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ATF and Jun transcription factors, acting through an Ets/CRE promoter module, mediate lipopolysaccharide inducibility of the chemokine RANTES in monocytic Mono Mac 6 cells

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The chemokine RANTES is produced by a variety of tissues, including cells of the monocyte/macrophage lineage. RANTES expression is rapidly and transiently up-regulated in primary monocytes and the monocytic cell line Mono Mac 6 in response to stimulation by the bacterial product lipopolysaccharide (LPS). Transient transfection of Mono Mac 6 cells with RANTES reporter-promoter deletion constructs, in conjunction with DNase I footprinting and heterologous reporter gene assays, allowed identification of an LPS-responsive region within the RANTES promoter. Electrophoretic mobility shift assays (EMSA), methylation interference and EMSA supershift experiments were used to characterize sequences and transcription factors responsible for this LPS inducibility. The region, termed RANTES site G [R(G)], contains consensus sites for Ets and CRE/AP-1-like elements. Site-directed mutagenesis of the Ets site resulted in a loss of only 15 % of promoter activity, while mutation of the CRE/AP-1 site led to a loss of 40 % of LPS-induced promoter activity. The Ets site constitutively binds the Ets family member PU.1. LPS stimulation leads to an induction of ATF-3 and JunD factor binding to the CRE/AP-1 site. Thus, LPS induction of RANTES transcription is mediated, in part, through the activation and selective binding of ATF and Jun nuclear factors to the R(G) promoter module.

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1 Introduction

The cytokine regulated upon activation, normal T cell expressed, and presumably secreted (RANTES) is a member of the CC- or β subfamily of chemokines [1]. RANTES acts as a potent chemoattractant for monocytes, T lymphocytes, eosinophils, basophils and NK cells [2]. In addition to its role as a chemoattractant, RANTES has been shown to directly activate basophils to release histamine [3, 4] and to stimulate eosinophils to release eosinophil cationic protein [5]. RANTES protein is sequestered in the α-granules of platelets and is released by thrombin activation during acute inflammatory responses [6]. The expression of RANTES can be induced in a variety of tissues in response to diverse pro-inflammatory stimuli [2, 7]. Given the expression of RANTES seen *in vivo*, and its actions *in vitro*, this chemokine is thought to play a central role in the pathology of a variety of inflammatory diseases [8, 9].

Monocyte/macrophage cells act as important immune effector cells. Their actions include antigen presentation, control of lymphocyte activation, tissue repair and host defense against infection [10]. The outer cell wall of gram-negative bacteria is the source for LPS, a potent stimulatory agent [11]. The response of macrophage cells to LPS helps protect the host from bacterial infection. However, overstimulation by LPS can lead to tissue injury, cachexia, circulatory collapse and death through septic shock [11]. The major LPS receptor on the monocyte/macrophage cell is the glycosylphosphatidylin-

[I 19578]

Abbreviations: RANTES: Regulated upon activation, normal T cell expressed, and presumably secreted **EMSA:** Electrophoretic mobility shift assay **MAPK:** Mitogen-activated protein kinase **MIP:** Macrophage inflammatory protein **MM6:** Mono Mac 6 **R(G):** RANTES site G **IRF:** Interferon regulatory factor

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ositol-anchored glycoprotein CD14 [12]. CD14 is associated with Gi/Go heterotrimeric G proteins that specifically regulate CD14-mediated mitogen-activated protein kinase (MAPK) activation in response to LPS [13]. In addition, phosphorylation of protein tyrosine kinases and the release of arachidonic acid metabolites are associated with LPS-mediated cell activation [14]. Recently it was shown that members of the Toll-like receptor family can also mediate CD14-dependent LPS stimulation [15]. This activation of one or more signal transduction pathways results in cytokine production that includes induction of RANTES gene expression in primary monocyte/macrophage cells [16, 17].

The expression of RANTES by monocytes may also play a role in the pathobiology of HIV-1 infection. Monocyte/macrophage cells are among the first tissues to become infected by HIV-1 and they act as a vital reservoir for the virus during progression of the infection. Monocytes may also contribute to containment of HIV-1 infection through the release of factors that suppress HIV fusion or replication [18]. HIV-1 expression in monocytes/macrophages infected *in vitro* can be inhibited by stimulation with LPS [19]. This inhibition is thought to be mediated in part through LPS-induced expression of the chemokines RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β, all ligands for the chemokine receptor CCR5, a co-receptor for strains of HIV-1 that infect cells of the monocyte/macrophage lineage [19]. The local production of specific chemokines, such as RANTES, may be an important determinant in the control of HIV infection and propagation *in vivo*.

Understanding the mechanisms regulating RANTES transcription in monocyte/macrophage cells will provide insights into the molecular control of inflammatory processes and the activation of monocyte/macrophage effector function, issues important for understanding of the immune response and, potentially, the dynamics of HIV infection and AIDS. We describe here the identification and characterization of a novel promoter module within the human RANTES promoter responsive to LPS in a human monocytic cell lines.

2 Results

2.1 The chemokine RANTES is rapidly induced in peripheral blood monocytes and the monocyte cell line MM6 following stimulation with LPS

The kinetics of expression of RANTES mRNA in monocytes was determined by Northern blot analysis. Total RNA was isolated from freshly isolated primary monocytes and Mono Mac 6 (MM6) cells following stimulation with either LPS or TNF-α. Primary monocytes were treated with LPS or TNF-α for up to 60 h. A strong transient induction of RANTES mRNA up-regulation was seen in response to LPS. Peak mRNA levels were reached by 6–9 h after stimulation (Fig. 1 a). TNF-α had a positive effect on the viability of the primary monocytes, but did not significantly effect RANTES mRNA levels (data not shown and Fig. 1 a). The expression of RANTES in MM6 cells was assayed over a 24-h period. LPS stimulation resulted in a rapid and transient increase in RANTES mRNA levels analogous to that seen in the primary monocytes, with maximal levels reached by 6–9 h after stimulation (Fig. 1 b). Stimulation with TNF-α (Fig. 1 b), IFN-γ or IL-1β (data not shown) did not significantly influence levels of RANTES mRNA either alone or in combination. A basal expression of RANTES mRNA was seen in both the primary monocytes and the MM6 cells. Due to difficulties in transient transfection of primary monocytes, the MM6 cells were then used as a model cell for LPS-induced RANTES expression in human monocytic cells.

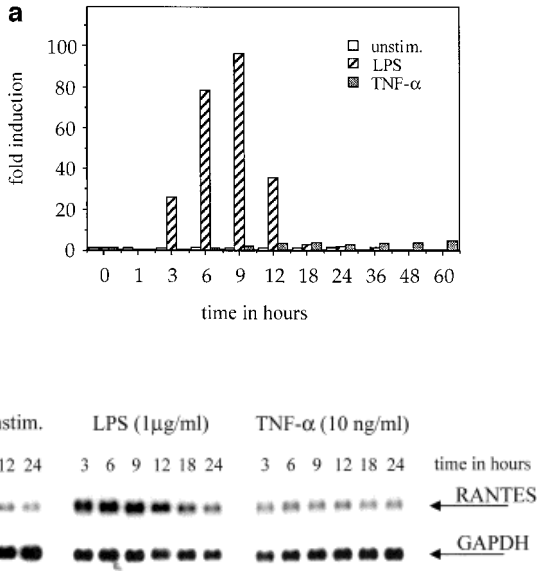

Fig. 1. (a) Northern blot of RANTES expression in primary human monocytes stimulated with either 1 μg/ml LPS or 10 ng/ml TNF-α. (b) Northern blot of MM6 cells stimulated with either 1 μg LPS or 10 ng/ml TNF-α. The blots were probed sequentially with a cDNA probe for RANTES and GAPDH. In (a) the results were quantitated using a Molecular Dynamics Phosphoimager and the RANTES values were normalized to GAPDH levels. These results are representative of more than three separate experiments.

2.2 Transient transfection of the MM6 cell line with a series of 5'-3' RANTES promoter-reporter deletion constructs defines the optimal promoter sequence

To study the transcriptional control of the RANTES gene in monocytic cells, MM6 cells were transiently transfected using DEAE-dextran. A series of RANTES promoter constructs derived from a DNA fragment containing 974 nucleotides of the RANTES immediate upstream region and the complete 5' untranslated region were used (Fig. 2 a) [20]. Transient transfection of the pGL3/-974 construct into MM6 cells showed constitutive reporter gene activity that was inducible by approximately three- to fivefold in response to LPS stimulation. Deletion of the promoter region to – 195 resulted in a loss of approximately 40 % of LPS-inducible luciferase activity (Fig. 2 a). A second group of transfection experiments using RANTES promoter deletions between – 434 and – 195 demonstrated that the LPS-induced promoter activity was lost between the – 228 and – 194 promoter deletions (Fig. 2 b).

2.3 DNase I footprinting of the RANTES promoter identified potential control elements between – 228 and – 184

DNase I footprinting was performed to identify potential control sequences proximal to the region functionally identified in Fig. 2 b. Analysis of both "sense" and "antisense" strands revealed the presence of slightly overlapping footprints that were equally present using unstimulated and LPS-stimulated MM6 nuclear extracts. Nucleotides spanning – 199 to – 187 were protected from DNase I digestion on the "sense" strand of the promoter sequence (Fig. 3 a). On the opposite DNA strand, a second footprint, spanning – 211 to – 200, was mapped (Fig. 3 b). This region from – 211 to – 187 was then designated RANTES site G [R(G)].

The DNase I footprinting experiments also identified an additional protected region downstream of R(G) (Fig. 3 a and b). This site corresponds to two previously described regions in the RANTES promoter [21, 22].

2.4 Region R(G) imparts LPS inducibility to a heterologous promoter-reporter gene construct

To further asses the LPS inducibility of region R(G), the sequence was cloned as a trimer, in both sense and antisense orientations, into the "enhancerless" minimal SV40 pGL3-Promoter reporter vector (Promega) (Fig. 4). Transient transfection of control and R(G) trimer constructs into MM6 cells demonstrated that the R(G) region mediates LPS inducibility independent of the orientation of the R(G) sequence (Fig. 4).

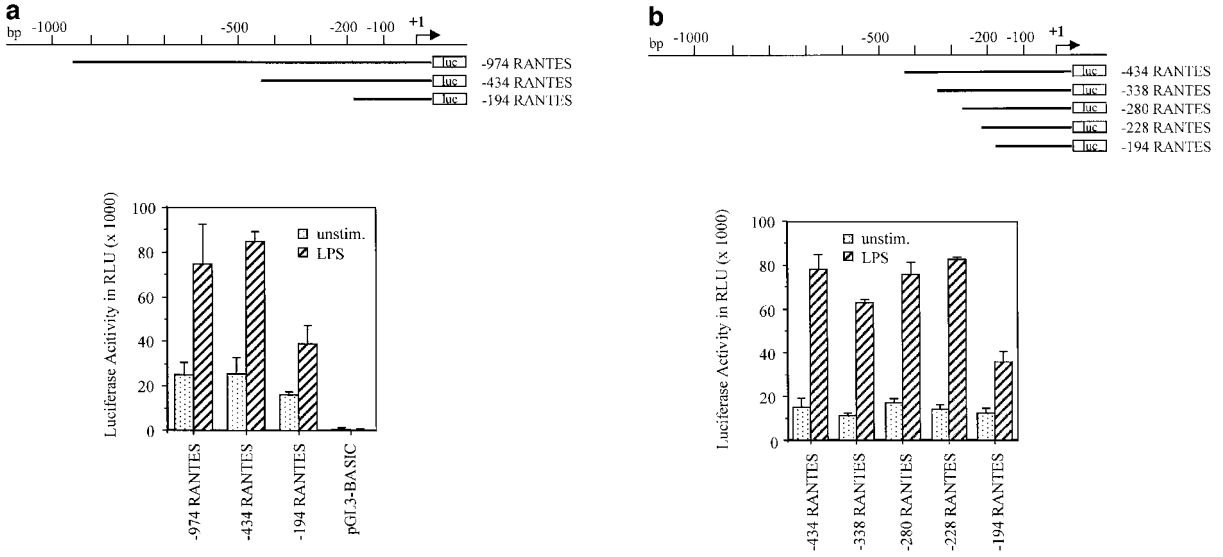

Fig. 2. (a) Transient transfection of 5' to 3' RANTES promoter-luciferase reporter gene deletions into MM6 cells (with RSV-βgal control plasmid). (b) Transient transfection of 5' to 3' RANTES promoter-luciferase reporter gene deletions spanning the – 434 to – 194 region into the MM6 cell line (with control RSV-βgal construct). Luciferase values were normalized to βgal activity. The results are representative of more than three experiments.

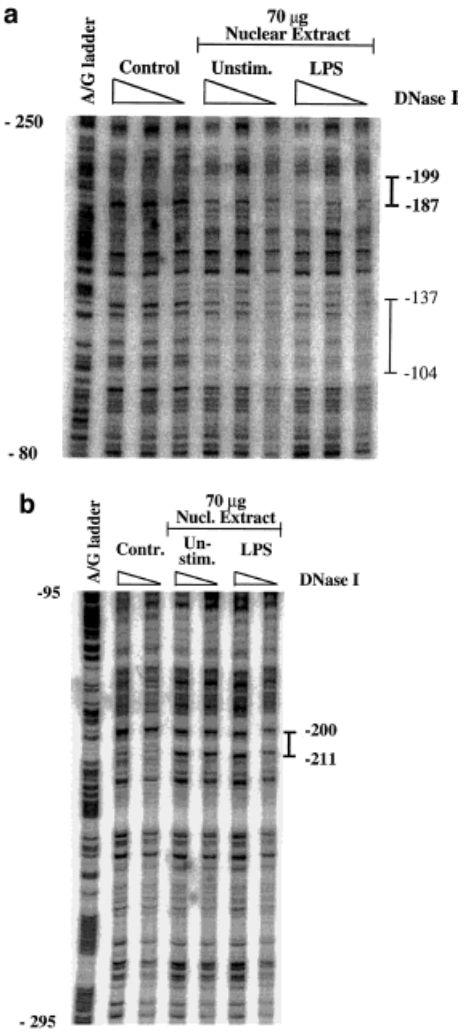

Fig. 3. DNase I footprinting was used to characterize the region responsible for LPS-induced activity. (a) A promoter fragment labeled on the "sense"-DNA strand was incubated with nuclear extracts isolated from unstimulated and LPS-stimulated MM6 cells and treated with increasing concentrations of DNase I. (b) DNase I footprinting was performed on the opposite DNA strand under identical conditions used in (a).

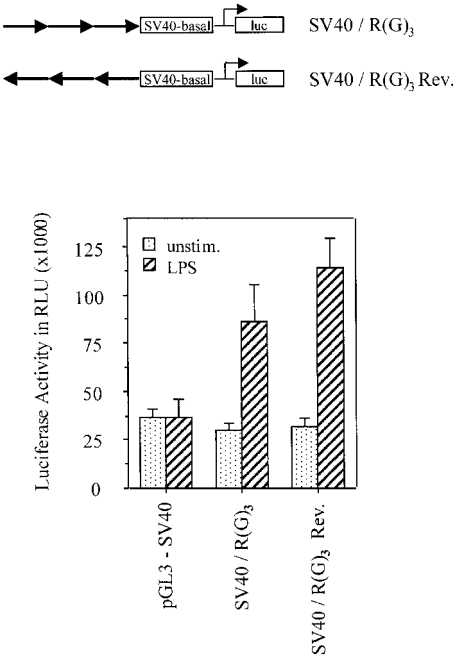

Fig. 4. The R(G) region identified by DNase I footprinting was cloned as a trimer into pGL3/SV40 "enhancer trap" vector (pGL3-Promoter, Promega) and tested for functionality in transient transfections of MM6 cells. The results depicted are representative of three separate experiments.

2.5 R(G) binds nuclear protein complexes in EMSA

Electrophoretic mobility shift assays (EMSA) were performed to study nuclear complex formation at R(G). An oligonucleotide probe containing the promoter sequence from – 219 to – 187 was incubated with nuclear protein extracts isolated from unstimulated and LPS-stimulated MM6 cells. Four complexes were found to associate with the R(G) oligonucleotide (Fig. 5 a). Complexes 1, 3 and 4 were present in unstimulated and stimulated cells, while complex 2, forming a broad diffuse band, was induced upon LPS stimulation. MatInspector analysis of the R(G) region sequence revealed potential consensus binding sites for Ets (AAGAGGAAACTGAT) family members and NFAT (AAGAGGAAACTG), starting at – 209. Slightly downstream from the potential Ets binding site at – 197 to – 190 a degenerate consensus sequence for AP-1 and CREB transcription factors was identified (TGATGAGCTCACTC) (MatInspector AP-1 score: 0.747, CREB/ATF score: 0.730).

The two most prominent EMSA bands (labeled with asterisks in Fig. 5 a) were further analyzed by methylation interference assay to characterize G and A residues important for complex formation. Nuclear extracts from LPS-stimulated MM6 cells, and sense and antisense R(G) probes were used. Comparison of alkaline cleavage ladders of the sense strand revealed an identical stretch of seven adjacent G and A residues (positions – 211 to – 205) involved in the formation of both EMSA complex 3 and 4. The digestion pattern of one representative complex is shown in Fig. 5 b. Cleavage at position – 199 was partially reduced and the corresponding G residue was scored as a partial "contact" residue. Analysis of the antisense strand identified one additional partial "contact" residue at position – 201 (Fig. 5 b). The residues identified by methylation interference included the "Ets-

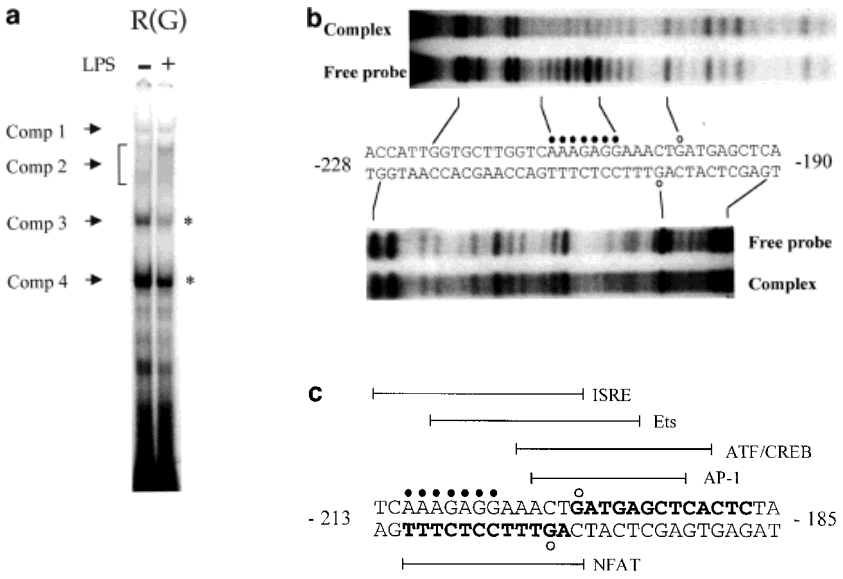

Fig. 5. (a) Comparison of binding activities by EMSA using nuclear extracts isolated from unstimulated and LPS-stimulated MM6 cells and R(G) sequence from – 219 to – 187 as probe. (b) G and A residue required for complex formation at R(G) were identified using methylation interference assay. A probe coding for the RANTES sequence from – 228 to – 183 was analyzed on both strands using nuclear protein from LPS-stimulated MM6 cells. Two EMSA bands corresponding to complex 3 and 4 in (a) (marked with asterisk) were analyzed with the free probe. Identical results were found for both of the EMSA bands studied; only one representative set of digestions is shown. (c) Summary of DNase I footprint analysis, methylation interference and sequence analysis of the R(G) region. Residues given in bold were protected from DNase I digestion. Positions labeled with filled circles were identified as full "contact" residues, empty circles represent partial "contact" residues. Sequence analysis (MatInspector) of R(G) revealed several consensus binding sites within R(G).

like" consensus sequence (– 209 to – 195). A summary, of the results obtained from the DNase I footprinting, methylation interference and sequence analysis by transcription factor weight matrices is shown in Fig. 5 c.

2.6 Functional analysis of the Ets- and CRE/AP-1 binding sites in R(G)

EMSA competition experiments using Ets, CRE, AP-1 and NFAT consensus and mutant oligonucleotide sequences were used to study complex formation at R(G) (Fig. 6 a). CRE and AP-1 consensus competitors specifically reduced formation of complex 2. The CRE consensus competitor was generally more effective than the AP-1 consensus competitor. Complex 4 was completely and selectively competed for with an oligonucleotide for Ets consensus sequences. Complex 1 and 3 were not influenced by any of the competitors studied.

The functional importance of the Ets and CRE/AP-1 binding sites were analyzed by introducing specific mutations into the Ets and/or CRE/AP-1 binding sites and studying the effects on EMSA (Fig. 6 b). Disruption of the Ets-like region (based upon the methylation interference data) led to a reduced formation of complexes 1 and 3 and a complete loss of complex 4 (Fig. 6 c). The reduction seen in complex 1 in this experiment was not reproducible in all experiments. Mutation of the CRE/AP-1-like sequence (based on MatInspector data) resulted in a specific loss of complex 2 (Fig. 6 c). Mutation of both the Ets and CRE/AP-1 region allowed only weak complex 1 formation.

The specific mutations in the Ets and CRE/AP-1 binding were then introduced into the – 974 RANTES promoter-reporter gene construct (Fig. 6 b) and tested in transient transfection experiments. Mutation of the Ets consensus sequence resulted in only a slight, but reproducible, loss of approximately 15 % of LPS-induced reporter gene expression (Fig. 6 d). By contrast, mutation of the CRE/AP-1 region led to a reduction of approximately 40 % in the promoter-reporter gene activity in the LPS-stimulated cells. Thus, complex 2 appears to contain factors that are, to a significant extent, responsible for LPS-induced RANTES expression in the monocytic MM6 cells directed through R(G). The formation of complexes 1, 3 and 4, which requires an intact Ets-like consensus

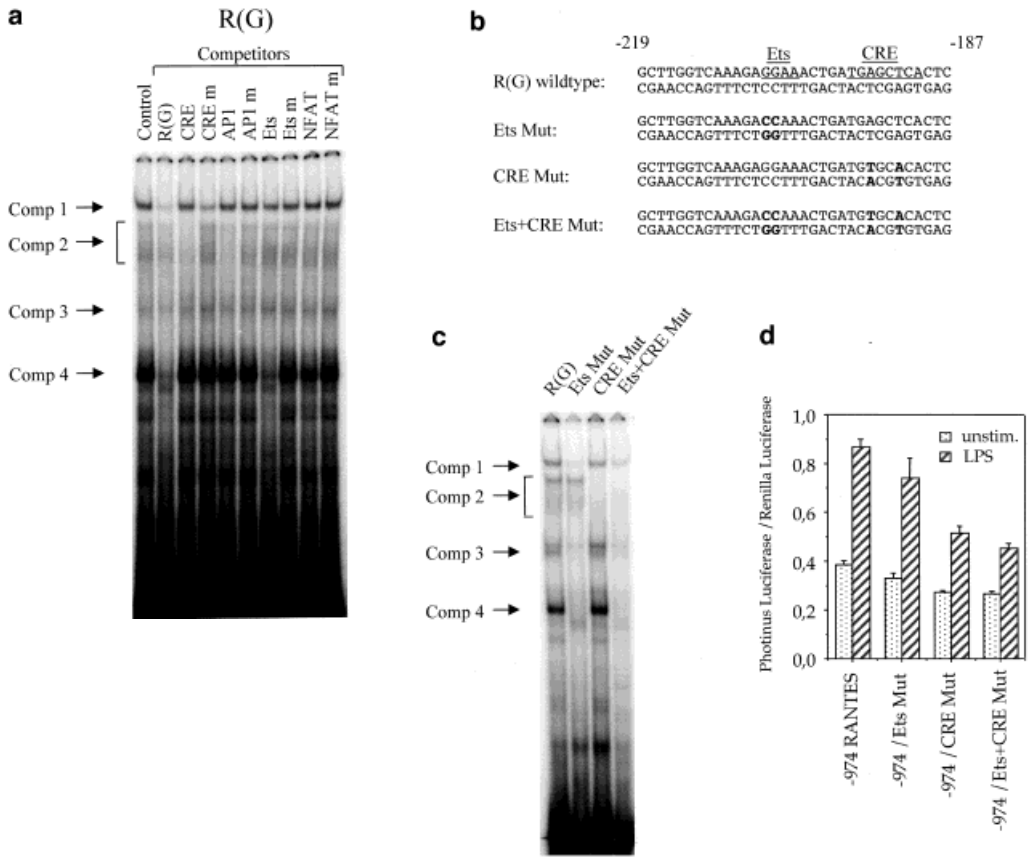

Fig. 6. (a) EMSA competition experiments using cold probe, consensus and mutant oligonucleotides. (b) Specific mutations in the Ets and/or CRE/AP-1 consensus binding sites in R(G) were studies. Mutated residues are given in bold. (c) The mutations were analyzed in EMSA for their effects on complex formation (LPS-stimulated extracts). (d) The mutants were introduced into the pGL-3 (– 974) RANTES promoter-reporter gene constructs and tested in transient transfection of MM6 cells with a pRL-TK control construct. Luciferase values were normalized to *Renilla* activity and are given as ratio of *Photinus*/*Renilla* luciferase. The results are representative of three independent experiments.

sequence, does not appear to contribute significantly to either constitutive or LPS-induced reporter gene activity in the MM6 cells.

2.7 Characterization of R(G) binding specificity

To better characterize the factors interacting at R(G), overlapping oligonucleotide probes were generated that contained only the Ets or the CRE/AP-1 binding site (Fig. 7 a). These probes, designated R(G)Ets and R(G)CRE, were tested in EMSA. The R(G)Ets probe was found to mediate formation of complex 4, while the R(G)CRE probe showed a strong complex 2 shift (Fig. 7 b). Complex 1 and 3 were not efficiently formed on the shortened probes, suggesting a requirement for both the Ets and CRE/AP-like sequences. The transcription factors present in complex 2 and 4 were further characterized using transcription factor-specific supershift/blocking antibody reagents (Fig. 7 c and d). Complex 4 was slightly inhibited (approximately 50 %) by a “pan” Ets antibody, while addition of a mAb specific for the Ets family member PU.1 resulted in a complete supershift (Fig. 7 c).

Complex 2 formation on the R(G)CRE probe was specifically competed with a CRE consensus oligonucleotide (Fig. 7 d). An AP-1 consensus oligonucleotide also reduced formation of complex 2, but appeared to have a stronger effect on the lower portion of this complex.

A series of supershift antibody reagents specific for CREB, ATF and AP-1 family proteins were used to characterize transcription factors present in complex 2 (Fig. 7 d). Antibody reagents directed against CREB1, CREB2, ATF1, ATF2 and ATF4 transcription factors showed no effect on the formation of complex 2 EMSA band patterns. However, antibody specific for ATF3 reproducibly displayed a supershift. A pan-Jun antibody

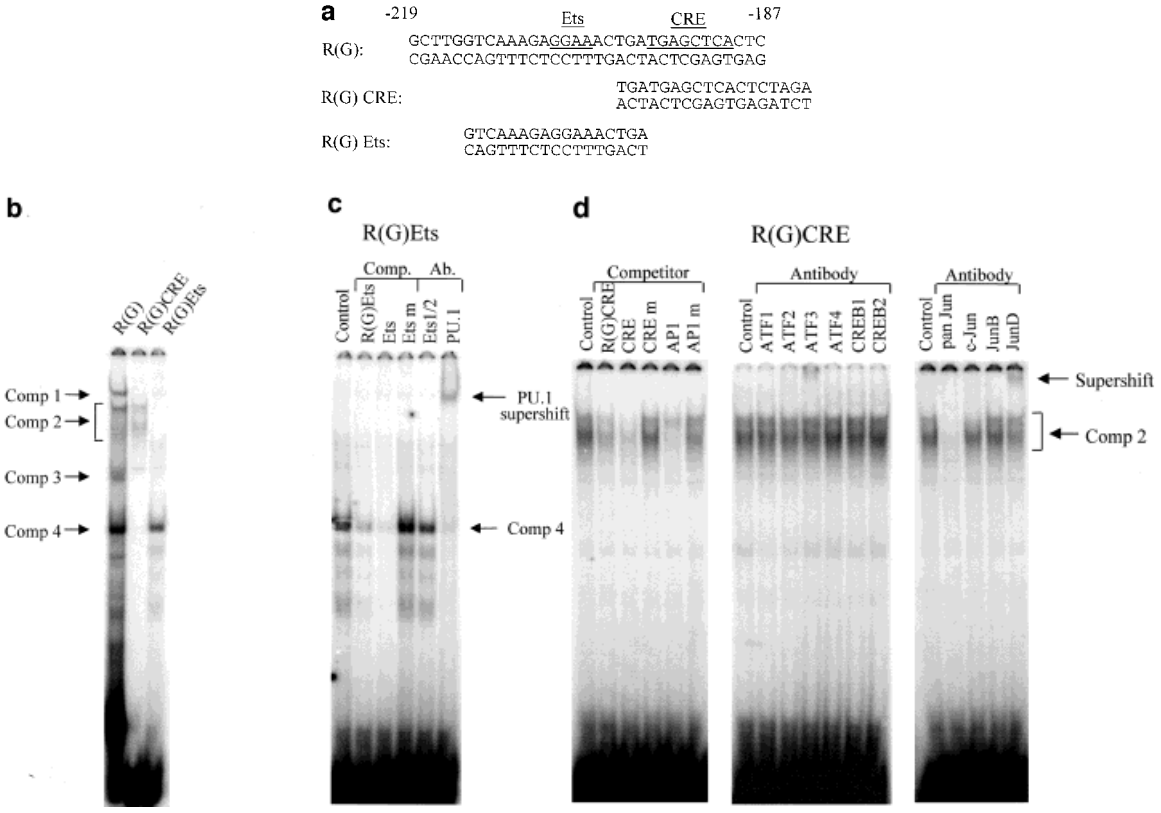

Fig. 7. Characterization of proteins present in complex 2 and 4 using nuclear extracts from LPS-induced MM6 cells and primary human monocytes. (a) Short overlapping oligonucleotides containing only the Ets (R(G)Ets) or CRE/AP-1 (R(G)CRE) binding sites were used. (b) EMSA results using the probes depicted in (a). (c) Competition and supershift experiment using R(G)Ets as probe. Cold R(G)Ets oligonucleotide and competitors as well as supershift reagents specific for Ets family members were analyzed. (d) Competition and supershift experiment using R(G)CRE as probe. Oligonucleotide competitors specific for CRE and AP-1 and a panel of antibodies directed against CREB, ATF, and Jun family members were used. The results are representative of more than three indepenent experiments.

completely blocked formation of complex 2. A blockage of the upper portion of complex 2 with an antisera specific for c-Jun and a supershift of the lower portion of complex 2 with an antiserum directed against JunD was demonstrated. Antibodies directed against JunB, c-Fos, FosB, Fra1 and Fra2 had no influence on complex 2 formation (Fig. 7 d and data not shown).

To corroborate the EMSA results found using the monocytic MM6 model cell line with normal cells, nuclear extracts were isolated from control and LPS-stimulated primary human monocytes. Results using the R(G)Ets probe showed that PU.1 constitutively bound to R(G) in primary monocyte cells (Fig. 8 a). EMSA using the R(G)CRE probe showed that complex 2 is induced by primary monocytes in response to LPS stimulation (Fig. 8 b). Analogous to what was found in the MM6 cell line, the pan-Jun antibody effectively eliminated complex 2, antibody reagent specific for JunD reduced the complex, and antibody directed against ATF3 resulted in an EMSA supershift (Fig. 8 c). Unlike the MM6 cells, no c-Jun protein could be demonstrated in the LPS-stimulated primary monocyte-derived complex (Fig. 8 c).

3 Discussion

In response to LPS stimulation monocytes express RANTES as an immediate early gene. The expression is rapid and transient, with peak expression seen by 6 to 9 h. Unlike epithelial cells, fibroblasts or astrocytes, the expression of RANTES in monocytes is not influenced by stimulation with the pro-inflammatory cytokines TNF-α, Il-1β or γ-IFN [2]. In contrast to monocytes, T cells appear to up-regulate RANTES in a “developmentally” controlled manner during the functional maturation seen 3 to 7 days after activation of resting peripheral blood T cells with mitogen [20, 23, 24].

In this report, we characterized aspects of the immediate early control of RANTES expression in human monocytic

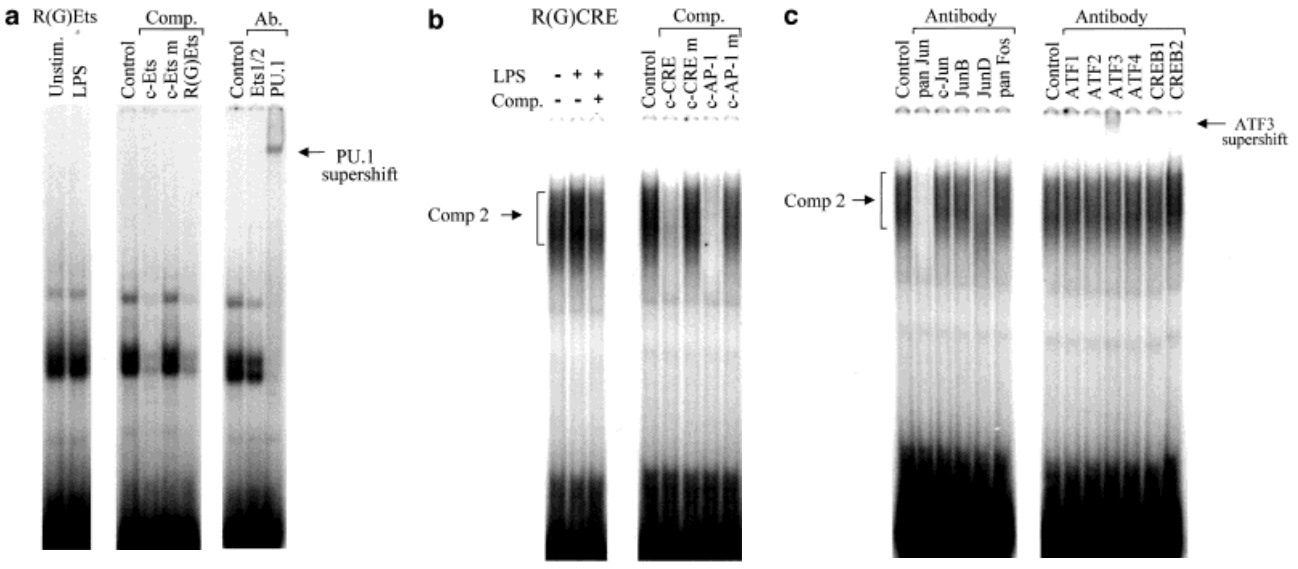

Fig. 8. Characterization of proteins present in complex 2 and 4 using nuclear extracts from control and LPS-induced primary human monocytes. (a) Competition and supershift experiments using R(G)Ets as probe. (b) Competition and (c) supershift experiments using R(G)CRE as probe. The results are representative of more than three experiments.

MM6 cells, a model cell line for LPS-induced RANTES expression. Transient transfection of MM6 using a series of 5' to 3' promoter-reporter deletion constructs demonstrated that the promoter gene sequence from – 228 to the translational start site (+ 49) acts as a minimal optimal promoter in these cells. The promoter sequence spanning – 211 to – 187 was found to contain a LPS-responsive promoter module called R(G). Computer analysis revealed Ets, NFAT, IRF-1 and a weak CRE/AP-1 binding site within R(G). Both the Ets and CRE/AP-1 binding sites are required for formation of EMSA complex 1 and 3, suggesting the presence of an NFAT-like protein complex [25]. However, these bands could not be competed with NFAT consensus oligonucleotides [26]. Regardless, complex 1 and 3 do not appear to play a significant role in the LPS-induced effects detailed here (Fig. 6 b, c, d).

The Ets family member PU.1 binds constitutively to the Ets-1-like site in R(G) in MM6 cells and in primary human monocytes. Mutation of the PU.1 site results in a slight but reproducible loss of reporter gene activity in MM6 cells. PU.1 has been shown to play a pivotal role in the differentiation of hematopoetic cells, especially monocytes and B cells [27], and contributes to the regulation of expression of cytokines, cytokine receptors and intgrins [28]. LPS stimulation of murine macrophages has been shown to result in conformational changes in the PU.1 protein brought about by phosphorylation of PU.1 at serine 148. These changes result in stronger transactivating effects of PU.1 while the DNA affinity remains unchanged [29]. PU.1 has been shown to assist in the recruitment of other transcription factors (*e. g.* IRF family members) to binding sites proximal to the PU.1 consensus sequence [29, 30]. An increase in transactivation seems to result from more efficient protein/protein interactions with other transcription factors. Based upon computer predictions, region R(G) also contains a potential IRF binding site that overlaps with the Ets/PU.1 binding site (Fig. 5 c), although no binding of IRF factors to this site was demonstrated. In addition, PU.1 and AP-1 factors can show cooperative binding at adjacent sites [31]. Thus, functional effects of PU.1 at RANTES site R(G) may lie in the recruitment of factors binding to the CRE/AP-1 consensus sequences and may also explain the functional importance of the weak AP-1 binding site present in R(G).

The transcription factors present in complex 2 appear to be key players mediating LPS induction directed through region R(G). Complex 2 is up-regulated upon LPS stimulation and mutation of the CRE/AP-1 region results in a loss of complex 2 formation and a dramatic reduction in the LPS inducibility of reporter gene activity. The transcription factors identified in complex 2 include c-Jun, JunD and ATF3 in MM6 cells, and JunD and ATF3 in primary monocytes. These proteins belong to the AP-1 and CREB/ATF families of transcription factors and are capable of forming selective interfamily heterodimers [32]. Our results suggest that complex 2 contains different ATF3/Jun heterodimers or Jun homodimers, respectively. The ATF factors do not appear to form homodi-

mers, as formation of complex 2 was completely blocked in presence of a pan-Jun antibody reagent. Fos proteins could not be detected in complex 2.

The transactivating effects of ATF, and Jun transcription factors have been found to be influenced at several levels, including by the direct phosphorylation by PKC and MAPK [33]. LPS signaling through CD14 induces MAPK activity [13]. The c-Jun N-terminal kinase (JNK) cascade is involved in LPS-mediated activation of c-Jun. In the mouse, the disruption of this pathway was shown to reduce RANTES expression in monocytes [34, 35]. In addition, the coactivator protein "CREB binding protein" (CBP) can interact with CREB transcription factors as well as some ATF proteins. CBP binding can potentiate the transactivating effect of ATF2 [36]. CBP may help amplify the moderate LPS-induced increase of factor binding in complex 2 by interaction with ATF proteins.

The overlapping binding sites in region R(G) exhibit the characteristics of a promoter module [37]. The R(G) module consists of four overlapping transcription factor binding sites (Fig. 5 c). When translocated into the context of another promoter, the function of R(G) (in this instance LPS-inducibility) is transferred as well, confirming the module. Promoter modules are known to contribute to the regulation of complex processes such as those involved in initiation of the immune response [37]. In computer-assisted sequence analysis using the FastM modeling software [37], sequences containing the R(G) module were identified in the human genes coding for α-D-galactosidase A, 4-hydroxyphenylpyruvatedioxygenase and pregnancy-specific beta-1-glycoprotein. Since the R(G)-like sequences are usually located in introns of these genes, the R(G) element is likely to be an enhancer module. This is also supported by the fact that R(G) function is independent of its orientation (Fig. 4).

While the R(G) region can mediate LPS induction in monocytic MM6 cells, additional elements also play important roles in LPS control of RANTES expression (Boehlk et al., submitted). These findings illustrate the complexitiy of the biochemical mechanisms involved in the regulation of RANTES transcription in monocyte/macrophage cells. Understanding these interactions provides insight into the biochemical control of inflammatory processes and may help in understanding how factors like RANTES may influence replication of HIV in individuals infected with HIV.

4 Materials and methods

4.1 Cells

Primary monocytes were isolated and cultured as described [38]. The human tumor cell line MM6 shows a surface phenotype that resembles that of mature monocytes. MM6 was cultured in RPMI 1640 with 10 % FCS supplemented as described [39]. Media and sera were routinely tested for low endotoxin content.

4.2 Northern blot analysis

Primary human monocytes or MM6 cells were stimulated with 1 μg/ml LPS or 10 ng/ml TNF-α for the time indicated and total RNA was isolated using acid phenol extraction [40]. Seven micrograms (primary monocytes) or 5 μg (MM6) total RNA was separated on a 1 % formaldehyde gel and transferred onto a nylon membrane (Gene Screen DuPont) according to the manufacturer's directions. A 410-bp ^{32}P-labeled ApaI/EcoR1 fragment of the human RANTES cDNA was used as a hybridization probe [20]. For normalization, the membrane was stripped and reprobed with a 780-bp PstI/XbaI fragment from the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The data were quantitated and normalized using a phosphorimater (Molecular Dynamics Storm 840) or by autoradiography.

4.3 Plasmids

The human RANTES promoter sequence from – 974 to + 49 was subcloned from previously described plasmids [20] into the reporter gene vector pGL3 (Promega, Mannheim, Germany) via SmaI/XhoI restriction sites. Constructs with RANTES promoter deletions from – 434 to – 194 were subcloned by PCR into XhoI/HindIII restriction sites of pGL3-Basic. Point mutations were introduced in pGL3/– 974 RANTES by site-directed mutagenesis using the "QuikChange Site-Directed Mutagenesis Kit" (Stratagene, Heidelberg, Germany). The R(G) trimer constructs (sequence from – 213 to – 187) were cloned into the XhoI site in the pGL3-Promoter vector (Promega, Mannheim, Germany). The expression plasmids pRSV-lacZII (Waltraud Ankenbauer, Heidelberg, Germany) or pRL-TK (Promega, Mannheim, Germany) were used as transfection controls. Plasmid DNA used in transfection experiments was prepared using either two CsCl gradients and subsequent LPS extraction ("LPS extraction Kit", Qiagen, Hilden, Germany) or the "EndoFree Maxi Plasmid Kit" (Qiagen, Hilden, Germany).

4.4 Transient transfection and luciferase reporter gene assays

Proliferating MM6 cells (10×10^6 cells per data point) were transfected in 1 ml of plain RPMI with 62.5 μg/ml DEAE-dextran (Pharmacia, Piscataway, NJ), 5 μg reporter gene construct and 1.0 μg of either pRSV-lacZII or pRL-TK (Promega, Mannheim, Germany) plasmid for normalization. After incubation for 90 min at 37 °C and DMSO shock (10 % final concentration for 3 min at room temperature) cells were washed twice in RPMI (without supplements) and seeded in

24-well plates in RPMI 1640 supplemented as described above. Twelve to eighteen hours after transfection the cells were stimulated with 1 μg/ml LPS for 9 h. *Photinus* luciferase and β-galactosidase activities were quantitated in a luminometer (Berthold, Lumat LB9501, Bad Wildbad, Germany) using the Luciferase assay system with reporter lysis buffer (Promega, Mannheim, Germany) or the Galacto light kit (Tropix, Bedford, MA), respectively. For quantitation of *Photinus*- and *Renilla* Luciferase values the Dual-Luciferase Reporter Assay (Promega, Mannheim, Germany) was carried out in a Lumat LB9507 Luminometer (Berthold, Bad Wildbad, Germany). All pGL3 constructs were tested in triplicates and normalized. The results are presented as total *Photinus* luciferase values or as a ratio of *Photinus*/*Renilla* luciferase. Only plasmids prepared at the same time were directly compared in reporter gene assays.

4.5 Nuclear extracts and EMSA

MM6 cells or primary human monocytes were stimulated with 1 μg/ml LPS for 1.5 h and nuclear protein was isolated using the high-salt extraction as described [41]. For EMSA, 7–14 μg protein were incubated for 45 min on ice with 30 kcpm ^{32}P-labeled oligonucleotide coding for the region of interest. Binding conditions were as described [42] except that 5 mM DTT was used. For oligonucleotide competitions and antibody supershift/blocking experiments, the gel shift mixture was preincubated at room temperature for 20 min with 20–40 ng specific oligonucleotide competitor or 1–2 μg antibody reagent.

4.6 Antibodies and gel shift oligonucleotides

Transcription factor-specific antibody reagents, antisera and mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The reagents included: Ets1/Ets2 (sc-112X), PU.1 (sc-352X), CREB1 (s-186X), CREB2 (sc-200X), ATF1 (sc-241X), ATF2 (sc-187X), ATF3 (sc-188X), ATF4 (sc-244X), pan-Jun (sc-44X), C-Jun (sc-1694X), JunB (sc-46X), JunD (sc-74X), pan-Fos (sc-413X), c-Fos (sc-52X), FosB (sc-48), Fra-1 (sc-605X) and Fra-2 (sc-171X). Oligonucleotide sequences for consensus and mutant DNA binding domains were either purchased from Santa Cruz Biotechnology [Ets (sc-2555/sc-2556), CRE (sc-2504/sc-2517), AP-1 (sc-2501/sc-2514), NFAT (sc-2577/sc-2578)] or were produced in house (oligonucleotides specific for consensus and mutated NFAT binding sites of the TNF-α promoter [26]).

4.7 DNase I footprinting and methylation interference

DNase I footprinting analysis and methylation interference were carried out essentially as described [21, 42] with minor modifications. For DNase I footprinting, the amount of DNase I was optimized for each of the end-labeled probes and ranged from 150 to 2.5 ng. In methylation interference experiments, the EMSA bands were cut out and the DNA was isolated using isotachophoresis. For cleavage of the DNA, the samples were resuspended in 30 μl A+G cleavage buffer (10 mM phosphate buffer, pH 6.8; 1 mM EDTA), incubated at 92 °C for 15 min and after addition of 3 μl 1 M NaOH kept at 92 °C for an additional 30 min. DNA was ethanol precipitated after the addition of 320 μl stop buffer (500 mM NaCl, 50 mg/ml tRNA). The resultant pellet was washed with 70 % ethanol and air-dried.

4.8 DNA sequence analysis

Consensus sites were analyzed using MatInspector software (Genomatix, Munich, Germany: http://genomatix.gsf.de). Mutations were routinely analyzed to verify that mutagenesis did not result in generation of new potential binding sites. For analysis of promoter modules the FastM computer model was applied (Genomatix, Munich, Germany).

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