# A non-classical ISRE/ISGF3 pathway mediates induction of RANTES gene transcription by type I IFNs

Isabelle Cremer[a], Jacques Ghysdael[b], Vincent Vieillard[c,*]

[a] *Laboratoire d'Immunologie Cellulaire et Clinique, INSERM U255, Université Paris-6, 75005 Paris, France*
[b] *Régulations Cellulaires et Oncogénèse, CNRS UMR 146, Institut Curie, 91405 Orsay, France*
[c] *Laboratoire d'Immunologie Cellulaire et Tissulaire, INSERM U543, Hôpital Pitié-Salpétrière, 75013 Paris, France*

Received 30 August 2001; revised 5 November 2001; accepted 28 November 2001

First published online 19 December 2001

Edited by Masayuki Miyasaka

**Abstract RANTES (regulated upon activation normal T cell expressed and secreted) is a chemoattractant cytokine important in the generation of inflammatory responses and human immunodeficiency virus resistance. In hematopoietic cells, RANTES is over-expressed by type I interferons (IFN-α and IFN-β). The upstream region of the RANTES gene promoter contains a distal low affinity IFN-stimulated response element (ISRE). Specific mutagenesis in this ISRE-like motif abolished the activation of RANTES transcription by type I IFNs. Examination of the ISRE binding factors strongly suggested that signal transducer and activator of transcription (Stat)-2 and p48/IFN-stimulated gene factor 3γ (ISGF3γ) are not required for the induction of RANTES by type I IFNs. The specific requirement of Stat-1 was demonstrated using Stat-1-deficient U3A cells. These results revealed a non-classical ISRE/ISGF3 signal transduction pathway for the induction of RANTES by type I IFNs.** 

*Key words:* Signal transduction; Interferon-α/β; Regulated upon activation normal T cell expressed and secreted; Signal transducer and activator of transcription-1

## 1. Introduction

Transcriptional responses to interferon (IFN) are mediated by tyrosine phosphorylation of nuclear factors of the signal transducer and activator of transcription (Stat) family [1]. The Stat-1 and Stat-2 proteins, present in the cytoplasm of untreated cells, become rapidly activated through tyrosine phosphorylation by two receptors of the Janus kinase (Jak) family, Jak1 and Tyk2, upon stimulation with type I IFNs (IFN-α and IFN-β) [2,3]. This activation subsequently allows Stat-1 and Stat-2 to assemble together within IFN-stimulated gene factor 3α (ISGF3α) [4]. This ISGF3α complex interacts with the p48/ISGF3γ protein, which activates transcription of IFN-stimulated genes through binding interactions with an IFN-stimulated response element (ISRE) present in many promoters activated by type I IFNs [2].

*Corresponding author. Fax: (33) 1 42 17 74 90.
*E-mail address:* vincent.vieillard@chups.jussieu.fr (V. Vieillard).

*Abbreviations:* IFN, interferon; RANTES, regulated upon activation normal T cell expressed and secreted; Stat, signal transducer and activator of transcription; Jak, Janus kinase; ISRE, interferon-stimulated response element; EMSA, electrophoretic mobility shift assay

RANTES (regulated upon activation normal T cell expressed and secreted) is a basic 8-kDa polypeptide of the CC-chemokine subfamily, produced by various cell types, including $CD8^+$ T cells, $CD4^+$ T cells, and macrophages [5,6]. The functions of RANTES include attracting blood leukocytes to the site of inflammation, regulating leukocytes maturation, trafficking and homing, and the development of lymphoid tissues [6]. In addition, RANTES plays a pivotal role during human immunodeficiency virus (HIV) infection. This chemokine highly expressed in some HIV-infected patients that do not progress to AIDS inhibits HIV infection [7]. Previously, we have observed the induction of RANTES production in several cell types (T lymphocytes, macrophages and dendritic cells) that constitutively express an IFN-β transgene [8–10]. In macrophages, the resistance against HIV, markedly enhanced by IFN-β, was virtually suppressed by RANTES-neutralizing antibody [9]. Similarly, in HIV-1-specific CTL cells, the induction of RANTES by IFN-β increased significantly the level of the specific lysis via the HIV entry coreceptor CCR3, and this effect is abolished in the presence of anti-RANTES antibody [11,12].

Thus, understanding the molecular basis for control of RANTES gene expression may help in the design and development of novel immunotherapies for a variety of diseases. In this study, we report that type I IFNs increase RANTES mRNA transcription and we provide the first evidence that RANTES gene is regulated in response to type I IFNs and that it requires an ISRE-like sequence, through the signaling protein Stat-1, in the absence of Stat-2 and p48/ISGF3γ proteins.

## 2. Materials and methods

### 2.1. Nuclear run-on

HPB-ALL and CEM cells were cultured for 4 h in the presence of IFN-α2 or IFN-β (1000 U/ml), and nuclei were harvested, as previously described [13]. Transcription initiated in intact cells was allowed to complete in the presence of [α-$^{32}$P]UTP, and the RNA was isolated and hybridized to slot-blotted probe (1 μg of DNA per slot) containing specific cDNA [13].

### 2.2. DNA construct

A 602-bp and a 1016-bp 5′-non-coding fragment upstream of the 6-16 and RANTES genes, respectively, were isolated by PCR amplification of genomic DNA derived from HPB-ALL cells. These amplified fragments were cloned into the pXP-2 luciferase reporter plasmid by using *Hin*dIII and *Kpn*I restriction sites. The reporter plasmid pΔRANTES was constructed by inserting the ISRE-like element (−672 to −616) of RANTES gene promoter upstream of the minimal sequence promoter of RANTES from −80 to +3. Punctual mutations

0014-5793/02/$22.00 
PII: S0014-5793(01)03276-8

in the ISRE-like element of the RANTES gene promoter were introduced using the Site-Directed Mutagenesis kit (Clontech). All of the constructions were verified by sequence analysis.

*2.3. Transfection and luciferase assay*

Forty million of CEM, Jurkat, HPB-ALL and U937 cells were transiently transfected by electroporation using a Cellject electroporator (Eurogentec), with 20 μg of plasmid DNA. The electroporator was set at 2100 μF capacitance, and 240 V. After electroporation, the cells were resuspended in RPMI 1640 supplemented with 10% SVF. 2fTGH, U1A, U2A and U3A cells (kindly provided from I. Kerr, Imperial Cancer Research Foundation, London, UK, and S. Pellegrini, Institut Pasteur, Paris, France) were transfected using calcium phosphate–DNA precipitates performed according to Bailly et al. [14]. Two days after transfection, luciferase activity was assayed using a Luciferase Assay System kit (Promega) and was measured in a Berthold counter (CliniLumat). As control, the plasmid pEFBosLacZ was cotransfected to normalize the transfection efficiency. Galactosidase activity was assayed using the Galacto-start kit (Tropix).

*2.4. Mobility shift assay and supershift*

Nuclear extracts were prepared as previously described [15]. For binding reaction, nuclear extracts (10 μg of protein) were incubated in buffer containing 20 mM HEPES (pH 8), 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 0.2 mg/ml bovine serum albumin and 1 μg of poly-deoxyinosinic-deoxycytidylic acid, for 15 min at 4°C. Double-stranded oligonucleotides were prepared by annealing the complementary single strands and were radiolabeled using the Klenow fragment of DNA polymerase I and [α-$^{32}$P]dATP in a fill-in reaction for 5′-protruding ends. The following oligonucleotides were used: ISRE from ISG15 gene promoter, as previously described [15]; ISRE-like from RANTES gene promoter: 5′-gatcCTTTCTAAACAAGGAAATAGAACTCAAAGACAT-3′ and 3′-GAAATATTTGTTCCTTTATCTTGAGTTTCTGTActag-5′; mutant ISRE-like from RANTES gene promoter: 5′-gatcCTTTCTAAACAAGGATATAGATCTCAAAGACAT-3′ and 3′-GAAATATTTGTTCCATATCTAGAGTTTCTGTActag-5′; GAS from ISGF2 gene promoter [16]: 5′-ctgaTTTCCCCGAAAtgac-3′ and 3′-gactAAAGGGGTTTactg-5′. The $^{32}$P-labeled oligonucleotide (0.1 pmol) was then added to the reaction mixture and incubated for 30 min at room temperature. In some experiments, antibodies against Stat-1α/p91, (kindly provided from B. Williams, The Cleveland Clinic Foundation, OH, USA), Stat-2/p113, Stat-3, Stat-5, ISGF3γ/p48, IRF-1, ICSBP and p300 (purchased from Santa Cruz Biotechnology) were included in the reaction mixture. The reaction products were analyzed by electrophoresis in a 5% non-denaturing polyacrylamide gel with 0.25% TBE buffer. The gel was dried and analyzed by autoradiography.

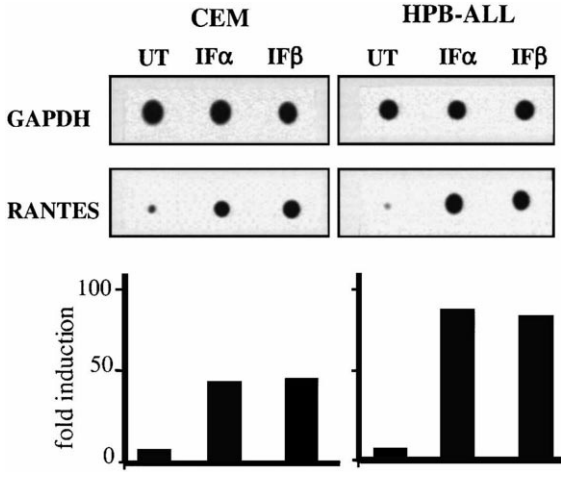


Fig. 1. Induction of RANTES gene transcription by IFN-α2 and IFN-β. CEM and HPB-ALL cells were untreated or treated with IFN-α2 (IFα) or IFN-β (IFβ) at 1000 U/ml for 90 min. Nuclei were harvested and transcriptional activity was assayed by nuclear run-on analysis. $^{32}$P-labeled RNA transcripts were hybridized with dot-blotted cDNA encoding RANTES or GAPDH, as indicated. Similar results were obtained in two separate experiments.

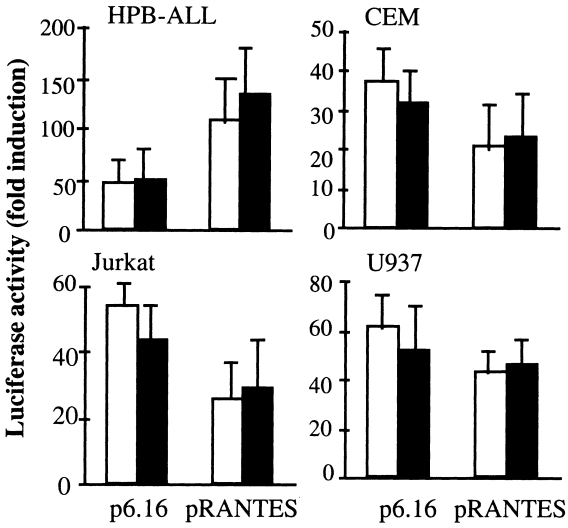


Fig. 2. IFN-α2 and IFN-β up-regulate the RANTES gene promoter activity. HPB-ALL, CEM, Jurkat and U937 cells were transiently transfected with the indicated constructs (p6.16 or pRANTES) and then treated with 1000 U/ml IFN-α2 (open bars) or IFN-β (closed bars). Levels of luciferase activity, determined 24 h later, were expressed as fold induction relative to untreated cells. Results are means ± S.D. of three independent experiments.

## 3. Results and discussion

Previously, we have shown that type I IFNs induced RANTES production in various human hematopoietic cells [9–12]. Therefore, we investigated whether up-regulation of RANTES production by type I IFNs occurred at the transcriptional level. The level of run-on transcripts was respectively 40- and 80-fold higher in CEM and HPB-ALL cells, respectively, after treatment with IFN-α or IFN-β, as compared to untreated cells (Fig. 1), indicating that type I IFNs induce the rate of RANTES gene transcription.

To analyze the effect of type I IFNs on RANTES gene promoter activity in several hematopoietic cell lines, HPB-ALL, CEM, Jurkat and U937 cells were transiently transfected with luciferase reporter constructs containing either the 6-16 gene promoter (p6-16), as a positive control [17], or the RANTES gene promoter (pRANTES). As shown in Fig. 2, the activity of the p6-16 gene promoter was induced more than 30-fold following treatment with either IFN-α2 or IFN-β, irrespective of the cell type analyzed. Similarly, the activity of the RANTES promoter construct was induced 20–100-fold. Furthermore, a dose–response curve of IFN-β suggested that the induction of the RANTES gene promoter activity is correlated with the dose of type I IFNs (data not shown).

These results suggest the presence of a type I IFN-responsive sequence in the RANTES gene promoter. Inspection of this sequence reveals a motif, 5′-TTCTAAACAAGGAAATAGAAC-3′ at position −653 relative to the ATG start codon, containing an ISRE-like element (Fig. 3A) [18]. Another ISRE-like element was previously described in the RANTES gene promoter, between nucleotides −123 and −96 [19]. The interaction of the IRF-3/IRF-7 factors with this proximal ISRE-like motif induces specifically RANTES production after virus infection, in the absence of type I IFNs, indicating that this proximal ISRE-like element is not implicated in the direct induction of RANTES expression by type I IFNs

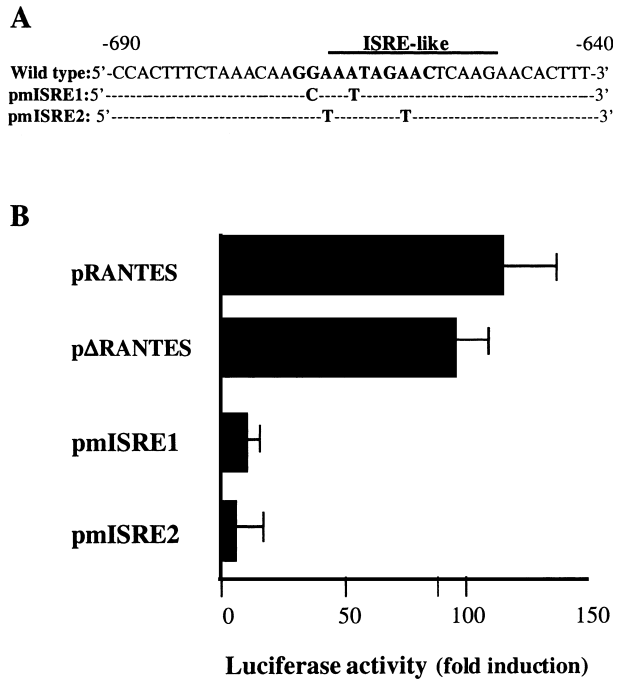


Fig. 3. Implication of an ISRE-like element in the induction of RANTES by type I IFNs. A: Site-directed mutagenesis of ISRE-like element in the RANTES gene promoter. The number above the sequence refers to the nucleotide position relative to the transcription site of the RANTES gene promoter [17]. The boldface types represent the nucleotide sequence for the ISRE-like element in the RANTES gene promoter. Two mutants of the ISRE-like element were obtained by oligonucleotide-directed mutagenesis: pmISRE1 and pmISRE2. The mutated sites are indicated. B: HPB-ALL cells were transfected with the indicated plasmid (pRANTES, pΔRANTES, pmISRE1, or pmISRE2). Cells were then treated with 1000 U/ml of IFN-β, 24 h before the luciferase activity assay. The relative luciferase activity was expressed as fold induction relative to untreated cultures. Results are means ± S.D. of three independent experiments.

[19,20]. To determine the specific function of the distal ISRE-like element in type I IFNs induction, we obtained a RANTES gene promoter construct (pΔRANTES) containing only the distal ISRE-like element (−672 to −616). This construction was transfected in HPB-ALL cells, and the luciferase activity was determined after IFN-β treatment. As shown in Fig. 3B, this distal ISRE-like motif is sufficient to respond to type I IFNs. Furthermore, two mutants of the ISRE-like element (mISRE1 and mISRE2) failed to respond to type I IFNs stimulation (Fig. 3B). Taken together, these results indicate that the integrity of this distal ISRE-like element is critical and sufficient for the response of the RANTES gene promoter to type I IFNs. Using a nucleotide alignment program, any similar combination of ISRE-like motifs was identified in the gene promoter of the other chemokines induced by type I IFNs; MIP-1α, MCP-1 or MCP-3 [21,22]. This observation suggests the concept that chemokine genes may be regulated by different mechanisms in response to type I IFNs.

To demonstrate that transcription factors specifically interact with this ISRE-like element, electrophoretic mobility shift assay (EMSA) was performed on nuclear extracts from untreated or IFN-β-treated HPB-ALL cells using a $^{32}$P-labeled double-stranded oligonucleotide DNA probe corresponding to the ISRE-like element of the RANTES gene promoter. At least three IFN-β-inducible complexes (R1, R2 and R3) were found associated with the RANTES ISRE-like oligonucleotide probe (Fig. 4A, compare lanes 1 and 2). These complexes appeared within 15 min of IFN-β stimulation, were still present 2 h after stimulation, and decreased after 4 h (data not shown). To assess the binding specificity of these complexes, we next performed a DNA competition assay in the presence of IFN-β. Addition of a 100-fold molar excess of unlabeled oligonucleotides, used as competitors, displaced all of the complexes. In contrast, a 100-fold excess of a mutated ISRE-like oligonucleotide failed to displace the complex R1 (Fig. 4A, compare lanes 4 and 5), indicating that only this

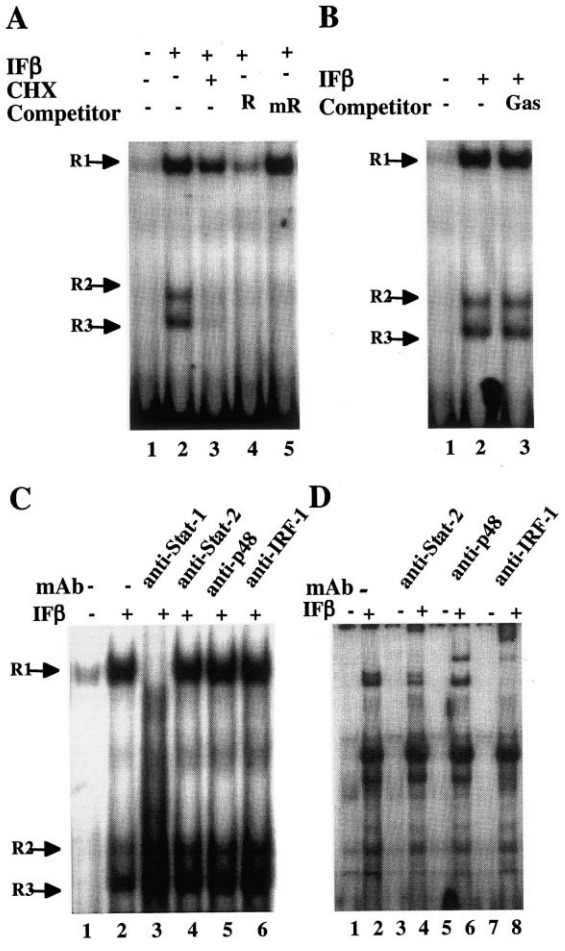


Fig. 4. DNA binding activity in HPB-ALL cells treated with IFN-β. A: HPB-ALL cells were untreated or treated with IFN-β (IFβ) at 1000 U/ml, for 2 h before the preparation of nuclear extracts. A: 30 μg of each nuclear extract was assayed for DNA binding activity by EMSA with a $^{32}$P-labeled oligonucleotide from the ISRE-like element from the RANTES gene promoter, in the presence or not of a 100-fold molecular excess of the unlabeled oligonucleotide probe from wild-type ISRE-like element (R) or mISRE (mR) from RANTES gene promoter. B: Competition assay using a 100-fold molecular excess of the unlabeled GAS (Gas) oligonucleotides probe from ISGF2 gene promoter. C: Identification of IFN-β-induced ISRE binding complexes from the RANTES gene promoter. D: Identification of IFN-β-induced ISRE binding complexes from the ISG15 gene promoter. 30 μg of nuclear extracts from untreated, IFN-β-treated (1000 U/ml) HPB-ALL cells was incubated in the presence of anti-Stat-1α, anti-Stat-2, anti-p48, or anti-IRF-1 antibodies.

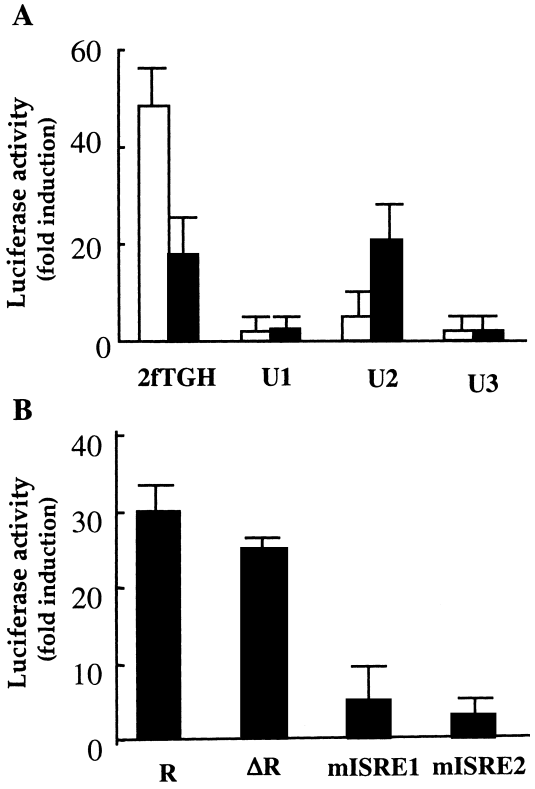


Fig. 5. Analysis of RANTES gene promoter induction in the parental cell line 2fTGH or in the mutant cell lines U1A, U2A, and U3A. A: Cells were transiently transfected with the p6.16 (open bars) or the pRANTES (closed bars) constructs. Cells were then treated with 1000 U/ml of IFN-β, 24 h before the luciferase activity assay. B: U2A cells were transiently transfected with the pRANTES (R), pΔRANTES (ΔR), pmISRE1 or pmISRE2 constructs before treatment with 1000 U/ml of IFN-β for 24 h. The relative luciferase activity was expressed as fold induction relative to untreated cultures. Results are means ± S.D. of three independent experiments.

complex is specifically implicate in the RANTES ISRE-like motif. Furthermore, as control, competition assay with an unlabeled GAS oligonucleotide from ISGF2 [16] failed to displace all of the complexes (Fig. 4B). In the presence of cycloheximide, we observed that the complex R1 is only slightly displace (Fig. 4A, lane 3), suggesting that the formation of the complex R1 did not require de novo protein synthesis. Similar results were obtained following treatment with IFN-α2 (data not shown).

To further identify the nuclear factors involved in the formation of the complexes on the ISRE-like element, antibodies specific of various members of the IRF and Stat protein families involved in IFN signaling were tested [1]. As shown in Fig. 4B, antibodies specific of Stat-2, ISGF3γ/p48 or IRF-1 failed to affect the electrophoretic mobility of the complex R1. Similar results were obtained with antibodies against Stat-3, Stat-5, ICSBP or p300 (data not shown). In contrast the formation of the complex R1 was specifically inhibited by anti-Stat-1-specific antibody (Fig. 4B), demonstrating that Stat-1 binds to the RANTES oligoprobe in response to type I IFNs. Of note, the pattern observed using the ISRE-like element of RANTES was distinct from that observed using the classical ISRE element of the ISG15 gene (Fig. 4C) [23]. These data revealed that the activation of RANTES gene transcription by type I IFNs is unlikely to implicate the classical ISRE/ISGF3 pathway.

Additional evidence for a non-classical pathway was obtained using several mutant cell lines of the ISRE/ISGF3 signaling pathway [24]. U1A, U2A, and U3A cells lacking functional Tyk2, p48/ISGF3γ, and Stat-1, respectively, were transiently transfected either with the p6-16 or the pRANTES gene promoter constructs, and luciferase activity was determined after IFN-β treatment. In the parental cell line 2fTGH transfected with either the p6-16 or pRANTES promoter construct, treatment with IFN-β markedly induced promoter activity (52- or 13-fold, respectively), as compared to untreated cells (Fig. 5A). Similar induction of luciferase activity was observed in p48-deficient U2A cells transfected either with the pRANTES or pΔRANTES constructs. In contrast, U2A cells transfected by pmISRE1 and pmISRE2 failed to respond to IFN-β (Fig. 5B). In line with the regulation of the p6-16 promoter by conventional ISGF3, the p6-16 construct was not inducible in p48-deficient cells (Fig. 5A). This demonstrates that p48/ISGF3γ transcription factor is not essential for the response of RANTES gene promoter to IFN-β. In contrast, both U1A and U3A cells transfected by the pRANTES promoter construct failed to respond to IFN-β (Fig. 5A). These data demonstrate that Stat-1 activation via a Tyk2-dependent but p48/ISGF3γ-independent pathway is critical for induction of RANTES gene promoter activity by the type I IFNs.

The results outlined in this study give evidence that RANTES regulation in response to type I IFNs occurs at the transcriptional level, and identify a novel IFN-responsive element (ISRE-like) in the promoter region of RANTES. We further identified the transcription factors required for the ISRE-like binding activity. Supershift analysis demonstrated that DNA–protein complexes contained Stat-1 protein, but not Stat-2 or p48/ISGF3γ proteins. The signaling protein Stat-1 is a key element activated by type I IFNs; several results obtained in Stat-1-deficient mice demonstrated that Stat-1 plays an obligate role in promoting most IFN-dependent biologic responses [25,26]. Our data revealed that the activation of RANTES gene transcription by type I IFNs is unlikely to implicate the classical ISRE/ISGF3 pathway.

## References

[1] Darnell Jr., J.E. (1997) Science 277, 1630–1635.
[2] Schindler, C. and Darnell Jr., J.E. (1995) Annu. Rev. Biochem. 64, 621–651.
[3] Velazquez, L., Fellous, M., Stark, G.R. and Pellegrini, S. (1992) Cell 70, 313–322.
[4] Schall, T.J. and Bacon, K.B. (1994) Curr. Opin. Immunol. 6, 865–873.
[5] Nelson, P.J., Kim, H.T., Manning, W.C., Goralski, T.J. and Krensky, A.M. (1993) J. Immunol. 151, 2601–2612.
[6] Taub, D.D., Turcovski-Corrales, S.M., Key, M.L., Longo, D.L. and Murphy, W.J. (1996) J. Immunol. 156, 2095–2103.
[7] Galo, R.C., Garzimo-Demo, A. and DeVico, A.L. (1999) J. Clin. Immunol. 19, 293–299.
[8] Vieillard, V., Jouveshomme, S., Leflour, N., Jean-Pierre, E., Debré, P., De Maeyer, E. and Autran, B. (1999) J. Virol. 73, 10281–10288.
[9] Cremer, I., Vieillard, V. and De Maeyer, E. (2000) J. Immunol. 164, 1582–1587.
[10] Cremer, I., Vieillard, V., Sautes-Fridman, C. and De Maeyer, E. (2000) Hum. Gene Ther. 11, 1695–1703.
[11] Hadida, F., Vieillard, V., Autran, B., Clark-Lewis, I., Baggiolini, M. and Debré, P. (1998) J. Exp. Med. 188, 609–614.
[12] Hadida, F., De Maeyer, E., Cremer, I., Autran, B., Baggiolini,

M., Debré, P. and Vieillard, V. (1999) Hum. Gene Ther. 10, 1803–1810.
[13] Valente, A.J., Xie, J.F., Abramova, M.A., Wenzel, U.O., Abboud, H.E. and Graves, D.T. (1999) J. Immunol. 161, 3719–3728.
[14] Bailly, R.A., Bosselut, R., Zucman, J.L., Cormier, F., Delattre, O., Roussel, M., Thomas, G. and Ghysdael, J. (1994) Mol. Cell. Biol. 14, 3230–3241.
[15] Kessler, D.S., Levy, D.E. and Darnell Jr., J.E. (1988) Proc. Natl. Acad. Sci. USA 85, 8521–8525.
[16] Pine, R., Cannova, A. and Schindler, C. (1994) EMBO J. 13, 158–167.
[17] Darnell Jr., J.E., Kerr, I.M. and Stark, G.R. (1994) Science 264, 1415–1421.
[18] Nelson, P.J., Kim, H.T., Manning, W.C., Goralski, T.J. and Krensky, A.M. (1993) J. Immunol. 153, 2601–2612.
[19] Lin, R., Heylbroeck, C., Genin, P., Pitha, P.M. and Hiscott, J. (1999) Mol. Cell. Biol. 19, 959–966.
[20] Genin, P., Algarte, M., Roof, P., Lin, R. and Hiscott, J. (2000) J. Immunol. 164, 5352–5361.
[21] Bug, G., Aman, M.J., Tretter, T., Huber, C. and Peschel, C. (1998) Exp. Hematol. 26, 117–123.
[22] Menten, P., Proost, P., Struyf, F., Van Coillie, E., Put, W., Lenaerts, J.P., Conings, R., Jaspar, J.M., De Groote, D., Billiau, A., Opdenakker, G. and Van Damme, J. (1999) Eur. J. Immunol. 29, 678–685.
[23] Improta, T., Pine, R. and Pfeffer, L.M. (1992) J. Interferon Res. 12, 87–94.
[24] Pellegrini, S., John, J., Shearer, M., Kerr, I.M. and Stark, G.R. (1989) Mol. Cell. Biol. 9, 4609–4612.
[25] Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Greenlund, A.C., Campbell, D., Carver-Moore, K., DuBois, R.N., Clark, R., Aguet, M. and Schreiber, R.D. (1996) Cell 84, 431–442.
[26] Durbin, J.E., Hackenmiller, R., Simon, M.C. and Levy, D.E. (1996) Cell 84, 443–450.