therapy of CML and chronic viral hepatitis. The third cell line chosen, i.e. RH-30 cells, represents a model of rhabdomyosarcoma, a cancer for which the use of IFNα2a has been proposed.[3]

The scheme of treatment, namely 5 hours of incubation in the presence of cycloheximide, was selected, as in our previous experiments,[15] in order to identify genes which: (i) are modulated early after the addition of the molecule and (ii) are directly (i.e. *in cis*) controlled, without the involvement of *de novo* synthesized proteins. Indeed, the addition of a protein synthesis inhibitor allowed us to characterize the *in cis* targets of IFNα2a transcriptional effects. The incubation time of 5 hours was chosen in order to minimize the toxic effects of cycloheximide. Although it is likely that the level of each mRNA might peak at distinct times, this period of treatment was chosen since previous studies selected a similar experimental point for investigating the effects of interferon α2a.[11] Finally, the concentration of IFN employed was the same as that used in the majority of *in vitro* investigations on this cytokine.[6-9] However, it should be stressed that our experimental scheme is different from those generally employed in previous analyses of interferon-dependent expression profiles for two main reasons: (i) the contemporaneous use of different cell models and (ii) the identification of solely in cis modulated genes.

A potential bias of our approach is that cycloheximide might cause increases or decreases of specific mRNA because of the possible absence of protein(s) involved in mRNA metabolism. However, the validation of results (at least for the investigated genes) by real-time PCR (see below) suggests that our findings do represent mainly the effect of IFNα2a on gene expression.

Tables 1 and 2 show the genes mostly up- or down-regulated *in cis* by the cytokine. The genes reported were selected on the basis of two constraints, namely: (i) that they were modulated in all three cell models, and (ii) that their expression was altered, more than two-fold, in at least one cell line. Thus, these genes likely represent a core response to IFNα2a that is conserved across cell types.

It should be said that although other microarray experiments have been performed with a higher cut-off, which obviously yields a stronger significance, a 2-fold variation is an acceptable level of difference in expression as indicated by several studies.[18,19] The findings reported in Table 1 show that a low number of genes are modulated in all the three models and that the degree of variation is rarely more than 3-fold. These findings are strongly in variance with other published data.[6-13] Interestingly, we did not observe remarkable differences among each experiment and among the different cell lines, the expression profiles being almost similar in all the cells (each cell line was analyzed in

Table 1. Human genes transcriptionally up-regulated by interferon α2a.

HUGO Symbol	*GenBank Accession No.*	*Description*	*Fold decrease*		
			RH-30	*HepG2*	*K562*
STAT1	AA478534	Signal transducer and activator of transcription 1.91kD	1.8±0.2	8.5±1.1	4.1±0.5
STAT2	U18671	Signal transducer and activator of transcription 2.113kD	1.9±0.2	2.4±0.4	2.1±0.4
ISGF3G	M87503	Interferon-stimulated transcription factor 3, γ(ISG3γ)	1.6±0.2	3.5±0.6	2.5±0.5
IRF1	X14454	Interferon regulatory factor 1	2.1±0.2	1.6±0.3	1.8±0.4
IRF7	U53830	Interferon regulatory factor 7	1.7±0.2	3.1±0.6	2.1±0.5
IFITM1	AA428847	Interferon-induced transmembrane protein 1 (9-27)	1.7±0.2	3.1±0.5	2.4±0.5
IFIT1	NM_001548	Interferon-induced protein 56	1.6±0.2	4.7±0.5	5.3±0.5
G1P3	BE407364	Interferon, α-inducible protein (clone IFI-6-16)	1.8±0.2	2.0±0.7	2.4±0.7
MAL	M15800	Mal, T-cell differentiation protein	1.7±0.2	3.1±0.4	2.1±0.5
TAP1	X57522	ATP-binding cassette, sub-family B (MDR/TAP), member 2	1.7±0.2	2.4±0.5	2.8±0.7
SSA1	M62800	Sjögren's syndrome antigen A1 (52kD, SS-A/Ro)	1.4±0.2	3.3±0.6	2.9±0.6
IFITM3	X57352	Interferon-induced transmembrane protein 3 (1-8U)	1.5±0.2	2.4±0.5	1.5±0.5
SLC35A1	AL049697	Solute carrier family 35 (CMP-sialic acid transporter)	1.4±0.2	2.1±0.4	1.5±0.4
IFI16	S75433	Interferon, γ-inducible protein 16	1.8±0.2	3.1±0.6	1.8±0.4
OAS2	M87284	2'-5'oligoadenylate synthetase 2	1.6±0.2	2.7±0.5	1.8±0.5
GCNT2	Z19550	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	1.5±0.2	3.2±0.7	1.6±0.4
PLSCR1	AB006746	Phospholipid scramblase 1	1.9±0.3	4.1±0.6	2.4±0.6
TNFS14	AF064090	Tumor necrosis factor (ligand) superfamily, member 14	1.8±0.2	3.8±0.7	1.7±0.5
GBP1	M55542	Guanylate binding protein 1, interferon-inducible, 67kD	1.4±0.2	3.2±0.6	1.4±0.3
CASP4	Z48810	Caspase 4, apoptosis-related cysteine protease	1.5±0.2	1.8±0.4	2.1±0.5
SP110	L22343	Interferon-induced protein 75, 52kD	1.4±0.2	4.1±0.7	2.4±0.5

The cell lines were treated for 5 hours with or without 1000 units/mL IFNα2a in the presence of 36 μM cycloheximide. Total RNA was isolated and analyzed as reported in the Design and Methods section. Data are the mean of three different experiments.

three independent experiments). Several of the activated genes belong to families whose interplay with IFNα2a has already been reported in the literature. However, some of them have never been correlated to the cytokine activity. Table 3 describes the (putative) function of the identified genes.

Discussion

A first interesting observation is that three of the transcriptionally induced genes, STAT1, STAT2 and ISGF3G (Table 1), encode components of the ISGF3 complex which binds to a 15-bp element, designated as the ISRE (interferon-stimulated response element), and activates ISG. As described in the Introduction, ISGF3 is formed by two elements, one defined as ISGF-3α, formed by STAT1 and STAT2 and one called ISGF-3γ of 48 kDa. Our observation that IFNα2a transcriptionally modulates all these proteins (Table 1) raises the possibility of an initial amplifying process, which might increase the response to the cytokine enormously.

Similar results were obtained by Melen *et al.*[11] although in that study no distinction between *in trans* and *in cis* regulation was reported. Thus, our findings, to the best of our knowledge, represent the first discovery that such a response might represent a transcriptional *in cis* event after IFNα2a treatment.

A second observation is that two pivotal transcription factors (interferon regulatory factor 1, IRF1 and interferon regulatory factor 7, IRF7) are directly up-regulated by IFNα2a. The targets of these genes are extremely important: for example IRF1, which is envisioned as a tumor suppressor gene, up-regulates $p21^{Cip120}$ and cas-

Table 2. Human genes transcriptionally down-regulated by interferon α2a.

HUGO Symbol	*GenBank Accession No.*	*Description*	*Fold decrease*		
			RH-30	*HepG2*	*K562*
FSTL3	NM_005860	Follistatin-like 3 (secreted glycoprotein)	3.2±0.5	4.1±0.6	2.3±0.6
PBX3	NM_006195	Pre-B-cell leukemia transcription factor 3	1.6±0.4	2.1±0.6	1.8±0.4
AKR1C4	S68287	Aldo-keto reductase family 1, member C4	1.6±0.3	2.3±0.4	1.8±0.5
ARPC5	AF006088	Actin-related protein 2/3 complex, subunit 5 (16 kD)	2.5±0.3	1.6±0.4	1.6±0.5

The experiments were carried out as described in the legend to Table 1. Data are the mean of three different experiments.

Table 3. Putative functions of interferon α2a-modulated genes.

HUGO Symbol	*Gene Function*
Up-regulated	
ISGF3G	Transcription factor
IFITM1	Cell-cell interaction, germ cell specification
IFIT1	2'-5' oligoadenylate synthetase like protein
G1P3	Not clarified
MAL	Cell differentiation
IRF1	Transcription factor and tumor suppressor
TAP1	Peptide/antigen presentation
SSA1	Ribonucleoprote in constituent
IFITM3	Transmembrane mucin glycoprotein
STAT1	Transcription factor
SLC35A1	CMP-sialic acid transporter
IRF7	Transcription factor
IFI16	GTPase
OAS2	2'-5'oligoadenylate synthetase
STAT2	Transcription factor
GCNT2	Branched poly-N-acetyllactosaminoglycans synthesis
PLSCR1	Lipid membrane composition modification
TNFSF14	Growth factor
GBP1	Involved in embryo implantation
CASP4	Caspase
SP110	Nuclear protein, ribosomal biogenesis
Down-regulated	
PBX3	Transcription factor
AKR1C4	Reduction of steroids, bile acids and pesticides
ARPC5	Control of actin polymerization
FSTL3	Morphogen-interacting protein

pase 8 expression and down-regulates cdk2 transcription.[21] Moreover, IRF1 modulates cathepsins B, D, L and S,[22] which are cysteine proteases participating in the lysosomal degradation of proteins and cell death responses, and hinders Sp1 activity.[23] These genes, which are reported by others as regulated by IFNα2a,[6-8] did not appear in any of our cell line expression profiles and thus probably represent a secondary response to the cytokine. The important and pleiotropic activity of IRF1 might also explain *inter alia* part of the IFNα2a activities. It should be stressed that the expression of IRF1 is regulated by a STAT1/STAT2 complex or STAT1 homodimers[23] and, thus, IRF1 expression is further activated by the above-described up-regulation of STAT1 and STAT2. Taken together, the hypothesized amplification loop and the up-regulation of the transcription factors might explain the large differences in intensity of activation and number of genes observed in experiments which do not employ cycloheximide.[6-8]

The effect on IRF1 merits a further brief discussion. Several papers describe a noticeable IFNα2a effect on the expression of this gene. Conversely, Melen *et al.*[11] reported that IFNα2a was unable to up-regulate IRF1 transcription. Our results, which indicate a moderately positive effect of IFNα2a on the expression of IRF1 partially confirm the data reported by Melen, and highlight the difficulty of comparing different studies.

IFNα2a also regulates cell death by transcriptionally modulating *in cis* the expression of additional genes (Table 1). Indeed, it induces scramblase, which has a well documented key role in the apoptosis program by flipping phosphatidylserine from the inner to the outer leaf of the cell membrane.[24] Exposed phosphatidylserine serves as a surface marker for recruited phagocytes to recognize and eliminate apoptotic bodies. Therefore, increased levels of phospholipid scramblase in response to IFNα2a might serve as a mechanism enabling more efficient removal of virus-infected cells undergoing apoptosis. In addition, the cytokine increases the levels of caspase 4, an enzyme involved in the cell death cascade. The interferon antiviral response is also related todirect modulation of 2'-5' oligoadenylate synthase 2 and 2'-5' oligoadenylate synthase-like protein (Tables 1 and 3), which are essential parts of the cytokine's antiviral response.[25] These enzymes catalyze the synthesis of 2'-5' oligoadenylate molecules which bind to and activate RNase L, leading to RNAs degradation and impairment of viral replication.[25] In addition, the synthesized oligomers have been implicated in the control of cell growth, differentiation, and apoptosis.[22] An interplay between interferon and 2'-5' oligoadenylate synthase-like protein has never been previously described.

One intriguing observation was the effect of IFNα2a on the transcription of Sjögren's syndrome antigen A1 (SSA1). Although this upregulation has already been reported,[6,26] the expression of the gene appears to be an unpredicted part of the core *in cis* transcriptional response to the interferon molecule. Sjögren's syndrome antigen protein A1, also known as RoSSA, is encoded by a gene member of the tripartite motif (TRIM) family.[27] The TRIM includes three zinc-binding domains, a RING, a B-box type 1 and a B-box type 2, and a coiled-coil region. This protein is part of the ribonucleoprotein complex which includes RoSSA polypeptide and one of four small RNA molecules.[28] The RoSSA particle localizes to both the cytoplasm and the nucleus. RoSSA interacts with autoantigens in patients with Sjögren's syndrome and systemic lupus erythematosus. The precise function of the RoSSA particle has not been determined, although ribonucleoproteins are believed to play a role in important events such as cell proliferation and differentiation.

The demonstrated effect of IFNα2a on RoSSA is extremely interesting since it might help in explaining the occurrence of autoimmune diseases that complicate the use of this cytokine in therapy.[29-31] The observed

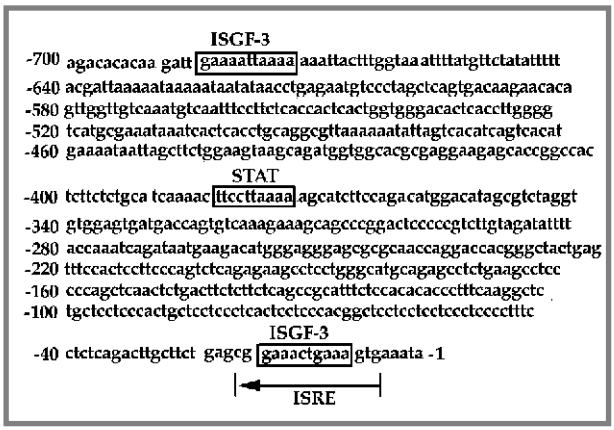

Figure 1. Structure of Sjögren's syndrome antigen A1 promoter. The 700 bp of Sjögren's syndrome antigen A1 promoter (before the starting transcription point) are shown. The localtions of one STAT and two ISGF3 responsive elements are evidenced in the boxes. An ISRE is shown as the complementary sequence to the region evidenced by the arrow. The direction of the arrow indicates the direction of the ISRE.

diseases include systemic lupus erythematosus and Sjögren's syndrome, both characterized by a high titer of anti-RoSSA serum antibodies. Finally, a computer-aided analysis of RoSSA gene promoter, carried out by two different software programs (see Design and Methods) demonstrated the occurrence of one ISRE, one STAT and two ISGF3 responsive elements (Figure 1). Additional studies are currently being developed to evaluate the importance of these sequences in the response of Sjögren's syndrome antigen A1 gene to IFNα2a treatment.

Another gene regulated by IFNα2a in all the investigated cellular models was Mal (myelin and lymphocyte) gene. So far, only one report[8] describes a connection betweeninterferon and Mal. The Mal gene comprises 4 exons, each encoding a hydrophobic, presumably membrane-associated, segment and its adjacent hydrophilic sequence.[32] The gene was originally identified in a study aimed at characterizing cDNA clones present in mature T cells and not expressed earlier.[33] Epitope-tagged Mal is localized in epithelial cells, mainly in glycolipid- and cholesterol-enriched membrane microdomains belonging to the trans-Golgi network, and at low levels in early endosomes.[34] Mal is also a component of the detergent-resistant membrane microdomains in T lymphocytes, thus suggesting that the protein plays a role in modulating the function of these cellular structures during T-cell differentiation. In addition, subsequent data on Mal indicated that the protein plays a role in neuronal differentiation.[35] Since Mal is involved in lymphocyte growth arrest, its IFNα2a-dependent increase might be responsible for part of the cytokine effect. Studying the Mal gene promoter region, we identified one STAT responsive element (at position –444 with respect to the transcription starting point) which could represent the sequence modulated by IFNα2a. A number of genes reported in Table 1 have never been, to the best of our knowledge, directly related to IFNα2a activity. These include: solute carrier family 35 (CMP-sialic acid transporter), glucosaminyl (N-acetyl) transferase 2, I-branching enzyme, and tumor necrosis factor (ligand) superfamily member.[14] Although, the precise role of these genes in cell physiology is not clear, at least some of them seem to play functions which might participate in the effects of interferon α2a.

Solute carrier family 35 (CMP-sialic acid transporter)[36] and glucosaminyl (N-acetyl) transferase 2, I-branching enzyme[34] are two proteins which probably play a sequential role. Indeed, the former is involved in the transport of CMP-sialic acid inside the Golgi apparatus while the latter, an enzyme localized in the Golgi system, uses CMP-sialic acid as its substrate.[36] Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme is capable of adding sialic acid to several different proteins.[37] The two proteins might play a role in the ability of IFNα2a to modify the composition of cell membrane MHC. Potential IFNα2a-responding elements have been identified by us on these two gene promoters. In detail, an ISRE at -565 was found in solute carrier family 35 (CMP-sialic acid transporter), and two ISRE (at -105 and -320) and a STAT responsive element (at -384) in glucosaminyl (N-acetyl) transferase 2, I-branching enzyme.

The data reported in Table 2, on the genes down-regulated after IFNα2a treatment, are completely new information. We again selected genes modified (in this case inhibited) in all three cell lines, with at least a two-fold change in expression in at least one cell line. Their function in connection with the complexity of the interferon phenotype is not clear. However, some inferences might be suggested. For example, the follistatin-like 3 is gene encodes a protein overexpressed during leukemogenesis;[38] its down-regulation might be relevant in explaining the efficacy of IFNα2a in the treatment of CML.

Table 4 reports the results of quantitative RT-PCR experiments carried out to confirm some of the data reported in Tables 1 and 2. In this set of analyses we evaluated the expression of 4 up-regulated genes and 2 down-regulated ones, selecting some of the most intriguing (STAT2, STAT2 and ISGF3G) and least investigated (SSA1, PBX and AKR1C4) ones. Three conditions were employed: addition of interferon without cycloheximide, addition of interferon plus cycloheximide, and the addition of cycloheximide alone. Moreover, two time intervals were considered, i.e. 2.5 and 5 hours. The results obtained confirm the findings reported in Table 1 and 2, although some minor differences were

Table 4. Real time PCR analysis of interferon-α2a-modulated gene expression in K562 cells.

HUGO Symbol	*Fold increase*					
	IFNα2a (2.5 hours)	*Cx (2.5 hours)*	*IFNα2a + Cx (2.5 hours)*	*IFNa2a (5 hours)*	*Cx (5 hours)*	*IFNα2a + Cx (5 hours)*
STAT1	5.5±0.5	1.2±0.2	4.8±0.5	5.8±0.5	1.5±0.5	6.2±0.5
STAT2	1.9±0.3	1.1±0.2	2.3±0.3	4.5±0.5	1.4±0.5	4.3±0.5
ISGF3G	3.8±0.5	1.7±0.5	3.2±0.5	5.2±0.5	1.7±0.5	3.8±0.5
SSA1	2.2±0.3	1.2±0.5	2.2±0.3	4.3±0.5	1.8±0.5	4.2±0.5
	Fold increase					
	IFNα2a (2.5 hours)	*Cx (2.5 hours)*	*IFNα2a + Cx (2.5 hours)*	*IFNα2a (5 hours)*	*Cx (5 hours)*	*IFNα2a + Cx (5 hours)*
PBX3	3.6±0.5	1.5±0.2	5.2±0.5	3.8±0.4	1.8±0.2	6.2±0.5
AKR1C4	3.1±0.5	0.9±0.2	3.2±0.4	4.5±0.5	1.0±0.2	4.2±0.5

K562 cells were treated for the indicated time periods with or without 1000 units/ml IFNα2a in the presence or absence of 36 mM cycloheximide (Cx). Total RNA was isolated and analyzed as reported in Materials and Methods by qRT-PCR. All the data refer to the control (without IFNα2a and Cx) which corresponds to 1. Data are the mean of three different experiments.

observed due to the different methodology employed.

Although, as discussed before, it is totally possible that our study might underevaluate the number of genes modulated *in cis* by IFNα2a as well as the degree of modulation, we believe that a consistent part of the core direct transcriptional effect of the cytokine has been definitely identified. Moreover, the discovery of new targets of IFNα2a activity as well as the confirmation of genes scarcely taken into account (such as MAL and SSA1) during cytokine studies highlight the necessity of further investigations.

FDR and AI designed the cellular and molecular studies and wrote the paper. AB, VC, VC, and SI performed the cellular cultures and treatment, an RNA preparation and critically reviewed the draft. LG, AM, VS. performed molecular experiments and critically reviewed the draft. SV and PG carried out the statistical analyses and contributed to their critical interpretation.

All the authors gave final approval for submission of the paper and are listed according to a criterion of decreasing individual contribution to the work.

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