MOLECULAR AND CELLULAR BIOLOGY, Aug. 1994, p. 5023–5031
0270-7306/94/$04.00+0

Vol. 14, No. 8

Involvement of the Transcription Factor PU.1/Spi-1 in Myeloid Cell-Restricted Expression of an Interferon-Inducible Gene Encoding the Human High-Affinity Fcγ Receptor

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Received 14 April 1994/Accepted 5 May 1994

Induction by gamma interferon (IFN-γ) of the gene encoding the human high-affinity Fcγ receptor (FcγR1) in myeloid cells requires an IFN-γ response region (GRR) and a myeloid cell-activating transcription element (MATE). GRR and MATE interact with factors to form, respectively, an IFN-γ-activating complex (GIRE-BP), depending on the phosphorylation of the 91-kDa protein (subunit of ISGF3), and a cell-type-specific complex (MATE-BP). Although GIRE-BP is detected in cells of different origins after IFN-γ treatment, the presence of MATE-BP was found to be restricted to B- and myeloid cell lines. Sequence analysis of a cDNA encoding a polypeptide recognizing specifically the MATE motif led to the identification of this product as the proto-oncogene PU.1/Spi-1, a transcriptional activator expressed in myeloid and B cells. Expression of this factor in nonhematopoietic cells allowed IFN-γ-induced expression of a reporter gene under control of the GRR and MATE sequences. The presence of these motifs in other gene promoters indicates that the binding of PU.1/Spi-1 and IFN regulatory proteins to their respective motifs could be part of a general mechanism leading to cell-type-restricted and IFN-induced gene expression.

Recent reports have shown that cell-type-specific transcriptional regulators are likely implicated in restricted gene expression in a particular hematopoietic cell lineage. Factors such as hGATA-1 (44, 45) involved in the transcriptional activation of the globin gene appear to be essential for erythroid cell development. In cells of myeloid origin, several factors, such as PU.1/Spi-1 (9, 18, 22, 35) and Spi-B (34) detected in B cells and monocytes, a zinc finger protein found only in granulocytic cells (13), and NF-M, a member of the C/EBP family, restricted to monocytes (11), have been identified. The cognate DNA-binding sites of these factors are widely distributed among promoter and enhancer sequences of genes expressed in their respective cells.

Our approach to characterization of lineage-specific transcription factors was to study the mechanisms of expression of a lineage-specific marker gene such as the gamma interferon (IFN-γ)-induced gene encoding the human high-affinity receptor for immunoglobulin G (FcγR1) (1). Studies of its promoter led to the identification of two *cis*-DNA elements, IFN-γ responsive region (GRR) (2, 28) and myeloid cell-activating transcription element (MATE) (31), involved, respectively, in its IFN-γ-induced and myeloid cell-restricted expression.

The GRR motif is a target for proteins forming an IFN-α- or IFN-γ-activated complex, GIRE-BP (29, 31, 49). At least, one IFN regulatory factor, the 91-kDa protein (7, 17, 21), was identified as a component of GIRE-BP (14, 29, 31). Tyrosine phosphorylation of this factor and of two other factors of 113 and 84 kDa (37) depends on the activation of Tyk2 (46) and JAK1 (23) following binding of IFN-α to its cell surface receptor. After translocation to the nucleus (17), these factors are found to be associated with a 48-kDa DNA-binding protein (21) targeting the IFN-stimulated response element (ISRE) to form a complex referred to as ISGF3 (7). Although GIRE-BP and ISGF3 share at least the 91-kDa protein, these complexes are different. In contrast to GIRE-BP, which is activated by both types of IFNs, ISGF3 is not activated by IFN-γ (21). Binding of IFN-γ to its receptor triggers the activation of JAK1 and JAK2 (48), leading to phosphorylation of the 91-kDa protein but not the 113-kDa protein (40). In addition to GIRE-BP and to GAF, a complex including the IFN-γ-activated sequence (GAS) of the guanylate-binding protein (GBP) gene (3, 4), the 91-kDa protein is involved in other complexes (8, 20, 32, 41). Recently, a palindromic sequence (pIRE) conferring IFN-γ responsiveness was identified in the genes encoding the IFN regulatory factors ICSBP and IRF1 (16, 32). Although the pIRE motif, which forms a complex containing the 91-kDa protein, was also found within the GAS and GRR sequences (16), it is difficult to determine whether or not these complexes are identical.

Previous studies on the FcγR1 promoter show that the GRR motif alone was able to confer IFN-γ responsiveness to a reporter gene under control of the thymidine kinase promoter in myeloid and nonmyeloid cells (29, 31). Such data concord with the observed wide cell distribution of the GIRE-BP complex (31). Downstream from GRR is a *cis*-DNA element, MATE, involved in cell-type-restricted FcγR1 gene expression (31). This motif carries the transcription initiation sites (2) and contains a pyrimidine-rich sequence also found in gene promoters expressed preferentially in cells of myeloid lineage (31). In line with these data, MATE interacts with constitutive factors leading to several DNA-binding complexes (MATE-BP1, MATE-BP2, and MATE-BP3) in different monocytic cell lines, but not in HeLa cells (31).

In order to gain more insight into the mechanisms leading to FcγR1 gene regulation, we therefore studied the distribution of MATE-BP among different cell lines. Nonhematopoietic and T-lymphoid cell lines did not express MATE-BP activity, which was, however, observed in all myeloid and B-lymphoid cell lines tested, with some differences in the relative abun-

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dances of MATE-BP1 and MATE-BP2. Footprinting experiments performed with a region containing the MATE motif demonstrated that protein-DNA interactions involved in the different MATE-BP complexes were identical. The presence of a common motif, AGAAAAG, within the MATE protected region and GRR (31) prompted us to investigate its implication in FcγR1 gene regulation. Cross-competition assays with MATE and GRR either as probes or as competitors suggested that one component involved in MATE-BP complexes might also have an affinity for the AGAAAAG sequence of the GRR region.

To clone the factors forming GIRE-BP and MATE-BP complexes, an in situ filter-binding technique (42) was performed. Using labeled MATE and GRR multimerized oligomers as probes, we screened a cDNA expression library derived from the THP1 myeloid cell line. One of the cDNA (λgta) encoded a protein exhibiting a DNA-binding affinity specific for the MATE probe. Sequence analysis of λgta cDNA demonstrated complete identity with the sequence of the gene encoding human PU.1/Spi-1 factor (35), a member of the *ets* proto-oncogene family (18). Moreover, cotransfection of nonhematopoietic cells with a PU.1/Spi-1 expression vector and a construct containing GRR and MATE motifs conferred an IFN-inducible response to the reporter gene. Antibodies raised against PU.1/Spi-1 protein inhibited the formation of MATE-BP complexes, confirming that this factor was at least one of those involved in MATE-BP complexes. In addition, PU.1 bound to the GRR motif to yield a complex distinct from GIRE-BP, migrating as MATE-BP1. These data demonstrated the role of PU.1/Spi-1 in the myeloid cell-restricted expression of the FcγR1 gene and also its potential affinity for the GRR motif, thus explaining the cross-competition observed.

MATERIALS AND METHODS

Cell culture. U937, THP1, RAW 264.7, HeLa, A-549, and Colo-205 cells were obtained from the American Type Culture Collection. Human monoblastic KG1 and its derivative KG-1A and pre-B (REH and KM3)-, B (WT46 and M28)-, and T-lymphocytic cell lines (Jurkatt, Molt4, and HPMBL) were provided by Djawad Mossalawi (CNRS URA 625, Hôpital Pitié Salpêtrière). All these hematopoietic cell lines were grown in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum. The megakaryocytic cell line Dami (10) was cultured in Iscove's modified Dulbecco's medium supplemented with 10% horse serum containing 1% phytohemagglutinin-stimulated leukocyte-conditioned medium. The eosinophilic leukemia cell line EOL-3 was grown in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (36). The nonhematopoietic cell lines such as HeLa, 2fTGH (30), A-549, Colo-205, and IVEC (47) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 5 μM L-glutamine.

Interferons. Recombinant human IFN-γ was a gift from Roussel Uclaf, and recombinant IFN-α was provided by Hoffmann-La Roche. Recombinant murine IFN-γ was produced by Genentech and provided by Boerhinger Ingelhein.

Cell extracts. Cells were grown to a concentration of 5×10^5/ml (and if required treated with IFN for 15 min) and then harvested for protein extraction. Cell extracts were prepared as previously described (31) and aliquoted at a protein concentration of 10 μg/μl.

Electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotides were labeled either by phosphate exchange reaction, by using [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) with T4 polynucleotide kinase (Pharmacia), or by completing the 5′ protruding sticky ends of an *Xba*I site present at both oligomer extremities by using [α-^{32}P]dCTP (3,000 Ci/mmol; NEN) and the Klenow fragment of DNA polymerase I (Appligene). Incubation of probes and extracts was performed as previously described (31). The samples were loaded on a 5% native polyacrylamide gel (39:1 acrylamide-bisacrylamide) and electrophoresed at 120 V in a 1× Tris-glycine buffer (50 mM Tris [pH 8.8], 200 mM glycine, 2 mM EDTA) at 4°C. For supershift experiments, 1 μl of a 1/10 dilution of sera raised against the 91- and 113-kDa proteins (31) or a 1-μl aliquot of a polyclonal antiserum raised against 111 N-terminal amino acids of murine PU.1/Spi-1 protein (5) was added to the extracts and preincubated for 15 min at 4°C prior to the addition of the probe.

Footprinting after cleavage with the 1,10-phenanthroline copper ion (OPCu). The −140 to −45 fragment of the FcγR1 gene (31) was cloned in the *Bam*HI site of the Bluescript II vector (Stratagene). The construct was then linearized either by *Eco*RI or *Xba*I digestion and labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; NEN), using the Klenow fragment of DNA polymerase I (Appligene). The insert was removed by a second digestion resulting in either an *Eco*RI-*Xba*I fragment (noncoding strand) labeled at the *Eco*RI site or an *Xba*I-*Eco*RI fragment (coding strand) labeled at the *Xba*I site. These probes incubated in the presence of THP1 cell extract were then submitted to EMSA. After autoradiography, bands corresponding to MATE-BP1, MATE-BP2, and the free probe were excised and exposed to a mixture containing 1,10-phenanthroline and 3-mercaptopropionic acid cupric ion (19). The samples were analyzed by electrophoresis on a 10% sequencing gel.

Transfections. Cells were plated at 5×10^6 cells per 100-mm-diameter tissue culture dish 1 day before transfection. HeLa cells and RAW 264.7 were transfected, respectively, by the calcium phosphate and DEAE-dextran methods, and chloramphenicol acetyltransferase (CAT) assays were performed as previously described (31). Transfected cells were treated with 200 U of IFN per ml for 8 h. CAT activities were adjusted for differences in the protein concentration of the extracts. β-Galactosidase activity in the cell extracts was assayed (31) by using *o*-nitrophenyl-β-D-galactopyranoside (Boehringer-Mannheim Biochemicals) as a substrate.

Preparation and screening of the cDNA expression library. Poly(A)$^+$ mRNA prepared from THP1 cells was used to synthesize cDNA according to the instructions of the You-Prime cDNA Synthesis Kit booklet of Pharmacia by adding random hexadeoxynucleotides and *Not*I-oligo(dT) primer in a 1/10 ratio (Pharmacia LKB Biotechnology). After ligation of *Eco*RI adaptors and *Not*I digestion, the cDNAs were cloned in the lambda gt11 *Sfi-Not* DNA (*Eco*RI-*Not*I arms) purchased from Promega Biotech Corporation. After packaging, infected host bacterial strain Y1090 was plated at 42°C for 3 h. Plates were then overlaid with nitrocellulose filters (Hybond-C; Amersham) saturated with isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for 4 h at 37°C. The β-galactosidase fusion proteins released from lytically infected cells were tested for their abilities to bind nick-translated hexamers of the GRR and MATE motifs by using an in situ filter-binding technique (42).

In vitro transcription/translation of PU.1 cDNA. PU.1/Spi-1 cDNA was cloned in the Bluescript vector (Stratagene). The translated product was synthesized by using the TnT Coupled Reticulocyte Lysate System as described by the manufacturer (Promega). Band shift analysis with a probe corresponding to the MATE motif was performed directly with 3 μl of the translation mixture as described above.

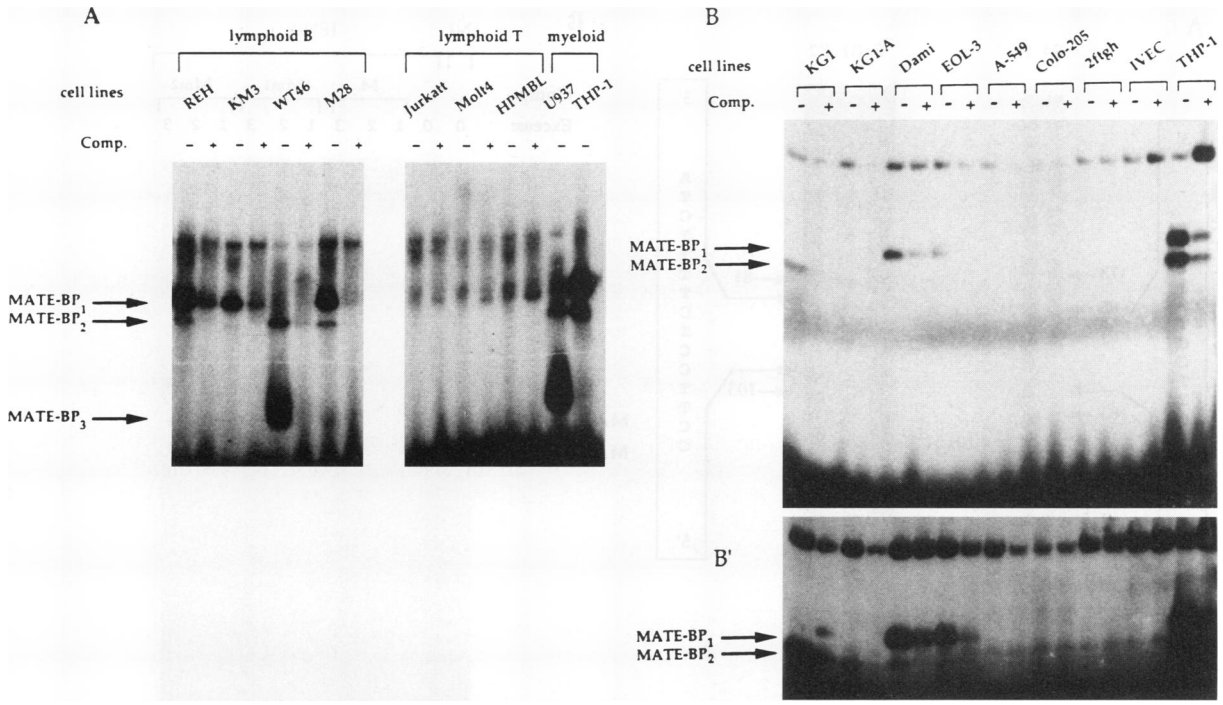

FIG. 1. Cell type distribution of MATE-BP. Whole-cell extracts and a ^{32}P-labeled MATE probe (see Fig. 3C) were submitted to an EMSA after incubation in the absence (−) or presence (+) of a 100-fold molar excess of MATE competitor (Comp.). (A) Hematopoietic lines. Lymphoid B- and T-cell lines were tested and compared with the promyelomonocytic U937 or the monocytic THP1 cell line. (B) Myeloid and nonhematopoietic lines. Binding activities were assayed with extracts prepared from immature myeloid (KG1 and KG1a), megakaryocytic (DAMI) and eosinophilic (EOL-3) cell lines. Nonhematopoietic cell lines such as epithelial (A-549), colocarcinoma (Colo-205), fibroblast (2fTGH), and endothelial (IVEC) were similarly tested. (B′) Overexposure of the same gel as in panel B. The abundance of MATE-BP complexes should be correlated to the abundance of the nonspecific upper band.

Transactivation of FcγR1 promoter in HeLa cells. Cells were cotransfected as mentioned above with 5 μg of the reporter plasmid and 5 μg of either the PU.1/Spi-1 expression vector ΔEB-Spi (34) or the ΔEB expression vector without PU.1 cDNA. The reporter plasmid $(GIRE)_3$-(140/45) CAT was built as follows: the fragment containing a trimer of the GRR sequence and the −140 to −45 region was amplified by PCR, using the $(GIRE)_3$-(140/45) TK CAT plasmid as a template (31). After purification, this insert was cloned in the *Hin*dIII-digested pCAT-Basic vector purchased from Promega.

RESULTS

MATE-BP activity is restricted to myeloid and B-cell lines. We have previously shown that MATE-BP activities are detected independently of IFN-γ treatment in different myeloid cell lines, such as THP1, HL60, and U937 (31). If the presence of MATE-BP complexes is relevant to the cell specificity of FcγR1 gene expression, one might expect to observe a limited cell distribution of the proteins which form these complexes. To approach this question, protein extracts prepared from a variety of cell lines were screened by EMSA for their ability to form MATE-BP complexes (Fig. 1). MATE-BP1 and MATE-BP2 complexes were detected in different B cells but not in T-cell lines (Fig. 1A). A complex migrating with the same electrophoretic mobility as MATE-BP1 was detected at low levels in T-cell lines (Fig. 1A) but was not specific since it was not affected by an excess of the homologous competitor. The relative abundance of this nonspecific complex in some cell lines might also explain the incomplete competition observed with REH and KM3 extracts (Fig. 1A). MATE-BP3, previously identified in the pre-myeloid cell lines HL60 and U937 (31), was observed in the Epstein-Barr virus-transformed B-cell line WT46 (Fig. 1A). By testing other myeloid and nonhematopoietic cell lines (Fig. 1B) or normal hematopoietic cells (data not shown), we confirmed that MATE-BP activities were restricted to myeloid and B cells. However, the MATE-BP1/MATE-BP2 ratio varied among the myeloid cell lines studied. In contrast to the undifferentiated myeloid KG1 cell lines, in which only MATE-BP2 was seen, extracts prepared from the megakaryocytic cell line Dami (10) or from the eosinophilic cell line EOL-3 (36) contained exclusively the MATE-BP1 complex (Fig. 1B and B′). Although it was difficult to predict the origin of these different complexes, they may reflect either several types of interactions or proteolytic derivative forms of the more slowly migrating complex, MATE-BP1.

Identification of the MATE-BP binding site. For further identification of MATE-BP1 and MATE-BP2 complexes, footprinting experiments (Fig. 2) were performed with a probe (−140 to −45) containing the MATE motif (Fig. 3). After EMSA, the different MATE-BP complexes were isolated and corresponding gel slices were exposed to a chemical degrading agent, OPCu (19). Analysis of the cleavage pattern of both strands revealed (Fig. 2A) that the protein(s) forming these two complexes protected the same region located between −84 and −100. This sequence comprised 17 bp of the 26-bp MATE region characterized by functional assays (31).

The importance of this 17-bp motif was confirmed (Fig. 2B) by using oligonucleotide competitors, Mm1 and Mm2, mutated respectively in the 3′ and 5′ moieties of this sequence (Fig. 3). Neither Mm1 nor Mm2 competed with the MATE probe to

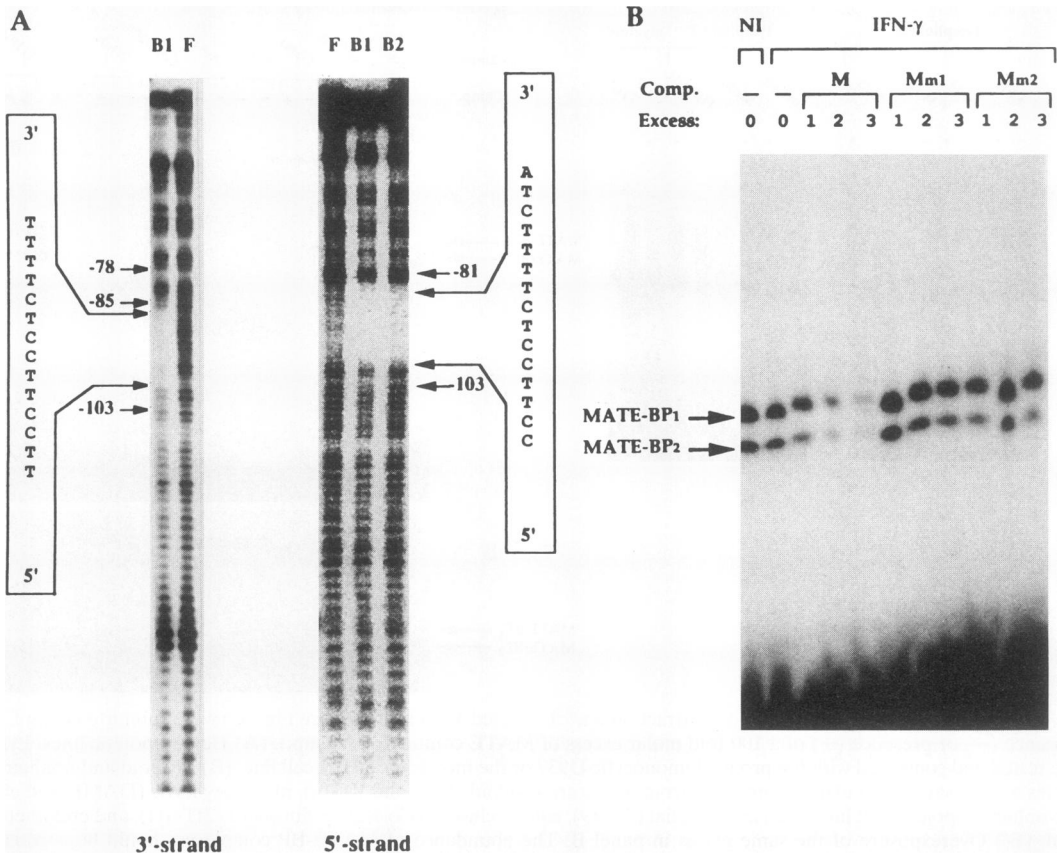

FIG. 2. Identification of the sequence involved in MATE-BP interactions. (A) Footprinting analysis. A coding and a noncoding strand FcγR1 promoter DNA fragment (−140 to −45) (see Fig. 3) were incubated with THP1 extract. After EMSA, bands corresponding to MATE-BP1 (B1), MATE-BP2 (B2), or free probe (F) were excised and submitted to phenanthroline-copper ion nuclease cleavage (see Materials and Methods). The products were then analyzed on a 10% sequencing gel. (B) Effects of mutated MATE motifs. EMSA with the MATE probe was performed in the absence (0) or presence of a 10-, 50- or 100-fold molar excess (1, 2, and 3, respectively) of either homologous (M) or mutated (Mm1 and Mm2, as described in Fig. 3) competitors (Comp.).

form the MATE-BP complexes (Fig. 2B). Moreover, since none of these mutated oligonucleotides led to the formation of MATE-BP (data not shown), we conclude that the 17-bp sequence is indeed required for its interaction with DNA-binding proteins.

A common motif within MATE and GRR contributes to the binding of MATE-BP with an efficiency which depends on the surrounding sequences. The presence of a repeated motif (AGAAAAG) located at several positions in the FcγR1 promoter (Fig. 3), and particularly within MATE and GRR, has

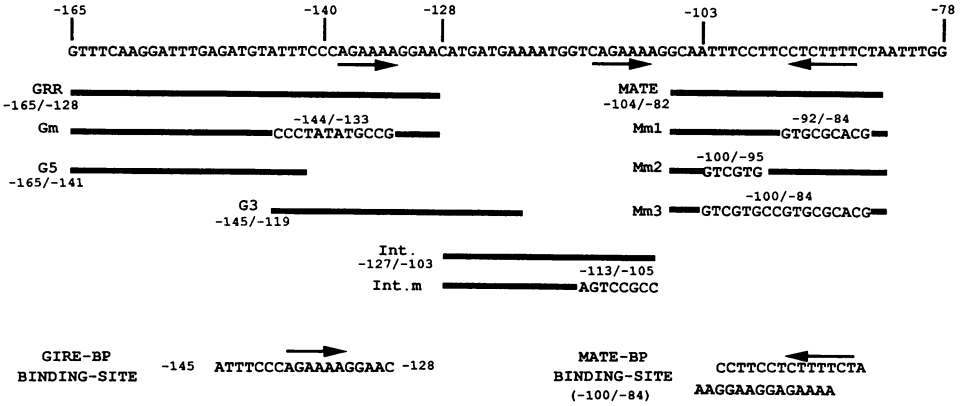

FIG. 3. Regulatory regions of the FcγRI gene promoter. The AGAAAAG motif is indicated by arrows. The different oligomers used as probes and/or competitors are listed. Unchanged nucleotide sequences are indicated by solid lines. The binding sites for the GIRE-BP and MATE-BP complexes deduced from our analysis are indicated.

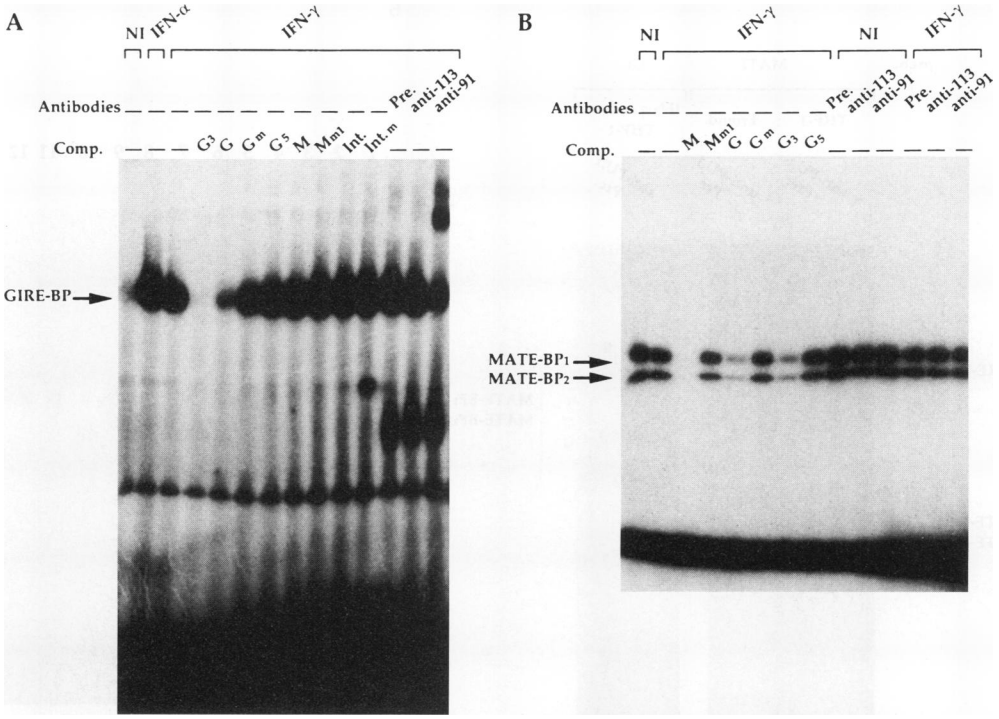

FIG. 4. A component of MATE-BP binds the GRR motif. Whole-cell extracts were prepared from the THP1 cell line untreated (NI) or treated with 200 U of IFN-α or IFN-γ per ml for 30 min. (A) Competition analysis of GRR-binding activity. Cell extract and a probe corresponding to the G3 sequence (Fig. 3) were incubated in the presence of a 100-fold molar excess of unlabeled sequences (Fig. 3C) (Comp.), of preimmune serum (Pre.), or of sera diluted 1:100 raised against the 113- or 91-kDa proteins (37). (B) Competition analysis of MATE-binding activity. Similar experiments to those in panel A were done, using the MATE motif as a probe.

been reported (31). To determine the potential of this motif in regulation of FcγR1 gene expression, oligonucleotides in which GRR was mutated (Gm) or truncated (G5 and G3) (Fig. 3) were used in EMSA either as probes or as competitors. Among these oligomers, only G3, which contained the AGAAAAG motif (Fig. 3) but not Gm or G5 (data not shown), was able to form a GIRE-BP complex after incubation with IFN-γ-induced cell extracts. Moreover, in contrast to a reporter gene containing a trimer of the G5 sequence cloned in front of the thymidine kinase promoter, a similar construct with a trimer of G3 led to IFN-γ-induced activity (data not shown). Whereas an excess of the GRR sequence competed with the G3 probe (although not so efficiently as unlabeled G3), no competition was observed with an excess of Gm or G5 (Fig. 4A). Therefore, the region involved in GIRE-BP formation and in the IFN-γ response depends on a 17-bp motif spanning the −144 to −128 region (Fig. 3). However, neither the MATE sequence nor the sequence between GRR and MATE (Int. [Fig. 3]), which contains a similar motif, was able to abolish the formation of GIRE-BP (Fig. 4A). In a similar experiment (Fig. 4B), these oligomers were tested as competitors when the MATE sequence was used as a probe. In this case, an excess of GRR and G3 sequences, but not of G5 and Gm (Fig. 4B) or Int. (data not shown), was able to reduce MATE-BP formation. The possibility that the 91-kDa protein, identified as a component of GIRE-BP (14, 29, 31), was also present in MATE-BP was ruled out by preincubation of THP1 cell extracts with antibodies raised against this protein. While such preincubation led to a supershift with the G3 probe (Fig. 4A), no effect on MATE-BP activities was observed when antibodies were included in the reaction medium (Fig. 4B).

These data suggest that the AGAAAAG motif, dependent on surrounding sequences, could be a potential target for a factor recognizing with different affinities the GRR and MATE motifs. Since the results were identical with uninduced and IFN-γ-induced extracts (data not shown), we can postulate that this factor is present independent of IFN treatment. These experiments also imply that at least one of the proteins leading to the MATE-BP complex is either restricted to myeloid and B cells or is specifically modified in such cells.

The transcription factor PU.1/Spi-1 is a component of the MATE-BP complex. In order to clone the cDNA encoding the DNA-binding factors involved, an in situ filter-binding technique (42) was performed. The cDNAs prepared from THP1 mRNAs were inserted by directional cloning into a λgt11 expression vector, and plaques were screened by using double-stranded multimerized MATE and GRR motifs as probes.

Expression of β-galactosidase fusion proteins led to the isolation of two different cDNA clones. Sequence analysis of one of these cDNA clones encoding a fusion protein recognizing the MATE motif revealed that this gene encodes the human PU.1/Spi-1 factor (35). Bacterially expressed PU.1 cDNA, although fused to the β-galactosidase encoding sequence, was able to bind MATE with an affinity and specificity similar to those of MATE-BP (data not shown). Moreover, Spi-1 antibodies (5) inhibited the formation of the MATE-BP1 and MATE-BP2 complexes (Fig. 5A), confirming that PU.1/Spi-1 is involved in these two complexes. The possibility that PU.1 was implicated in the GIRE-BP complex was ruled out by the absence of effect of the antibodies on this complex (Fig. 5A).

In contrast to unprogrammed lysate (data not shown), incubation of in vitro-translated PU.1/Spi-1 protein (Fig. 5B, lanes 9 to 12) with the MATE probe resulted in the formation

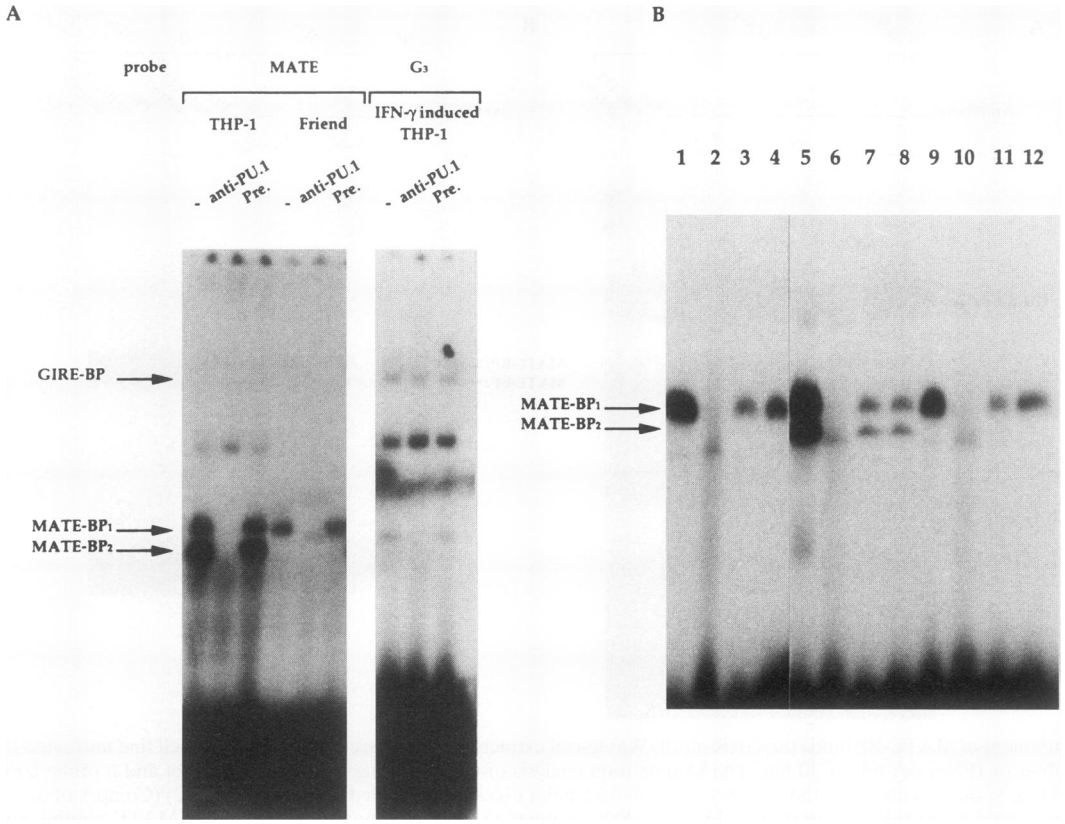

FIG. 5. Role of PU.1/Spi-1 transcription factor in MATE-BP formation and FcγRI gene expression. (A) Effects of anti-PU.1/Spi-1 antibodies (5) on MATE-BP and GIRE-BP formations. Cell extracts were preincubated in the absence (−) or presence of preimmune serum (Pre.) or with antibodies raised against murine Spi-1 protein (anti-PU.1). After addition of the probes corresponding to either the MATE or G3 sequences (Fig. 3), the mixture was submitted to EMSA. (B) Implication of PU.1/Spi-1 factor in the formation of MATE-BP. An in vitro-translated PU.1/Spi-1 protein (lanes 1 to 4 and 9 to 12) was added to HeLa cell extracts (lanes 1 to 4) or tested alone (lanes 9 to 12). THP1 cell extracts (lanes 5 to 8) were used as a control. Extracts were incubated in the absence (lanes 1, 5, and 9) or presence of a 100-fold molar excess of unlabeled MATE (lanes 2, 6, and 10), GRR (3, 7, and 11), or G3 (lanes 4, 8, and 12) competitors. An end-labeled MATE probe was then added to the mixture.

of one complex with the same electrophoretic mobility and sensitivity to competitors GRR (Fig. 5B, lane 11) and G3 (Fig. 5B, lane 12) as MATE-BP1 (Fig. 5B, lane 5). This factor also formed with the G3 probe a low-affinity complex which migrated with a mobility similar to that of MATE-BP1 (data not shown). Indeed, incubation of this probe with THP1 cell extracts allowed identification of a MATE-BP1-like complex whose formation was abolished by the addition of PU.1 antibodies (Fig. 5A). Thus, the binding of PU.1 to the AGAAAAG sequence within the GRR motif could explain the cross-competition observed (Fig. 4B). Since addition of the translated protein to HeLa cell extract led only to the detection of MATE-BP1 activity (Fig. 5B, lane 1), it seems likely that PU-1/Spi-1 could be the sole component involved in the myeloid and B-cell MATE-BP1 complex.

To confirm the role of PU.1/Spi-1 in FcγR1 gene expression, we cotransfected HeLa cells with or without a PU.1-expressing vector (35) and a reporter plasmid in which three copies of GRR were cloned in front of the −140 to −45 region containing the MATE motif (31). In the absence of PU.1/Spi-1 expression, the IFN-induced CAT activities detected with the construct containing GRR and MATE sequences were similar to those in noninduced cells (Fig. 6). However, in the presence of PU.1/Spi-1, the activity of the vector containing the GRR and MATE motifs increased significantly in IFN-γ-treated cells (Fig. 6). This induced activity was similar to that deduced

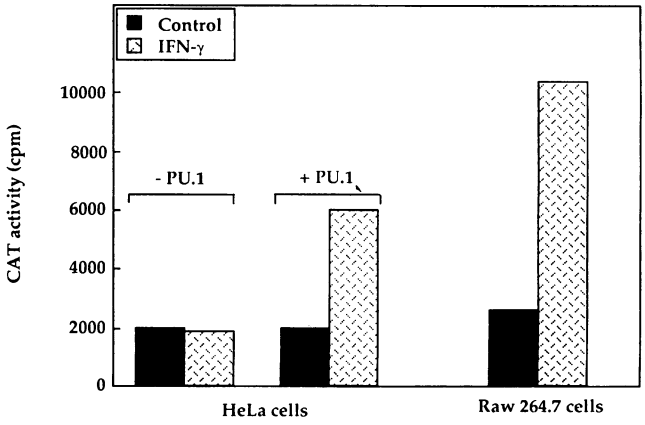

FIG. 6. PU.1/Spi-1 transactivates expression of a reporter gene controlled by GRR and MATE motifs. HeLa cells were cotransfected with an effector plasmid (34) expressing or not expressing PU.1 and with a reporter construct carrying three copies of GRR and a DNA fragment containing the −140 to −45 region (see Materials and Methods). The same reporter plasmid was used for transfection of the murine macrophagic cell line RAW 264.7 as a control. Transfected cells were treated with 200 U of human or murine IFN-γ per ml for 12 h. IFN-γ-induced CAT activities were then measured (see Materials and Methods). The results were determined by scintillation counting as the mean of at least five independent experiments showing standard deviations of less than 10%.

after transfection of the murine macrophagic cell line RAW 264.7 with the same construct (Fig. 6). These data therefore demonstrate that the mechanisms controlling FcγR1 promoter activity depend on activation of IFN-regulatory factors for IFN responsiveness and on the presence of the PU.1/Spi-1 factor which restricts FcγRI-induced expression to myeloid cells.

DISCUSSION

Although recent reports have improved our knowledge of the events involved in IFN-induced gene regulation (23, 24, 40, 46, 48), the mechanisms underlying the restricted expression of genes in a particular cell type upon IFN treatment are still poorly understood. To investigate such mechanisms, we and others (2, 28) have initiated a study of the gene encoding the human FcγR1. FcγR1 gene expression is IFN dependent and is limited to cells of the myeloid lineage (1). This regulatory process requires a GRR (2, 28) and a sequence, MATE, which confers myeloid cell-restricted expression (31). Both GRR and MATE motifs have been reported to be the target of factors leading to different DNA-binding activities: respectively, GIRE-BP (29, 31, 49) and MATE-BP (31). At least one component of GIRE-BP has been identified (14, 29, 31) as the 91-kDa protein (a subunit of ISGF3α) known to participate in different complexes (8, 16, 20, 32), such as ISGF3 involved in IFN-α-stimulated expression of early response genes (21) and GAF conferring IFN-γ responsiveness to the GBP gene (6, 40). In addition to the 91-kDa protein, participation of the 48-kDa subunit (ISGF3γ) in the GIRE-BP complex was suggested by its absence in extracts prepared from the cell mutant U2 (15, 30, 43) in which the 48-kDa protein is defective (43). However, this result was not supported by experiments showing that antibodies against the 48-kDa protein did not react with the GIRE-BP complex (29). Although we cannot exclude the possibility that the 48-kDa protein is involved indirectly in GIRE-BP formation, the U2 mutant might be defective in another gene encoding a component of GIRE-BP. An alternative explanation could be that the epitopes of the 48-kDa protein recognized preferentially by the antibodies are masked by a protein-protein interaction when this factor is engaged in the GIRE-BP complex.

Similarly, as reported for GIRE-BP (29, 31), the IFN-γ-activated factor (GAF) which binds to the GAS of the GBP gene is not inhibited by addition of an excess of ISRE (3). In contrast to the GAS motif (GAS-Ly6E) identified within the Ly6E gene promoter (29), GAS-GBP is not able to compete efficiently with GRR for the formation of GIRE-BP (31, 49). Although a palindromic region (pIRE) previously identified within the ICSBP gene enhancer was found in GAS-GBP and GRR (16), GAS-Ly6E (29) does not contain this sequence. Thus, depending on the nucleotide content of these motifs, the palindromic sequence by itself might not be an absolute requirement for binding the factors. The absence of GAF in cell extracts prepared either from cells treated with IFN-α (40) or from IFN-γ undifferentiated U937 (unless tetradecanoyl phorbol acetate was added) (6) could suggest that GIRE-BP and GAF are different. However, these differences and the absence of competition could be due to the fact that GAS-GBP is a very weak GAF site in contrast to GRR. Indeed, binding to the GAS motif might depend on the amount of 91-kDa protein available in the cells. This hypothesis is supported by data (6) showing that appearance of GAF activity is related to an increase in 91-kDa protein synthesis occurring during differentiation of U937 cells. The identity of GAF and GIRE-BP cannot, therefore, be excluded.

Although several questions remain to be clarified concerning regulation of the FcγR1 gene by IFN-γ, in this report we describe experiments leading to identification of the factor responsible for MATE-BP activities. Such activity was previously shown to be independent of IFN stimulation and was detected as two complexes in the monocytic cell line THP1 (31). First, we demonstrate that the presence of MATE-BP is restricted to myeloid and B-lymphoid cell lines (Fig. 1). Within a region including MATE, the sequence participating in the protein-DNA interaction (Fig. 2A) contained the AGAAAAG motif, which has been previously shown (31) to be present at several locations (Fig. 3) in the FcγR1 gene promoter and, in particular, in GRR (31). The importance of this motif was confirmed by its deletion and mutation within the MATE and GRR sequences (Fig. 4). Indeed, only reporter gene constructs containing a G3 region (−145 to −119) including this particular sequence was responsive to IFN-γ (data not shown), in agreement with a previous report (29) identifying the −145 to −134 sequence as required for complex formation. In addition, the fact that an excess of G3 specifically inhibited MATE-BP activities (Fig. 4B) suggested a potential affinity of at least one component of MATE-BP for the repeated motif in either a GRR or a MATE context.

Therefore, experiments were performed to characterize the factors providing the DNA-binding specificity for the GRR and MATE motifs. One cDNA clone was found to encode a fusion protein which specifically bound the MATE motif. Sequence analysis of this cDNA allowed us to identify the encoded product as the myeloid and B-cell transcription factor PU.1/Spi-1 (35). In our previous report (31), through analysis of upstream sequences of genes preferentially expressed in hematopoietic cells, we noted the presence of a MATE-like motif similar to the PU.1 box (18) in genes encoding cathepsin G, c-*fes*, CD11b, gp91-phox, and human elastase (31). Studies on the promoters of CD11b and CD11a genes (26, 27, 38) confirmed the participation of PU.1 in complexes binding to this consensus motif (31). PU.1/Spi-1 was also found to be involved in controlling the activity of the immunoglobulin κ and μ heavy-chain enhancers through its interaction with the B-cell-restricted factor, NF-EM5, and the *ets-1* oncogene, respectively (25, 33). In contrast, activation of J-chain gene transcription by PU.1 did not require protein-protein interaction with an adjacently bound nuclear factor (39). Similarly, the MATE-BP complex, like the complexes formed by the binding of PU.1 to the regions of the CD11b and macrophage colony-stimulating factor receptor promoters (27, 51), might also only depend on binding of the PU.1/Spi-1 factor to its cognate sequence. Several observations could favor this hypothesis. Addition of bacterially expressed PU.1 (data not shown) or of in vitro-translated PU.1/Spi-1 protein (Fig. 5B) to the binding mixture containing or not containing HeLa cell extract led to a DNA-binding activity identical to that of the MATE-BP1 complex. No complex migrating more slowly than MATE-BP1 was detected. Since in nonhematopoietic cells expression of PU.1/Spi-1 allowed transactivation of the FcγR1 promoter upon IFN induction (Fig. 6), no other cell-type-specific factor seems to be required for the expression of the FcγR1 gene in myeloid cells.

Although antibodies raised against PU.1/Spi-1 (5) abolished the formation of MATE-BP1 and MATE-BP2 in THP1 cells (Fig. 5A), they did not affect (data not shown) the presence of the faster complex, MATE-BP3, detected in long-term cultured premyelomonocytic HL60 and U937 cell lines (31). Several specific complexes which did not supershift with PU.1 antibodies and migrate faster than the one obtained with in vitro-translated PU.1 protein were reported (27, 39, 51). They represent DNA-binding complexes formed by proteolytic

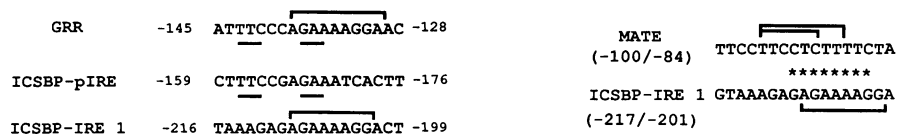

FIG. 7. Homology within FcγR1 and ICSBP promoter sequences. The palindromic sequences (pIRE) found in the IFN-γ-induced ICSBP gene promoter (16) and in the GRR motif are underlined. The putative PU boxes within GRR, MATE, and ICSBP-IRE 1 are indicated by brackets. Alignment for the conserved nucleotides between MATE and ICSBP-IRE 1 is noted by asterisks.

products of PU.1 which contains a PEST region implicated in targeting proteins for degradation (18). Since the antibodies used recognize only the N-terminal portion of the protein (5), a proteolytic product of PU.1 conserving the C-terminal DNA-binding site might therefore be implicated in formation of MATE-BP3 complex. A proteolytic mechanism leading to truncated forms of PU.1/Spi-1, corresponding to the different MATE-BP activities, could reflect potential homeostasis of the system. However, the physiological relevance of the proteolytic activity has yet to be demonstrated.

As defined by our footprinting experiments, the PU.1 DNA-binding site covered a region longer than the previously described consensus binding site for PU.1 and other *ets* family proteins (27). A similar protected region was also identified within the CD11b promoter (26, 27), suggesting that surrounding sequences of the core site could influence PU.1 binding. Competitions with Mm1 and Mm2 (Fig. 2B) support this hypothesis. As with CD11b, macrophage colony-stimulating factor receptor, and FcγR1 gene promoters (27, 31, 51), PU.1/Spi-1 binds either within or to a site just upstream the major transcriptional start site in a region lacking a TATAA box. Since the amino-terminal region of PU.1 binds the TFIID subunit of RNA polymerase II and subsequently other components of the transcriptional machinery (12), this factor could be involved in constitutive expression of the FcγR1 gene. It remains to be clarified whether the potential binding of PU.1 (Fig. 5A) on the AGAAAAG sequence located within the GRR motif acts on this constitutive expression.

It is tempting to speculate that the mechanism described in this report is involved in the regulation of other IFN-induced genes preferentially expressed in hematopoietic cells, such as genes encoding *mig* (monokine induced by IFN-γ) (50) and ICSBP (16). In addition to the palindromic sequence (pIRE) involved in the IFN-γ response (16), promoters of both genes contained potential PU.1 boxes (Fig. 7). In the ICSBP gene, upstream of ICSBP-pIRE, a PU.1/MATE-like motif (Fig. 7) was identified within a sequence designated ICSBP-IRE-1 (16). The function of ICSBP-IRE-1 has to be investigated, as it could represent a target for the PU.1 factor conferring cell-type-restricted expression to the ICSBP gene. Although the mechanisms involved in IFN-induced and cell-type-restricted gene expression are distinct, modulations of PU.1 activity could regulate the IFN responsiveness of these genes whose expression is restricted to B and myeloid cells.

ACKNOWLEDGMENTS

We thank Sandra Pellegrini for helpful advice in the preparation of the manuscript.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale and from the Association pour la Recherche sur le Cancer.

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