ELSEVIER

Journal of Immunological Methods 276 (2003) 207–222

JIM Journal of Immunological Methods

www.elsevier.com/locate/jim

Recombinant Technology

Differential expression of IFN-α subtypes in human PBMC: evaluation of novel real-time PCR assays

S. Löseke*, E. Grage-Griebenow, A. Wagner, K. Gehlhar, A. Bufe

Ruhr-University Bochum, Experimental Pneumology, University Hospital Bergmannsheil, BGFA XU 19, Bürkle-de-la-Camp-Platz 1, D-44789 Bochum, Germany

Received 19 February 2002; received in revised form 25 April 2002; accepted 10 February 2003

Abstract

Studies of the human IFN-α subtype system have been hampered by the lack of efficient procedures to quantify and differentiate the expression of the highly homologous IFN-α subtypes. Here we evaluate four novel real-time PCR assays for the specific detection and quantification of IFN-α mRNA for the subtypes α_2, α_6, α_8 and $\alpha_{1/13}$ in a combined assay in human peripheral blood mononuclear cells (PBMC). This included (a) the selection of β-glucuronidase (GUS) as a suitable housekeeping gene for relative quantification; (b) verification of the specificity by using human DNA of different IFN-α subtypes; and (c) comparison of the amplification efficiencies among the different assays. This highly sensitive method allows the detection of low-level, constitutive IFN-α mRNA and shows differences in the composition of constitutive IFN-α subtypes compared to other cell types (HeLa and HEp-2). The in vitro stimulation of PBMC with Newcastle disease virus (NDV), Respiratory syncytial virus (RSV) or an inactivated Herpes simplex (HSV) preparation leads to the transcriptional induction of all IFN-α subtypes investigated but to different expression levels. Among the subtypes detected, IFN-$\alpha_{13/1}$ and α_2 are the major transcripts followed by α_8, and finally α_6 as a minor transcribed subtype. Time-kinetics of IFN-α transcriptional activation also revealed variations in the course of IFN-α transcription between NDV, RSV or HSV. The data obtained from the real-time PCR assays correlated well with IFN-α_2 protein release. In conclusion, we have demonstrated the suitability and reliability of new real-time PCR assays for the rapid and efficient analysis of IFN-α subtype expression.
© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Real-time PCR; IFN-α subtypes; Quantification; Virus; Human PBMC

1. Introduction

After viral contact, the innate immune system reacts rapidly with the production of high amounts of type I interferons (IFNs) as a first line defense mechanism. In the human system, the type I IFN proteins are represented by a large family of structurally related genes. Their common properties are the intron-less structure and the co-localization in a

Abbreviations: Ct, threshold cycle; dsRNA, double stranded ribonucleic acid; FAM, 6-carboxyfluorescein; FCS, fetal calf serum; GUS, beta glucuronidase; HSV-1, herpes simplex type 1 virus; IFN, interferon; LPS, lipopolysaccharide; MOI, multiplicity of infection; NDV, Newcastle disease virus; Rn, normalized reporter signal; RSV, Respiratory syncytial virus; RT, reverse transcription; TAMRA, 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine; UNG, uracil-*N*-glycosylase.

* Corresponding author. Tel.: +49-234-3074-566.
E-mail address: stefan.loeseke@ruhr-uni-bochum.de (S. Löseke).

0022-1759/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0022-1759(03)00072-3

400-kb gene cluster on the short arm of chromosome 9. Excluding the pseudogenes, there are 13 fully translated IFN-α genes which share more than 80% identity with each other; additionally, a single IFN-β and IFN-ω gene and a recently discovered novel type I interferon called IFN-κ (LaFleur et al., 2001). Type I interferons bind to a common cellular surface receptor (IFN-α/β receptor) which mediates the major signaling pathway. The signaling leads to a broad range of gene induction that reflects the pleiotropic effects of IFN-α. It mediates antiviral activities and possesses antiproliferative properties (Goodbourn et al., 2000). Besides their beneficial use as adjuvants in antiviral or anticancer therapy, the type I IFNs possibly play a protective role in the context of allergic diseases (Bufe et al., 2002) because of their immunomodulatory properties (e.g. shifting the immune balance preferentially towards a Th1 response) (Ito et al., 2001). At present, the biological significance of the abundance of IFN-α genes is not fully understood. All IFN-α subtypes show antiviral and antiproliferative properties and bind to the same receptor; however, quantitative and qualitative differences between the single subtypes could be demonstrated. Some investigations describe quantitative differences between IFN-α subtypes in their potency to induce antiviral (Foster et al., 1996) and antiproliferative (Yanai et al., 2001a) effects. Regarding their immunologic effects, IFN-α subtypes can differ substantially in their stimulatory activity on NK cells (Verhagen et al., 1990) and in their effectiveness to induce the proliferation of primary B-cells (Hibbert and Foster, 1999). Depending on the cell type and the kind of stimulation, the expressed IFN-α subtype pattern is influenced by transcription factors of the interferon regulatory factor (IRF) family, that play a critical role in the induction of IFN-α gene transcription (Taniguchi et al., 2001). Molecular studies of differential IFN-α subtype expression under diverse experimental settings have been hampered by the lack of efficient procedures to differentiate and quantify the expression of IFN-α subtypes. The amount of total IFN-α protein could easily be detected by ELISA or by bioassays which measure the antiviral activity or read out the expression of a strictly type I interferon-induced protein (Lleonart et al., 1990). But a selective and specific determination of single IFN-α subtypes is difficult to achieve. At the protein level, most selective detection methods essentially depend on IFN-α subtype-specific antibodies. But the commercial availability of those antibodies is very limited and restricted to only a few subtypes. Such specific antibodies enable the detection of IFN-α subtypes with methods like radioimmunoassay (Greenway et al., 1992), immunocytometry or immunofluorescence-flow cytometry (Greenway et al., 1993). Antibody independent identification of IFN-α subtype proteins could only be achieved by intensive use of advanced biochemistry methods like reverse phase-HPLC, MALDI-TOF MS and protein sequencing (Nyman et al., 1998). The detection of different IFN-α subtype mRNAs could be achieved by the use of labeled and specific probes in combination with methods like S1-mapping (Hiscott et al., 1984), in situ hybridization (Gobl et al., 1988) or differential hybridization to subtype-specific oligonucleotides (Hughes et al., 1994). Besides the rare use of specific primers in PCR for amplification of distinct subtypes (Brandt et al., 1994), a more frequently used RT-PCR-based strategy is the amplification of all IFN-α subtypes together using consensus primers followed by cloning the PCR products. The sequence analysis of a great amount of clones containing individual IFN-α subtypes (Castelruiz et al., 1999; Yeow et al., 2001) allows conclusions to be drawn from the IFN-α subtype composition of the RNA mixture. Another more indirect way to analyze transcriptional IFN-α activation is the use of reporter-gene constructs under the control of individual IFN-α subtype promoters (Dent et al., 1996). All these methods possess one or more disadvantage. They may be insensitive, not quantitative, not very specific, possess low reproducibility, need highly specialized knowledge or the methods are time consuming and therefore not suitable for large-scale investigations. To overcome most of these problems, we have established and evaluated a sensitive and highly specific reverse-transcription polymerase chain reaction (RT-PCR) method based on the 5′ nuclease assay (Holland et al., 1991) for relative quantification and differentiation of mRNA-transcription for some IFN-α subtypes (α_2, α_6, α_8, $\alpha_{13/1}$). With this assay, we have analyzed the differential expression and kinetics of IFN-α subtypes in human PBMC after stimulation by different viruses.

2. Materials and methods

2.1. Cell lines and virus preparations

The lymphoblastoid Namalwa cell-line (DSM ACC24) was obtained from the German collection of microorganisms and animal cell cultures (Braunschweig, Germany). The two human cell-lines HeLa (ATCC CCL-2) and HEp-2 (ATCC CCL-23) were used to amplify the respiratory syncytial virus (RSV) and the Adenovirus type 3 (Adv3). Semi-confluent HeLa cultures were inoculated with 0.1 multiplicity of infection (MOI) of Adv3 and cultures of HEp-2 cells were infected with an initial 0.1 MOI of RSV. After 2 h of incubation in serum-free DMEM, the medium was changed and the cells were further incubated for 72 h in DMEM supplemented with 0.5% heat-inactivated fetal calf serum (FCS; Biochrom, Berlin, Germany). The RSV was further concentrated and separated by ultra-centrifugation of the RSV-containing culture supernatant for 2 h at $60{,}000 \times g$ using a 10% sucrose gradient in MHN buffer (1 M $MgSO_4$, 50 mM HEPES, 150 mM NaCl, pH 5.7) at 8 °C. The pelleted virus was resuspended in MHN buffer and stored immediately at − 70 °C. The virus titer for Adv3 and RSV was determined by immune detection after minimal dilution experiments. The Newcastle disease virus (NDV Ulster2c) was a kind gift from Dr. R. Zawatzky, Deutsches Krebsforschungszentrum, Heidelberg, Germany. The inactivated Herpes simplex type 1 (HSV-I) preparation and the respective HSV-I control antigen were purchased from Dade Behring, Marburg, Germany; and were dissolved in 1 ml PBS as recommended by the manufacturer and stored at − 70 °C.

2.2. Preparation of human PBMC

PBMC were isolated from heparinized whole blood after 1:1 dilution with Hanks' balanced saline solution (HBSS, Biochrom, Berlin, Germany) by Ficoll/Isopaque (1.077 g/ml, PAN Biotech, Aidenbach, Germany) density gradient centrifugation ($460 \times g$, 20 min, 25 °C). Cells harvested from the interphase were washed twice in HBSS at $180 \times g$ for 8 min at 4 °C and the pelleted cells were resuspended in RPMI-1640. The cell counts were performed using a Sysmex cell counter F-820 (Sysmex Medical Electronics, Norderstedt, Germany).

2.3. Cell cultures and stimulation

All cell cultures were performed in RPMI-1640 medium supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mmol/l) and 5% FCS, all obtained from Biochrom. For in vitro stimulation, the cells were cultured at a concentration of 5×10^5 cells in 250 μl of culture medium at 5% CO_2 and 37 °C in the presence or absence of different viruses, viral antigens or LPS in the following concentrations: (1) 3.2 HA/ml (HA, hemagglutinating units) NDV; (2) 1:100 dilution of HSV-I antigen solution; (3) 3 MOI RSV; (4) 1 MOI Adv3; (5) 10 ng/ml LPS (*Salmonella minnesota* Strain R7). The Namalwa cells were stimulated with 50 HA NDV in a high density culture (1×10^7 cells/ml RPMI-1640 without FCS) for 90 min at 37 °C and 5% CO_2 . Then the cells were seeded out at a density of 2×10^6 cells/ml in RPMI-1640 with 5% FCS and further incubated for 24 h. After incubation for 2–32 h all cultures were centrifuged at $380 \times g$ for 5 min at 4 °C. The pelleted cells were lysed in 350 μl of RLT lysis buffer (Qiagen, Hilden, Germany) and stored immediately at − 70 °C for RNA extraction. The supernatants were collected and stored at − 20 °C for determination of IFN-α_2 protein by enzyme-linked immunosorbent assay (ELISA).

2.4. Measurement of IFN-α in culture supernatants

The concentration of IFN-α_2 in supernatants was determined by the use of a quantitative ELISA, kindly provided by Dr. H. Gallati (Hoffmann-La Roche, Basel, Switzerland). The assay was carried out as recommended by the manufacturer (Hoffmann-LaRoche) and described by Gallati (1982).

2.5. Cloning of IFN-α subtypes

IFN-α subtypes DNA were generated by PCR with genomic DNA as a template using the following primer pairs (α_2: sense 5′-AGTCAAGCTGCTCTGTGG-GC, antisense 5′-GTGAGCTGGCATACGAATCA; α_4, α_7, α_{16}: sense 5′-CTCAGCTACAAATCCATC-TG, antisense 5′-AGTCTCTTCCACCCCAAC; A8:

sense 5′-ATATGGATCCTCAGCTACAAGTCATTCAGC, antisense 5′-ATATGGATCCTGATTTCTGCTCTGACAACC; α_5: sense 5′-GGTCACTCAATCTCAACAGC, antisense 5′ ATATGGATCCTGATTTCTGCTCTGACAACC; α_{13}: sense 5′-ATATCTATGATGGCCTCG, antisense 5′ ATATGGATCCTGATTTCTGCTCTGACAACC; α_{17}: sense 5′-ATATGGATCCGCAACATTTGCAACATCC, antisense 5′-ATATGGATCCATTCACCACAATGTAAAGG). The PCR products for α_2, α_4, α_5, α_7, α_{13}, α_{16} were cloned into the TOPO4 vector using the TOPO TA cloning kit (Invitrogen, Groningen, Netherlands) and the amplicons of α_8 and α_{17} were cloned after *Bam*HI digestion into the *Bam*HI linearized pBluescript SK(−) plasmid (Stratagene, La Jolla, CA, USA). To confirm the correct sequences, all inserts were sequenced using the Big Dye Prism Terminator Kit (Applied Biosystem, Foster City, CA, USA) and the ABI Prism 310 sequencer according to the manufacturer's protocol. All generated plasmids shown in Table 1 were used to control the specificities of the established real-time PCR assays. To check for possible cross-hybridization events between the different subtype detection reactions, 2.5 pg of each plasmid per 28 μl reaction was used as templates under standard assay conditions.

2.6. Extraction of RNA from cultured cells

Total RNA was isolated either from 5×10^6 PBMC lysed in 650 μl of RLT buffer or 5×10^5 PBMC lysed in 350 μl of RLT buffer with the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol including an on-column DNase I digestion step. The lysed cells were centrifuged through a Qiashredder column to shear the genomic DNA. Afterwards, the lysate was mixed with an equal volume of 70% ethanol and transferred to the RNeasy column and was further processed following the manufacturer's protocol. The DNA digestion step was performed on the column for 12 min at room temperature using the RNase-free DNase set (Qiagen). After the washing steps, the total RNA was eluted either with 30 μl (for 5×10^5 starting cells) or with 50 μl (for 5×10^6 starting cells) using RNase-free water and immediately stored at − 70 °C. The amount of total RNA from cultures with 5×10^6 cells was spectrophotometrically determined at 260 and 280 nm.

Table 1
Constructed plasmids containing IFN-α DNA

Plasmid name	Insert size	Location to CDS	IFN-α subtype
pCRIFNA2	567	50 to + 50 (3′UTR)	α_2
pCRIFNA4	356	40 to 396	α_4
pCRIFNA5	558	− 44 (5′UTR) to 514	α_5
pCRIFNA7	356	40 to 396	α_7
pCRIFNA8	474	40 to 514	α_8
pCRIFNA13	523	− 9 (5′UTR) to 514	α_{13}
pCRIFNA16	356	40 to 396	α_{16}
pCRIFNA17	884	− 20 (5′UTR) to + 294 (3′UTR)	α_{17}

Generation of plasmids was performed as described in Section 2.

2.7. Reverse transcription (RT) reaction

Ten microliters of total RNA solution extracted from 5×10^5 PBMC or 500 ng total RNA extracted from 5×10^6 cells was reverse transcribed in a final volume of 30 μl using all components from the TaqMan RT kit (Applied Biosystems). Prior to the RT reaction, a second DNase I digestion step was performed. The RNA was incubated for 20 min at 37 °C in 15 μl of a solution consisting of RT buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 5.5 mM $MgCl_2$, 0.7 U/μl RNase inhibitor and 0.2 U/μl DNase I (Rnase-free, Roche Diagnostics, Mannheim, Germany). Afterwards, the DNase I was heat-inactivated at 75 °C for 8 min. For the RT reaction, 15 μl of a premix was added to the sample, adjusting the compounds to a final concentration of 50 mM KCl, 10 mM Tris–HCl, 5.5 mM $MgCl_2$, 0.5 mM each dNTP, 2.5 μM random hexamers $[d(N)_6]$, 0.33 U/μl RNase inhibitor and 1.25 U/μl Multiscribe (MuLV) reverse transcriptase. To exclude genomic DNA contamination, a negative control was done from every sample by performing the RT reaction without reverse transcriptase. All RT reactions were done in a PCR thermocycler (PTC200 MJ Research, Watertown, MA, USA) with 10 min at 25 °C, 45 min at 48 °C and 5 min at 95 °C following the manufacturer's recommendations. Finally, the cDNA was diluted either with 50 or 100 μl of distilled water when using 500 ng total RNA in the RT reaction and stored at − 20 °C.

2.8. Primers and TaqMan probes

Primer sequences and target-specific fluorescence-labeled TaqMan probes were designed to meet specific criteria using Primer Express™ software version 1.0 (Applied Biosystems) following the standard program settings (Primer Tm: 58–60 °C with maximal 2 °C differences between forward and reverse primer; probe Tm: 67–70 °C and amplicon length ranging from 50 to 150 bp). The design of different IFN-α subtype assays using these uniform settings generates primer combinations with closely matched Tm and short amplicons (74–110 bp amplicon length for the different IFN-α subtype assays), resulting in equal amplification efficiencies and it guarantees comparable and homogenous annealing conditions for all primer/probe combinations. Therefore it is possible to use a standard cycle program for each subtype allowing a flexible combination of different subtype detections in one experiment. The sequence-specific probes were covalently labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA) as a quencher dye at the 3′ end. The sequences of PCR primers and specific probes for amplification and detection of different IFN-α subtypes are shown in Table 2. For detection of the housekeeping gene, β-glucuronidase (GUS), we used the pre-developed TaqMan assay primer/probe combination from Applied Biosystems.

2.9. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR analysis was performed with the ABI PRISM 5700 sequence detection system (Applied Biosystems). This system allows the detection of increasing amounts of amplicons at every individual PCR cycle (Kang et al., 2000). This is achieved by using a sequence-specific probe containing a reporter dye and a quencher molecule that absorbs light emitted by the reporter dye. If specific amplifications occur, the probe is cleaved by the 5′-nuclease activity of the Taq-polymerase, and the reporter dye is physically separated from the quencher (Holland et al., 1991). This leads to emission of fluorescence by the released reporter dye. The amount of fluorescence that is detected by a CCD camera correlates with the amount of amplicons generated.

All PCR reactions were set up in 96-well optical reaction plates (Applied Biosystems) with 14 μl of double concentrated TaqMan universal Mastermix (5 mM $MgCl_2$ final concentration; Applied Biosystems), 5 μl of diluted cDNA (diluted as described in Section 2.7), 200 nM probe and 300 nM of each primer

Table 2
Sequences of primers and probes for different IFN-α real-time PCR assays

IFN-α subtype	Accession number	Sequence (5′–3′)	Position in CDS	Amplicon length (bp)
α_2	J00207	For: CTTGAAGGACAGACATGACTTTGGA	156	74
		Rev: GGATGGTTTCAGCCTTTTGGA	229	
		P: TTCCCCAGGAGGAGTTTGGCAACC	182	
α_6	X02958	For: TCCATGAGGTGATTCAGCAGAC	239	108
		Rev: GCTGCTGGTAAAGTTCAGTATAGAGTTT	346	
		P: CTGTTGCTTGGGATGAGAGGCTTCTAGAC	290	
α_8	X03125	For: CCTTCTAGATGAATTCTACATCGAACTTG	309	86
		Rev: ACTCTATCACCCCCACTTCCTG	394	
		P: CAGCTGAATGACCTGGAGTCCTGTGTG	343	
$\alpha_{13/1}$	X75934 (α_{13})	For: CTTCAACCTCTTTACCACAAAAGATTC	261	84
	XM005504 (α_1)	Rev: TGCTGGTAGAGTTCGGTGCA	344	
		P: TGCTTGGGATGAGGACCTCCTAGACA	294	
AQ[a]	NM002169 (α_5)	For: GAAGAATCTCTCCTTTCTCCTGCC	134	110
	NM002171 (α_{10})	Rev: ATGGAGGACAGAGATGGCTTG	243	
	NM021268 (α_{17})	P: AGGAGTTTGATGGCAACCAGTTCCAGAAG	191	
	NM002175 (α_{21})			

For, forward primer; Rev, reverse primer; P, TaqMan probe.

[a] AQ assay was designed for the detection of α_5, α_{10}, α_{17}, α_{21}; but possesses minor cross-hybridization with other IFN-α isoforms as indicated in Table 3.

and were filled up with sterile distilled water to a final reaction volume of 28 μl. The standard PCR cycle program starts with a 2-min step at 50 °C to perform degradation of possible contamination carried over from previously generated PCR products by uracil-*N*-glycosylase (UNG; included in the TaqMan universal mastermix) cleavage followed by heat activation of AmpliTaqGold polymerase at 95 °C for 10 min. The following 40 cycles of a two-step PCR consist of a denaturation step for 15 s at 95 °C and a combined primer/probe annealing and elongation phase for 1 min at 60 °C. Each sample was run in duplicate. To exclude genomic DNA contamination the RT negative control of each sample was measured on the same plate.

2.10. Relative quantification of mRNA expression

The raw data were normalized by the software to the internal passive reference dye included in the TaqMan mastermix by dividing the reporter signal by the baseline average of the passive reference signal. This normalized reporter signal (Rn) is plotted against the cycle numbers forming the PCR amplification plot. After manual setting of a threshold line into the logarithmic phase of all amplification plots, the software calculates the threshold cycle (Ct value). The Ct value is the PCR cycle in which the amplification plot crosses the threshold line. The Ct values correlate with the initial amount of specific template (Higuchi et al., 1993). In a PCR system with 100% efficiency, the Ct value decreases by one cycle as the concentration of template doubles. The Ct values of each amplification reaction are used to calculate the difference (ΔCt value) between the housekeeping gene β-glucuronidase (GUS) and the target gene signal of the same sample ($\Delta Ct = Ct_{gus} - Ct_{target}$). This operation normalizes possible differences in quality and amount of input cDNA between different samples and allows the quantification and comparison of relative transcription levels in different samples.

2.11. Determination of housekeeping gene

To select a transcriptional, stable housekeeping gene for relative quantification under our stimulation conditions, we performed a screening of 11 putative housekeeping genes using the TaqMan human endogenous control assay (Applied Biosystems) following the manufacturer's recommendations. It consists of a 96-well plate preloaded with lyophilized primer/probe combinations for 11 most commonly used housekeeping genes and one internal control in duplicate. PBMC (5×10^6/ml of culture medium) was stimulated with LPS (10 ng/ml), RSV (MOI 3), NDV (3.2 HA/ml) or cultured without stimulus for 6 h. Afterwards, the cells were lysed and total RNA was isolated. The concentration of RNA was determined by measuring the absorbance at 260 and 280 nm and the integrity of the RNA was checked on a 1.2% denaturating agarose gel. Total RNA (400 ng) from each sample was reverse transcribed in a total volume of 30 μl (described in Section 2.7) and diluted afterwards with distilled water to a final volume of 140 μl. The PCR reactions were performed in a total reaction volume of 50 μl set up with the TaqMan universal mastermix and 5 μl of diluted cDNA as the template (cDNA generated from 14 ng of total RNA per well) on the TaqMan human endogenous control plate. The results of the endogenous control plate were calculated in relation to the unstimulated calibrator sample as ΔCt values ($Ct_{calibrator} - Ct_{housekeeping\ gene}$). Thus, the calibrator serves as a baseline for the assay, samples with positive and negative ΔCt values have higher and lower initial template concentrations than the calibrator sample, respectively. For all subsequent investigations, huGUS was selected as the housekeeping gene because it had the smallest deviations from the calibrator baseline under the different stimulation conditions.

2.12. Data analysis

Data were analyzed and processed with SigmaPlot Version 4.0 and SigmaStat Version 2.0 (SPSS) on a Windows NT operating system.

3. Results

3.1. Selection of housekeeping gene

When using the relative quantification strategy, the increase or decrease of cellular IFN-α mRNA levels has to be calculated in relation to a constantly expressed housekeeping gene. This kind of quantifi-

cation allows the compensation for minimal changes in the quantity and quality of different cDNA preparations. Fig. 1 shows the influence of active NDV, inactivated HSV and LPS as a non-viral control on the expression of common housekeeping genes. After stimulation with NDV, HSV and LPS, the expression of β-actin (BA, − 1.1 ΔCt for NDV, RSV or LPS) and phosphoglycerokinase (PGK, − 1.7 ΔCt for NDV and − 1.4 for RSV) was reduced, whereas transferin-receptor (TFR) mRNA increased (+ 1.9 ΔCt) only after LPS challenge. Other genes like 18S rRNA, cyclophilin (CYC) or the β-glucuronidase (GUS) showed only minor deviations (≤ 0.5 ΔCt) compared to the calibrator sample. We selected GUS as a good candidate for the housekeeping gene in our experimental setting, because it turned out to be constantly expressed. Furthermore, we evaluated the stability of GUS expression in PBMC over a culture period from 2 to 32 h. Fig. 2 indicates that there is no significant influence of the viral stimuli on the GUS expression during this period of time. The course of the regression lines under all conditions differs in a range of only 1 Ct due to intra- and inter-assay variations.

3.2. Specificity of the TaqMan primer/probe combinations

Due to the high homology between the different IFN-α genes, it is essential to control the specificity of the different PCR reactions. For this purpose, we constructed plasmids containing DNA fragments of some IFN-α subtypes (Table 1). The cloned fragments contained the annealing areas of all primer/probe combinations so that possible cross-hybridization events could be detected. The results of one representative experiment for the IFN-α_2 real-time PCR assay are shown in Fig. 3. Only the combination of the pCRIFNA2 plasmid as a template with the IFN-α_2-specific primers/probe led to a strong amplification, whereas all other plasmids were not detected. The investigation of the other primer/probe combinations (Table 3) demonstrated the highly specific character of

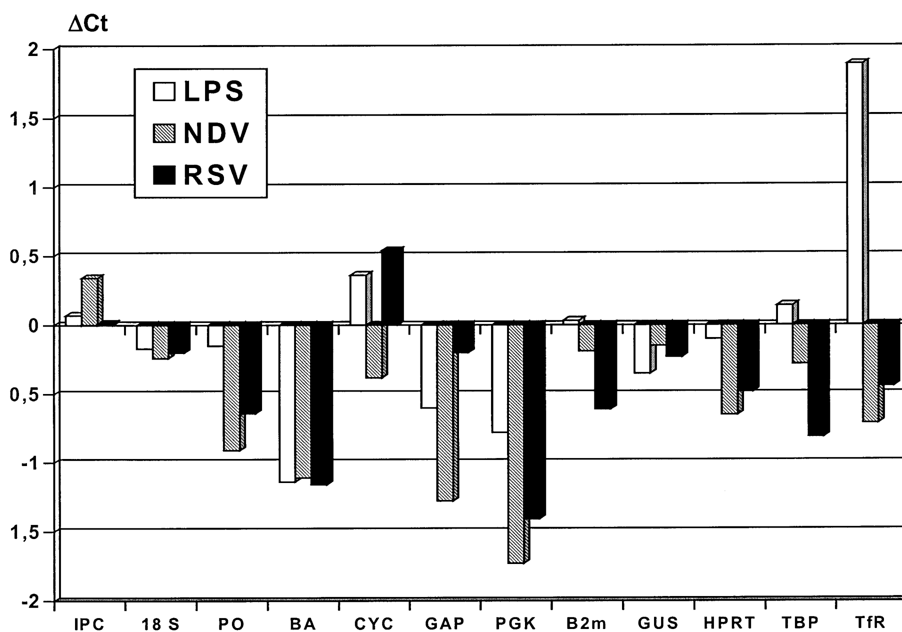


Fig. 1. Influence of different stimuli on housekeeping gene transcription in human PBMC. The transcriptional variations of 11 commonly used human housekeeping genes after stimulation of cultured PBMC with NDV (3.2 HA/ml), RSV (MOI 3) or LPS (10 ng/ml). 18*S*, 18*S* rRNA; PO, acidic ribosomal protein; BA, beta-actin; CYC, cyclophilin; GAP, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerokinase; B2m, β_2-microglobulin; GUS, β-glucuronidase; HPRT, hypoxanthine ribosyl-transferase; TBP, transcription factor IID; TfR, transferin-receptor; IPC, internal positive control. Negative Δ*Ct* deviations from the baseline indicate a decrease and positive deviations show an increase of transcription relative to the unstimulated calibrator sample.

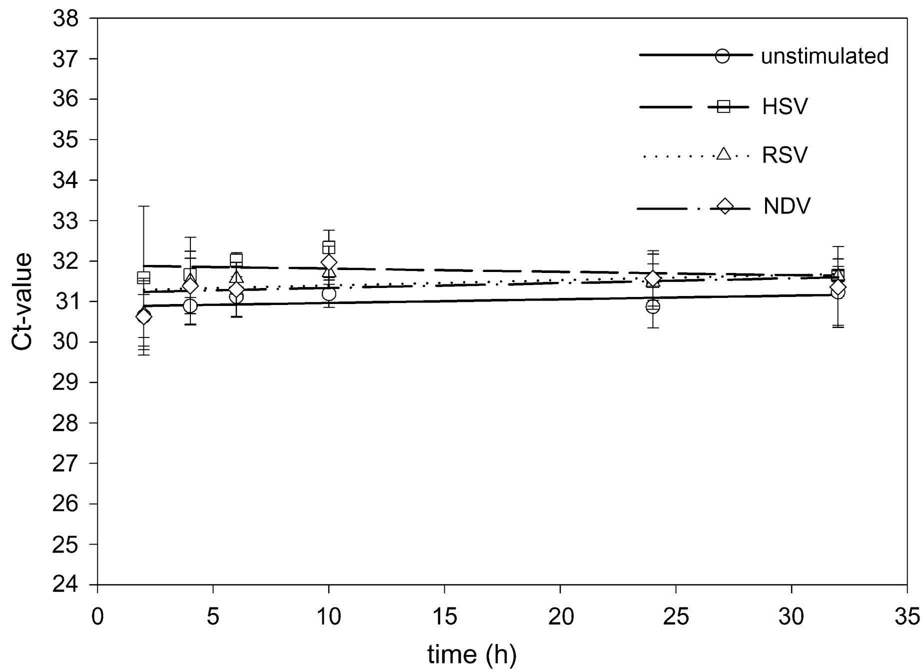


Fig. 2. Stability of GUS housekeeping gene transcription after viral stimulation. The glucuronidase (GUS) transcription was monitored by quantitative real-time PCR in 5×10^5 PBMC with or without viral stimulation [HSV (1:100), NDV (3.2 HA/ml) or RSV (MOI 3)] over an in vitro culture period from 2 to 32 h. The mean *Ct* values from three independent experiments are plotted versus the incubation time and the regression lines were calculated.

the assays. The PCR reactions for IFN-α_2, IFN-α_6 IFN-α_8 and IFN-$\alpha_{13/1}$ showed no unspecific amplification signals. The primer/probe combination of the AQ assay was designed for a broader range of detection capacity including the IFN-α subtypes α_5, α_{10}, α_{17} and α_{21}. Therefore, strong amplifications occurred by using plasmids containing IFN-α_5 and IFN-α_{17} DNA. Due to cross-hybridization events, the AQ assay led to amplification of four other subtype plasmids, but with much lower efficiencies (on average 6 Ct higher) compared to the specific PCR reaction. In summary, all IFN-α real-time assays, except AQ, were highly specific and were able to clearly discriminate between the different IFN-α subtype transcripts.

3.3. Comparability of different IFN-α real-time PCR reactions

An essential prerequisite for comparing the results of different IFN-α PCR reactions is the stability and comparability of their amplification efficiencies over the range of measurement. We examined this for the established IFN-α real-time PCR assays by using serial diluted genomic DNA (100–0.01 ng per reaction) as a template to perform standard curves (Fig. 4). The genomic DNA contains all human IFN-α genes in a single copy per haploid genome and therefore the relative amounts between the different IFN-α subtypes are equal. The standard curves generated for the IFN-α_2, IFN-α_6 and IFN-α_8 specific real-time PCR assays showed the same course within the effective range and produced equal Ct values (e.g. $Ct_{A2} = 32.3$, $Ct_{A6} = 32.8$, $Ct_{A8} = 32.4$ for 1 ng gDNA per reaction). Because the IFN-$\alpha_{13/1}$ assay detects both subtypes, IFN-α_{13} and IFN-α_1, the standard curve for this assay ran about 1.5 Ct values lower than the single subtype assays. Since the AQ assay showed an even broader detection spectrum, it ran about 2.5 Ct below the level of the three single subtype assays, IFN-α_2, IFN-α_6 and IFN-α_8. By analyzing the slopes of each standard curve, it was possible to make a predication regarding their relative amplification efficiencies. The slopes of all standard curves are within the same range (− 3.75

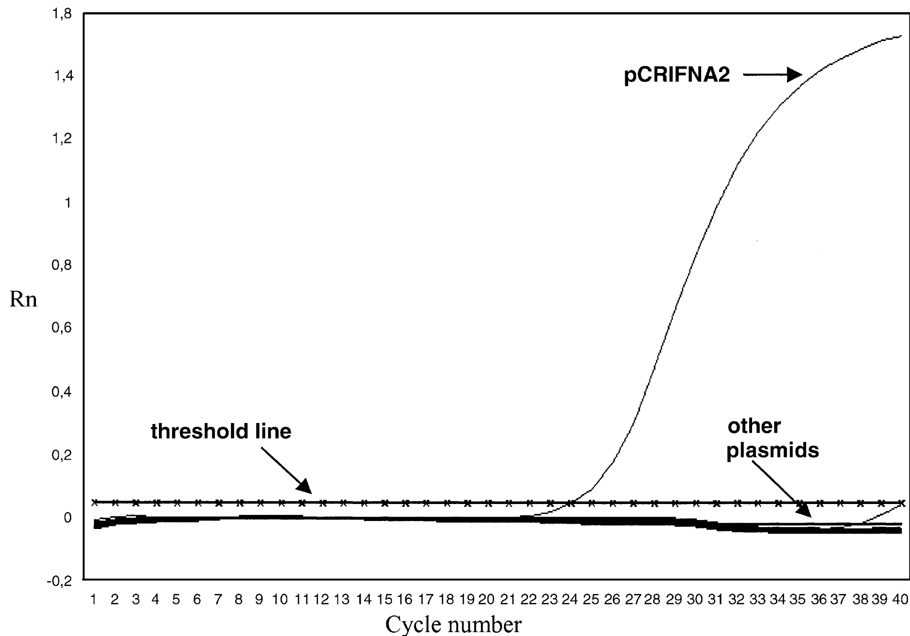


Fig. 3. Amplification plot of IFN-α_2 real-time PCR assay. A representative result of a control experiment for checking the specificity of the IFN-α_2 real-time PCR assay. The plasmid pCRIFNA2 as a template leads to a strong increase of the normalized reporter signal (Rn) that is plotted versus the cycle number. All other plasmids used (Table 1) were not be detected by the IFN-α_2 real-time PCR assay.

for IFN-α_2, − 3.57 for IFN-α_6, − 3.68 for IFN-α_8, − 3.87 for IFN-$\alpha_{13/1}$ and − 3.72 for AQ). These data clearly demonstrate equal amplification efficiencies within the effective range and the possibility to compare results of the different IFN-α real-time PCR assays with each other.

Table 3
Specificities of the established IFN-α real-time PCR assays

Plasmid	IFN-α_2 PCR assay	IFN-α_6 PCR assay	IFN-α_8 PCR assay	IFN-$\alpha_{13/1}$ PCR assay	IFN-α AQ PCR assay
pCRIFNA2	+	−	−	−	−
pCRIFNA4	−	−	−	−	+/−
pCRIFNA5	−	−	−	−	+
pCRIFNA7	−	−	−	−	+/−
pCRIFNA8	−	−	+	−	−
pCRIFNA13	−	−	−	+	+/−
pCRIFNA16	−	−	−	−	+/−
pCRIFNA17	−	−	−	−	+

The primer/probe combinations designed for the real-time assays α_2; α_6, α_8, $\alpha_{13/1}$ and AQ were checked for unspecific amplification of other IFN-α subtypes by performing real-time PCR reactions under standard assay conditions. For each PCR reaction, 2.5 pg of the plasmids shown above, containing defined IFN-α subtype DNA, was used as a template. +, specific amplification; −, no amplification; +/−, amplification with reduced efficiency caused by cross-hybridization events.

3.4. IFN-α transcription in unstimulated cells

Since it is known that cellular IFN-α production is strictly regulated on the transcriptional level, we have analyzed the non-induced state of PBMC in conjunction with the evaluation of the PCR assays . In freshly isolated or in vitro cultured, unstimulated cells, no IFN-α_2, α_6, α_8 and $\alpha_{13/1}$ transcripts could be detected by the real-time PCR. In contrast to this, we detected transcripts of the subtype group AQ (Table 4a). The observed Ct values were slightly above the detection limit of Ct 40. This constitutive transcription in human PBMC could be found in freshly isolated cells as well as in cultured cells during the whole incubation period. To exclude the possibility that the broader detection range of the AQ assay is responsible for the low-level detection of transcripts, we measured non-induced IFN-α transcription in two cell-lines (HEp-2 and HeLa) as a control. In contrast to PBMC, these

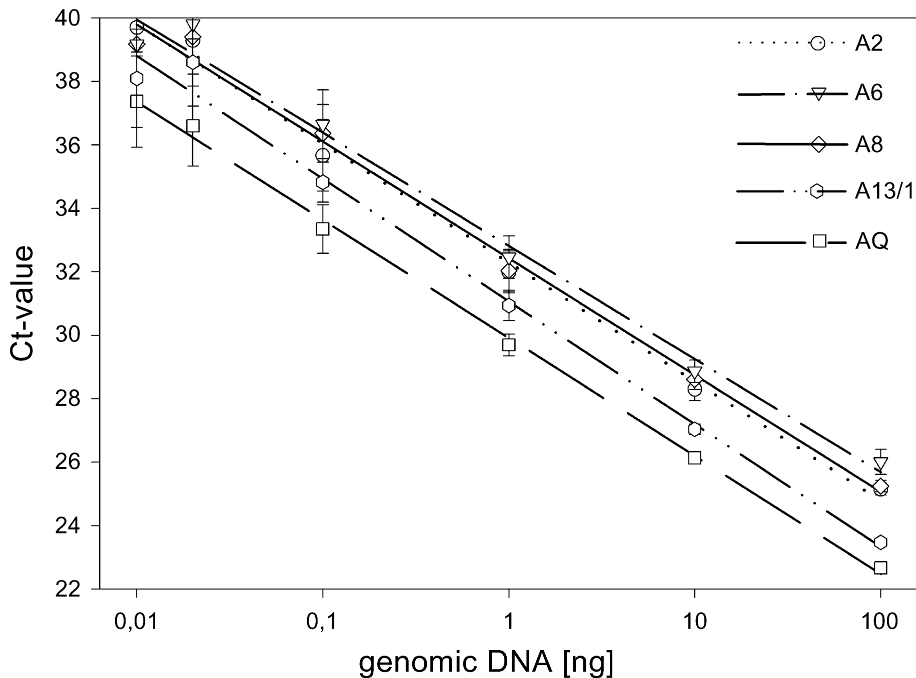


Fig. 4. Standard curves for IFN-α real-time PCR assays performed with serial diluted human genomic DNA. All five real-time assays were performed with tenfold serial diluted human genomic DNA in a range from 100 to 0.01 ng per reaction. The mean data from three independent experiments were plotted versus the input gDNA and regression lines were calculated. α_2: $f(x) = -3.75 \times x + 32.29$, $r^2 = 0.995$; α_6: $f(x) = -3.57 \times x + 32.81$, $r^2 = 0.988$; α_8: $f(x) = -3.68 \times x + 32.42$, $r^2 = 0.993$; $\alpha_{13/1}$ $f(x) = -3.87 \times x + 31.07$, $r^2 = 0.992$; AQ: $f(x) = -3.72 \times x + 29.91$, $r^2 = 0.998$.

cells possessed a constitutive transcription of IFN-α_{13} and/or IFN-α_1 and additionally of the subtype IFN-α_8, but not for AQ. These findings indicate a specific constitutive IFN-α subtype transcription pattern in different cells.

Table 4a
Constitutive IFN-α transcription in different cell types

	α_2	α_6	α_8	$\alpha_{13/1}$	AQ	Control
PBMC	–	–	–	–	36.3	–
	–	–	–	–	37.3	–
HEp-2	–	–	37.2	33.6	–	–
	–	–	36.9	34.1	–	–
HeLa	–	–	36.9	33.1	–	–
	–	–	38.4	32.5	–	–

Total RNA (500 ng) from freshly isolated PBMC or unstimulated HEp-2 and HeLa cells was reverse transcribed and IFN-α real-time PCR was performed. The results from two individual experiments are given as Ct values. –, no IFN-α transcripts detectable in all experiments; control, RT reaction without reverse transcriptase tested with AQ real-time PCR.

3.5. Specific induction of IFN-α expression

It is well accepted that certain, but not all, viruses induce a strong IFN-α transcription in vitro and in vivo whereas bacterial products such as LPS are not able to induce IFN-α. To prove whether this observation is true, when using our IFN-α real-time assays, we tested NDV as high and LPS and Adv3 as non-IFN-α inducers (Table 4b). The bacterial product LPS and adenovirus, as a different viral stimulus, induced no IFN-α transcription in PBMC. In contrast to this, NDV resulted in a strong transcriptional activation of all IFN-α subtypes investigated. Namalwa cells were used as a control and showed only a minor transcriptional activation of IFN-α mRNA after stimulation with NDV (Table 4b). However, the sensitivity of the real-time PCR allowed the quantification of different IFN-α subtypes in these cells. Whereas in PBMC the NDV-induced IFN-$\alpha_{13/1}$ expression level was always about

Table 4b
Specific IFN-α mRNA induction

	Namalwa	PBMC		
	NDV	NDV	LPS	Adv3
α_2	35.1	26.4	–	–
	33.7	25.1	–	–
α_6	–	31.7	–	–
	–	28.9	–	–
α_8	–	28.1	–	–
	38.9	26.6	–	–
$\alpha_{13/1}$	36.9	25.5	–	–
	34.6	24.4	–	–
AQ	36.4	24.6	37.3	36.2
	35.4	23.0	38.1	39.0
Control	–	–	–	–
	–	–	–	–

PBMC (5×10^5) was incubated in vitro with NDV (3.2 HA/ml), Adv3 (MOI 1) or LPS (10 ng/ml) for 6 h and 5×10^6 Namalwa cells were stimulated with NDV (50 HA/ml) for 24 h. Afterwards, IFN-α transcription was measured with the real-time PCR assays. The results are given as Ct values from two independent experiments. –, no IFN-α transcripts detectable in all experiments; control, RT reaction without reverse transcriptase tested with AQ assay.

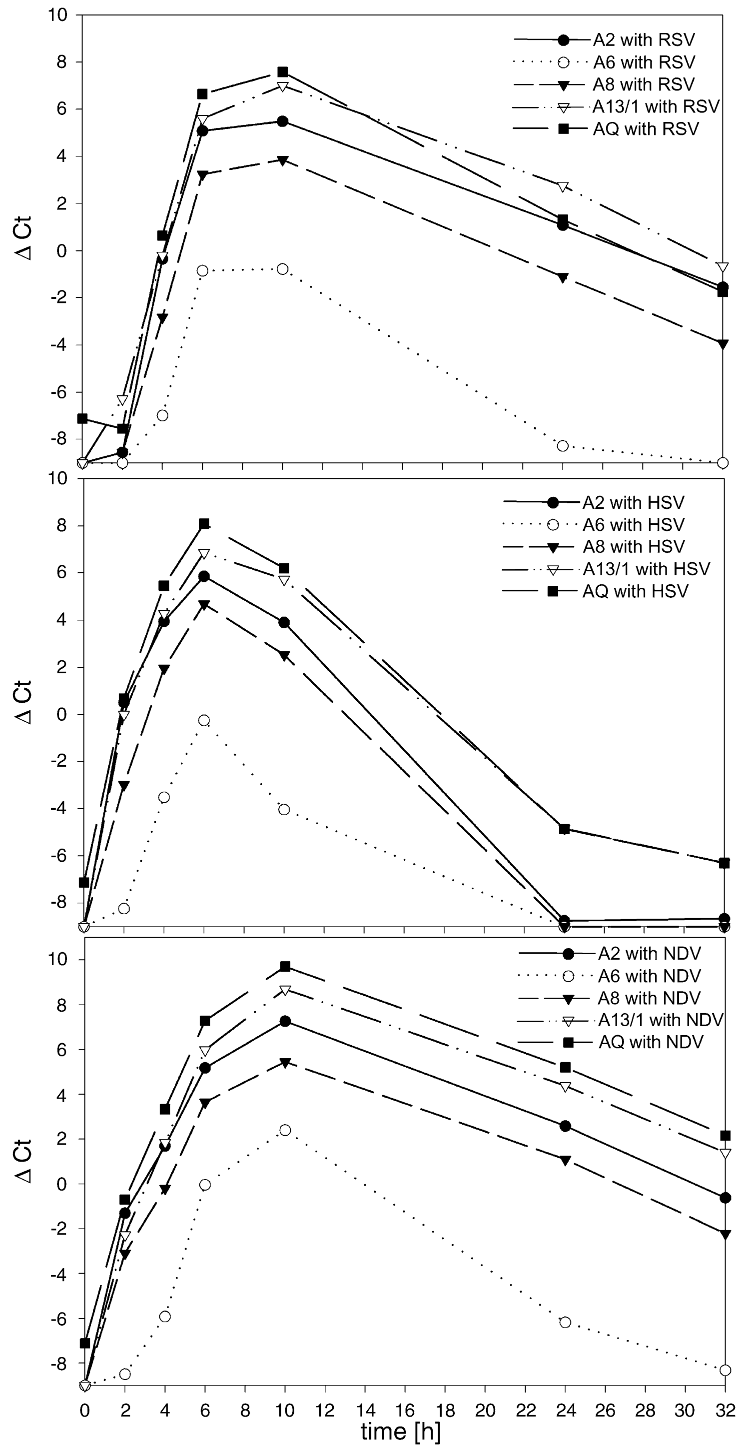
twice as much as for IFN-α_2 (mean $\Delta\Delta Ct_{\alpha_2} - \alpha_{13/1}$ value of 0.8), in Namalwa cells this relation was reversed (mean $\Delta\Delta Ct_{\alpha_2} - \alpha_{13/1}$ of -1.4). Furthermore, AQ detectable IFN-α transcripts always reached the highest level in PBMC and only medium level in Namalwa cells.

3.6. Time kinetics of IFN-α expression

The quantitative and specific character allowed a precise tracking of IFN-α subtype activation over the time and under different stimulation conditions. Throughout all time kinetics, we could find a stable and reproducible pattern of IFN-α activation (Fig. 5). The viral stimuli used (HSV, NDV and RSV) led to a rapid activation of IFN-α_2, $\alpha_{13/1}$ α_8, α_6 and AQ transcription. There are differences in the amount of mRNA induced between the IFN-α subtypes. The mean ΔΔCt value of 1.3 between α_2 and $\alpha_{13/1}$ at their maximum represents a 2.5 times higher transcript amount of $\alpha_{13/1}$ compared to α_2. The subtype α_8 shows a reduced transcription of 1.5 ΔΔCt (2.8 times less) compared to α_2 and 2.9 ΔΔCt (8 times less) compared to $\alpha_{13/1}$. The transcriptional differences between α_6 and the other isoforms are much higher and can be calculated to have an 18, 52 or 133 times difference towards α_8, α_2, or $\alpha_{13/1}$. Besides these common characteristics, some differences in the course of the kinetics could be observed. Comparing the maximum ΔCt values of IFN-α_2 induction caused by NDV (ΔCt_{max} at 10 h; 7.26), HSV (ΔCt_{max} at 6 h; 5.86) or RSV (ΔCt_{max} at 10 h; 5.49) the highest transcriptional activation could be obtained with NDV. In the respective peak, NDV induced nearly three times higher amounts of IFN-α_2 transcripts compared to RSV and HSV (1.8 $\Delta\Delta Ct_{NDV-RSV}$ and 1.4 $\Delta\Delta Ct_{NDV\text{-}HSV}$). Stimulation with NDV and RSV reached the maximum of transcriptional activation after 10 h. In contrast, HSV induced a more rapid activation resulting in a maximal response after 6 h followed by a rapid decline of expression. In the case of stimulation with NDV or RSV, the transcriptional activation continued over a longer period of time and the decrease was much slower. Furthermore, the induction of IFN-α transcription by RSV compared to HSV and NDV was significantly delayed by at least 2 h. In the first 2 h of incubation with RSV, only IFN-$\alpha_{13/1}$ transcription showed a slight increase and not even the AQ transcription is influenced.

Parallel to the analysis of transcriptional activation, protein release kinetics were performed by measurement of IFN-α_2 accumulation in the culture supernatants. The results (Fig. 6) correlate with the course of the real-time PCR kinetics (Fig. 5). The IFN-α_2 release with NDV stimulation reached much higher levels compared to RSV or HSV during the later phase of induction (10–32 h). The IFN-α release by HSV could be detected earlier and was higher than that induced by RSV in the early phase of induction (4–10 h). After an incubation time above 10 h, the IFN-α release induced by RSV clearly increased further, whereas the IFN-α release of HSV-induced cells increased only slightly. This correlated with the rapid decline of transcription in HSV-induced PBMC versus the longer activation in RSV-infected cells.

In conclusion, the IFN-α subtype-specific, real-time PCR assays allowed the detection of quantitative differences in the transcriptional activation of the subtypes investigated. Furthermore, the data demonstrated differences in the time course and strength of


A2 with RSV
A6 with RSV
A8 with RSV
A13/1 with RSV
AQ with RSV
Δ Ct
A2 with HSV
A6 with HSV
A8 with HSV
A13/1 with HSV
AQ with HSV
Δ Ct
A2 with NDV
A6 with NDV
A8 with NDV
A13/1 with NDV
AQ with NDV
Δ Ct
time [h]

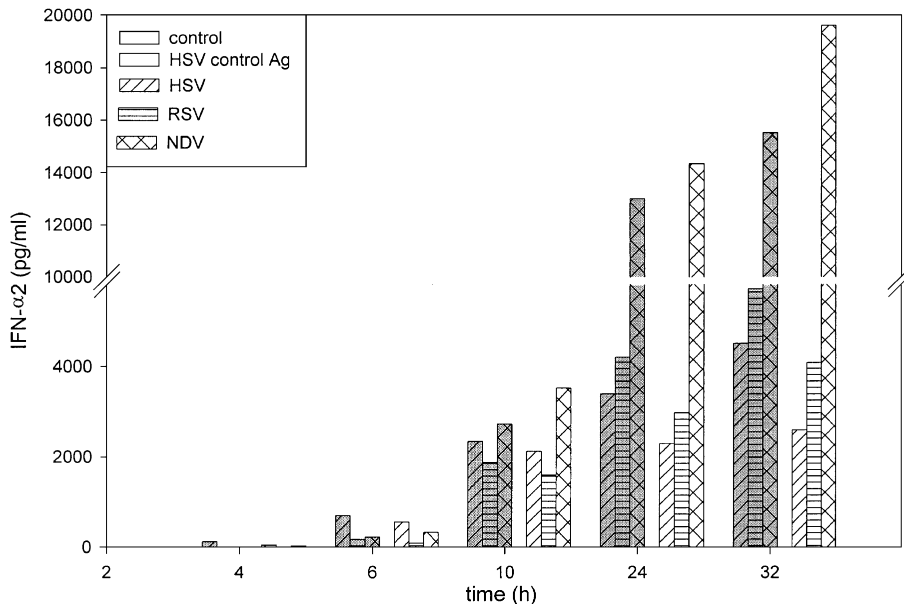


Fig. 6. Virus-induced IFN-α_2 protein release in PBMC. PBMC (5×10^5 in 250 μl medium) was cultured in the absence or presence of NDV, RSV, HSV or HSV control antigen for 2–32 h. IFN-α_2 release from two individual blood donors (gray and white bars) in the supernatant was analyzed by ELISA.

activation depending on the kind of viral stimulus used.

4. Discussion

Here, we have evaluated novel real-time PCR assays for the quantification and discrimination of individual members of the closely related IFN-α gene family. At present, only a few studies have used this method to monitor expression of closely related genes (Bleicher et al., 2001) especially of IFN-α expression (Remoli et al., 2002) or follow up the kinetics of gene expression (Stordeur et al., 2002). Most current methods do not discriminate easily among family members, are very labor intensive or only semi-quantitative. Time-consuming methods like cloning of total IFN-α cDNA followed by sequencing of clones (Castelruiz et al., 1999; Yeow et al., 2001) or the analysis of specific IFN-α mRNA by S1 mapping are not practical for larger investigations. Also, the analysis of IFN-α gene activation by using IFN-α promoter-reporter constructs is limited to transfected cell lines and is not applicable to ex vivo derived unmanipulated cells. With our new real-time PCR assays, we can overcome these problems and are able to track the induction and the course of transcriptional activation of three single IFN-α subtypes α_2, α_6, α_8 and, in a combined assay, the expression of IFN-α_{13} and IFN-α_1. Control experiments with plasmids, containing different highly homologous IFN-α DNAs, demonstrated the excellent discrimination properties of the IFN-α assays for α_2, α_6, α_8 and $\alpha_{13/1}$.

The quantification was done by the use of an internal control gene. This kind of quantification allows the correction of minor variations in sample quality and quantity by the normalization to a housekeeping gene. We demonstrated how important it is to prove the

Fig. 5. Time-dependent transcriptional activation of different IFN-α subtypes in human PBMC after viral stimulation. The combined real-time PCR data of two representative and independent experiments are shown. PBMC (5×10^5/250 μl of culture medium) was stimulated with NDV (3.2 HA/ml), RSV (MOI 3), HSV (1:100) or without any stimulus and cultured for 0–32 h. After RNA isolation and reverse transcription each sample was measured in duplicate and ΔCt values ($Ct_{gus} - Ct_{IFN\text{-}\alpha}$) were calculated. At 0 h, the freshly isolated cells were immediately lysed after setting up the stimulus.

suitability of a housekeeping gene under the specific experimental settings, since the expression of the classical housekeeping genes is influenced by culture or stimulation conditions and it can vary in relevant doses between different cell or tissue types (Schmittgen and Zakrajsek, 2000; Gorzelniak et al., 2001).

We decided to use GUS as a housekeeping gene not only for its stable expression, but because the constant transcriptional level of GUS (on average Ct 31.4) was in the mean of the range in which IFN-α is expressed (Ct 22–40). This is a prerequisite for a reliable comparison of gene expression by real-time PCR (Thellin et al., 1999).

The use of a standard cycle program for all real-time PCR assays allows the detection of mRNA from different IFN-α subtypes together on one plate. To compare the quantification results, the PCR efficiencies have to be nearly the same. These requirements are met by our assays, because of the comparable length of the amplicons and design of the primers and probes according to the same parameters. The comparability of PCR efficiencies is demonstrated by the equality of slopes of the standard curves using genomic DNA as a template.

Because of the high sensitivity of this IFN-α PCR method, it is possible to detect very low cellular transcriptional activity. In all unstimulated PBMC preparations investigated we observed a constitutive low-level IFN-α transcription. Interestingly, human cell-lines of different tissue origin also possessed a constitutive transcription. But in contrast to PBMC, other IFN-α subtypes were constitutively expressed in these cell lines. Other investigators also describe minor levels of IFN-α transcripts in uninduced human cells (Brandt et al., 1994; Greenway et al., 1995; Taniguchi and Takaoka, 2001), and recently it was suggested that this low level of constitutive IFN-α expression results in a basic IRF-7 level which is important for an efficient IFN-α/β gene induction by viruses (Hata et al., 2001). But nothing is known about the role of different constitutive subtypes pattern in different cell types.

With our real-time PCR assays, we can for the first time analyze time kinetics of expression of different IFN-α subtypes in human PBMC following viral stimulation. Our results are basically in line with the literature showing that IFN-α_1 and α_2 are two dominantly expressed subtypes, whereas IFN-α_8 mRNA is less transcribed (Hiscott et al., 1984). In contrast to PBMC, a different quantitative relation of IFN-α subtypes could be measured with the real-time PCR assays in Namalwa cells, a human lymphoblastoid cell line inducible for IFN-α expression. Our data are in good accordance with published results obtained from S1 mapping (Hiscott et al., 1984) or cDNA library screening experiments (Lund et al., 1985), showing that IFN-α_2 is the dominant subtype in Namalwa cells. Therefore the PCR assays confirm the biologic difference regarding the IFN-α subtype pattern between PBMC and Namalwa cells.

The sensitivity of our IFN-α PCR allows the detection of the IFN-α_6 subtype which is up to 52 times less activated than IFN-α_2. This observation may explain why only a few authors have detected IFN-α_6 transcripts with their methods (Megyeri et al., 1995). Since the strength of biological and immunological effects of the IFN-α subtypes was shown to be different (Fish et al., 1983; Foster and Finter, 1998), it will be very important to know the exact level and composition (Yanai et al., 2001b) of different subtype expression. The differentiation and quantification characteristics of our real-time PCR assays now enable those measurements.

Furthermore, the real-time PCR is ideal to trace the transcriptional activation of IFN-α as early as 1 h after stimulation and can follow up the course during the whole experiment. The comparison of real-time PCR data with the results of the IFN-α_2-specific ELISA demonstrated good correlation. Therefore, transcriptional activation can also be used for the interpretation of IFN-α protein release.

We found differences between the kinetics of IFN-α dependent on the kind of viral stimulus. Although the inactivated HSV preparation does not enter the cells with active virus particles and leads to an ongoing intracellular infection, it was able to induce IFN-α in human PBMC. Therefore induction mechanisms for IFN-α must exist, which are independent of the intracellular presence of viruses and the formation of double stranded RNA (dsRNA), which is described as a type I IFN inducer (Lepe-Zuniga et al., 1989; Der and Lau, 1995). The dsRNA is either provided by the viral genome or arises as an intermediate during viral replication. For HSV (Ankel et al., 1998) and a variety of other viruses (Yasuda et al., 1992; Francis and Meltzer, 1993; Wertz et al., 1994), it has been reported that

viral glycoproteins may be involved in the IFN-α induction. The cellular receptors that are able to recognize viral components and mediate an appropriate signaling have not yet been identified but some candidates have been discussed. These are toll-like receptors (Kadowaki et al., 2001), mannose receptors (Milone and Fitzgerald-Bocarsly, 1998) or CD4 (Francis and Meltzer, 1993). It is interesting to speculate that the observed earlier appearance and decrease in IFN-α transcription induced by the inactivated HSV preparation is indicative of an IFN-α activation mediated only by receptor-mediated recognition of viral proteins. In contrast, the prolonged IFN-α transcription seen with the active viruses NDV and RSV seems to additionally involve signals that are provided if the virus is present intracellularly. In conclusion, our newly established quantitative real-time PCR is a powerful tool to investigate expression of IFN-α subtypes. It is a rapid and sensitive method, which allows the specific detection and quantification of gene expression in many samples without any post-PCR analysis steps. The established real-time PCR assays are able to distinguish between some highly homologues IFN-α subtypes and they can be useful in investigations regarding the induction and regulation of IFN-α subtype expression.

Acknowledgements

We thank K. Brockmann for her excellent technical assistance and P. Rozynek for his skillful support in the sequencing procedures for the pCRIFNA constructs. This work was sponsored by the Bundesministerium für Bildung und Forschung (BMBF) FKZ 01GC 9802/8 and 01GC0101.

References

Ankel, H., Westra, D.F., Welling-Wester, S., Lebon, P., 1998. Induction of interferon-alpha by glycoprotein D of herpes simplex virus: a possible role of chemokine receptors. Virology 251, 317–326.

Bleicher, K.B., Pippert, T.R., Glaab, W.E., Skopek, T.R., Sina, J.F., Umbenhauer, D.R., 2001. Use of real-time gene-specific polymerase chain reaction to measure RNA expression of three family members of rat cytochrome P450 4A. J. Biochem. Mol. Toxicol. 15, 133–142.

Brandt, E.R., Linnane, A.W., Devenish, R.J., 1994. Expression of IFN A genes in subpopulations of peripheral blood cells. Br. J. Haematol. 86, 717–725.

Bufe, A., Gehlhar, K., Grage-Griebenow, E., Ernst, M., 2002. Atopic phenotype in children is associated with decreased virus induced interferon-α release. Int. Arch. Allergy Immunol. 127, 82–88.

Castelruiz, Y., Larrea, E., Boya, P., Civeira, M.P., Prieto, J., 1999. Interferon alfa subtypes and levels of type I interferons in the liver and peripheral mononuclear cells in patients with chronic hepatitis C and controls. Hepatology 29, 1900–1904.

Dent, C.L., Macbride, S.J., Sharp, N.A., Gewert, D.R., 1996. Relative transcriptional inducibility of the human interferon-alpha subtypes conferred by the virus-responsive enhancer sequence. J. Interferon Cytokine Res. 16, 99–107.

Der, S.D., Lau, A.S., 1995. Involvement of the double-stranded-RNA-dependent kinase PKR in interferon expression and interferon-mediated antiviral activity. Proc. Natl. Acad. Sci. U. S. A. 92, 8841–8845.

Fish, E.N., Banerjee, K., Stebbing, N., 1983. Human leukocyte interferon subtypes have different antiproliferative and antiviral activities on human cells. Biochem. Biophys. Res. Commun. 112, 537–546.

Foster, G.R., Finter, N.B., 1998. Are all type I human interferons equivalent? J. Viral Hepatitis 5, 143–152.

Foster, G.R., Rodrigues, O., Ghouze, F., Schulte-Frohlinde, E., Testa, D., Liao, M.J., Stark, G.R., Leadbeater, L., Thomas, H.C., 1996. Different relative activities of human cell-derived interferon-alpha subtypes: IFN-alpha 8 has very high antiviral potency. J. Interferon Cytokine Res. 16, 1027–1033.

Francis, M.L., Meltzer, M.S., 1993. Induction of IFN-alpha by HIV-1 in monocyte-enriched PBMC requires gp120-CD4 interaction but not virus replication. J. Immunol. 151, 2208–2216.

Gallati, H., 1982. Interferon: wesentlich vereinfachte, enzym-immunologische Bestimmung mit zwei monoklonalen Antikörpern. J. Clin. Chem. Biochem. 20, 907–914.

Gobl, A.E., Funa, K., Alm, G.V., 1988. Different induction patterns of mRNA for IFN-alpha and-beta in human mononuclear leukocytes after in vitro stimulation with herpes simplex virus-infected fibroblasts and Sendai virus. J. Immunol. 140, 3605–3609.

Goodbourn, S., Didcock, L., Randall, R.E., 2000. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J. Gen. Virol. 81 (Pt 10), 2341–2364.

Gorzelniak, K., Janke, J., Engeli, S., Sharma, A.M., 2001. Validation of endogenous controls for gene expression studies in human adipocytes and preadipocytes. Horm. Metab. Res. 33, 625–627.

Greenway, A.L., Overall, M.L., Sattayasai, N., Rowley, M.J., Hertzog, P.J., McMullen, G.L., Cheetham, B.F., Marzuki, S., 1992. Selective production of interferon-alpha subtypes by cultured peripheral blood mononuclear cells and lymphoblastoid cell lines. Immunology 75, 182–188.

Greenway, A.L., Brandt, E.R., Hertzog, P.J., Devenish, R.J., Linnane, A.W., 1993. Post-transcriptional regulation of interferon-alpha 4 subtype production by lymphoblastoid cells. Hematol. Oncol. 11, 7–21.

Greenway, A.L., Hertzog, P.J., Devenish, R.J., Linnane, A.W.,

1995. Constitutive and virus-induced interferon production by peripheral blood leukocytes. Exp. Hematol. 23, 229–235.

Hata, N., Sato, M., Takaoka, A., Asagiri, M., Tanaka, N., Taniguchi, T., 2001. Constitutive IFN-alpha/beta signal for efficient IFN-alpha/beta gene induction by virus. Biochem. Biophys. Res. Commun. 285, 518–525.

Hibbert, L., Foster, G.R., 1999. Human type I interferons differ greatly in their effects on the proliferation of primary B cells. J. Interferon Cytokine Res. 19, 309–318.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11, 1026–1030.

Hiscott, J., Cantell, K., Weissmann, C., 1984. Differential expression of human interferon genes. Nucleic Acids Res. 12, 3727–3746.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of Thermus aquaticus DNA polymerase. Proc. Natl. Acad. Sci. U. S. A. 88, 7276–7280.

Hughes Jr., T.K., Chin, R., Tyring, S.K., Rady, P.L., 1994. Distinction of mouse interferon-alpha subtypes by polymerase chain reaction utilizing consensus primers and type-specific oligonucleotide probes. J. Interferon Res. 14, 117–120.

Ito, T., Amakawa, R., Inaba, M., Ikehara, S., Inaba, K., Fukuhara, S., 2001. Differential regulation of human blood dendritic cell subsets by IFNs. J. Immunol. 166, 2961–2969.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R.W., Kastelein, R.A., Bazan, F., Liu, Y.J., 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. 194, 863–869.

Kang, J.J., Watson, R.M., Fisher, M.E., Higuchi, R., Gelfand, D.H., Holland, M.J., 2000. Transcript quantitation in total yeast cellular RNA using kinetic PCR. Nucleic Acids Res. 28, e2.

LaFleur, D.W., Nardelli, B., Tsareva, T., Mather, D., Feng, P., Semenuk, M., Taylor, K., Buergin, M., Chinchilla, D., Roshke, V., Chen, G., Ruben, S.M., Pitha, P.M., Coleman, T.A., Moore, P.A., 2001. Interferon-kappa, a novel type I interferon expressed in human keratinocytes. J. Biol. Chem. 276, 39765–39771.

Lepe-Zuniga, J.L., Rotbein, J., Gutterman, J.U., 1989. Production of interferon-alpha induced by dsRNA in human peripheral blood mononuclear cell cultures: role of priming by dsRNA-induced interferons-gamma and-beta. J. Interferon Res. 9, 445–456.

Lleonart, R., Naf, D., Browning, H., Weissmann, C., 1990. A novel, quantitative bioassay for type I interferon using a recombinant indicator cell line. Biotechnology 8, 1263–1267.

Lund, B., von Gabain, A., Edlund, T., Ny, T., Lundgren, E., 1985. Differential expression of interferon genes in a substrain of Namalwa cells. J. Interferon Res. 5, 229–238.

Megyeri, K., Au, W.C., Rosztoczy, I., Raj, N.B., Miller, R.L., Tomai, M.A., Pitha, P.M., 1995. Stimulation of interferon and cytokine gene expression by imiquimod and stimulation by Sendai virus utilize similar signal transduction pathways. Mol. Cell Biol. 15, 2207–2218.

Milone, M.C., Fitzgerald-Bocarsly, P., 1998. The mannose receptor mediates induction of IFN-alpha in peripheral blood dendritic cells by enveloped RNA and DNA viruses. J. Immunol. 161, 2391–2399.

Nyman, T.A., Tölö, H., Parkkinen, J., Kalkkinen, N., 1998. Identification of nine interferon-α subtypes produced by Sendai virus-induced human peripheral blood leucocytes. J. Biochem. 329, 295–302.

Remoli, M.E., Giacomini, E., Lutfalla, G., Dondi, E., Orefici, G., Battistini, A., Uze, G., Pellegrini, S., Coccia, E.M., 2002. Selective expression of type I IFN genes in human dendritic cells infected with *Mycobacterium tuberculosis*. J. Immunol. 169, 366–374.

Schmittgen, T.D., Zakrajsek, B.A., 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J. Biochem. Biophys. Methods 46, 69–81.

Stordeur, P., Poulin, L.F., Craciun, L., Zhou, L., Schandene, L., de Lavareille, A., Goriely, S., Goldman, M., 2002. Cytokine mRNA quantification by real-time PCR. J. Immunol. Methods 259, 55–64.

Taniguchi, T., Takaoka, A., 2001. A weak signal for strong responses: interferon-alpha/beta revisited. Nat. Rev. Mol. Cell Biol. 2, 378–386.

Taniguchi, T., Ogasawara, K., Takaoka, A., Tanaka, N., 2001. IRF family of transcription factors as regulators of host defense. Annu. Rev. Immunol. 19, 623–655.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A., Heinen, E., 1999. Housekeeping genes as internal standards: use and limits. J. Biotechnol. 75, 291–295.

Verhagen, A., Mackay, I.R., Rowley, M., Tymms, M., 1990. Comparison of augmentation of human natural killer cell cytotoxicity by interferon-alpha subtypes. Nat. Immun. Cell Growth Regul. 9, 325–333.

Wertz, K., Buttner, M., Mayr, A., Kaaden, O.R., 1994. More than one component of the Newcastle disease virus particle is capable of interferon induction. Vet. Microbiol. 39, 299–311.

Yanai, Y., Horie, S., Yamamoto, K., Yamauchi, H., Ikegami, H., Kurimoto, M., Kitamura, T., 2001a. Characterization of the antitumor activities of IFN-alpha8 on renal cell carcinoma cells in vitro. J. Interferon Cytokine Res. 21, 1129–1136.

Yanai, Y., Sanou, O., Kayano, T., Ariyasu, H., Yamamoto, K., Yamauchi, H., Ikegami, H., Kurimoto, M., 2001b. Analysis of the antiviral activities of natural IFN-alpha preparations and their subtype compositions. J. Interferon Cytokine Res. 21, 835–841.

Yasuda, Y., Ohgitani, E., Yamada, A., Hosaka, Y., Uno, K., Imanishi, J., Kishida, T., 1992. Induction of interferon-in human peripheral blood mononuclear cells by influenca virus: role of viral glycoproteins. J. Interferon Res. 12, 141.

Yeow, W.S., Au, W.C., Lowther, W.J., Pitha, P.M., 2001. Downregulation of IRF-3 levels by ribozyme modulates the profile of IFNα subtypes expressed in infected human cells. J. Virol. 75, 3021–3027.