Induction of Myc-intron-binding polypeptides MIBP1 and RFX1 during retinoic acid-mediated differentiation of haemopoietic cells

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Retinoic acid-mediated differentiation of HL60 cells is associated with an alteration of chromatin structure that maps to protein-binding sequences within intron I of the *c-myc* gene and with down-regulation of *c-myc* expression. By using HeLa cell extracts, we previously identified two polypeptides, designated MIBP1 (for Myc-intron-binding peptide) and RFX1, that interact *in vivo* and bind to the intron I element; we showed that tandem repeats of an MIBP1/RFX1-binding site can exhibit silencer activity on a heterologous promoter. Here we demonstrate that p160 MIBP1 and p130 RFX1 are absent from undifferentiated HL60 cells. In addition, we show that treatment with retinoic acid induces both MIBP1 and RFX1 protein, as well as their DNA-binding activity,

upon granulocytic differentiation of HL60 cells, with a gel mobility pattern identical to that of HeLa cells. In the absence of p160 MIBP1 and p130 RFX1, we observed that the altered gel mobility-shift pattern detected in undifferentiated HL60 cells reflects the binding of two novel polypeptides, p30 and p97, that can be cross-linked to the same recognition intron sequence. We also show that the time course of MIBP1 and RFX1 induction is inversely correlated with the down-regulation of *c-myc* levels during the treatment of HL60 cells with retinoic acid.

Key words: *c-myc* oncogene, granulocytic differentiation, HL60 cell line, RFX gene, transcription.

INTRODUCTION

A large body of evidence indicates that the deregulated expression of the *c-myc* gene is associated with cell proliferation and, under selected conditions, can trigger apoptosis [1–3]. In contrast, the tightly regulated expression of the *c-myc* gene is linked to the terminal differentiation and growth arrest of haemopoietic cells [4,5]. However, the mechanism underlying this process of terminal differentiation is not well understood. The HL60 leukaemia cell line provides an experimental model in which *c-myc* expression can be modulated with pharmacological agents known to induce differentiation along granulocytic or monocytic pathways [6–8]. When HL60 cells are induced to differentiate, the steady-state levels of *c-myc* RNA decrease rapidly and remain low [9,10]. This repression of *c-myc* mRNA levels has been proposed to operate in two stages. An initial ‘pausing’ of the RNA polymerase enzyme at the initiation complex, also referred to as a block of mRNA elongation, results in the rapid down-regulation of *c-myc* expression [6,11]. This is followed by a termination of transcription initiation that coincides with a marked change in chromatin structure upstream of both the P1 and P2 *myc* promoters [8]. In addition, a DNase-I-hypersensitive site is detected within *c-myc* intron I during retinoic acid-induced differentiation of HL60 cells [6]. This DNase-I-hypersensitive site maps to a region of protein-binding sequences, which was previously identified within intron I of the *c-myc* gene [12,13]. These results suggest that an alteration of chromatin structure in the regulatory regions of *c-myc* might be required to facilitate the interaction of protein-binding factors with their cognate sequence *in vivo* to regulate *c-myc* transcription during the differentiation of HL60 cells [6].

Several protein factors have been reported to bind to the intron I region of the *c-myc* gene [12–15]. Myc intron factor

(MIF-1) was originally identified as a nuclear phosphoprotein that binds to a 20 nt region within intron I [12,16], and it was shown that protein binding was blocked by point mutations frequently found in DNA isolated from Burkitt’s lymphoma cells [12]. Subsequently it was demonstrated that MIF-1 activity consists of at least two polypeptides, the Myc-intron-binding peptide (MIBP1) and the MHC-class-II-promoter-binding protein RFX1 [17]. RFX1 was initially identified as a protein that binds to the X-box site in the promoter of the MHC class II gene and subsequently shown to function as a transactivator of the hepatitis B virus (HBV) enhancer through its interaction with a binding sequence referred to as the EP element [18,19]. In addition, RFX1 was shown to regulate several other mammalian genes, including the human leukaemia (‘PCNA’) promoter and the interleukin 5 receptor α promoter [20,21]. RFX1 associates *in vivo* with MIBP1; it has been shown that this heterodimer binds to the MIF-1 recognition sequences in intron I of the *c-myc* gene, to the promoter region of MHC class II genes and to the regulatory regions of a several viral genes, including HBV, cytomegalovirus, Epstein–Barr virus and polyomavirus [17,22]. Both the MIF-1 and EP sites regulate the activity of a heterologous promoter in a position- and orientation-independent manner and function as a silencer in a panel of hepatocarcinoma cell lines [17,22].

The identification of an altered chromatin structure in the vicinity of the MIBP1/RFX1-binding site during the programmed differentiation of HL60 cells [6] prompted us to compare the MIBP1/RFX1 protein–DNA complexes between undifferentiated and retinoic acid-treated HL60 cells. Whereas MIBP1/RFX1 protein–DNA complexes were absent from undifferentiated cells, we have now identified two polypeptides, p30 and p97, that interact with the MIF-1-binding site in undifferentiated HL60 cells. In addition, we have demonstrated

Abbreviations used: EMSA, electrophoretic mobility-shift assay; HBV, hepatitis B virus; MIBP1, Myc-intron-binding peptide; MIF, Myc intron factor.

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Figure 1 Induction of MIF-1 binding activity after the differentiation of haemopoietic cells with retinoic acid

Upper panel: EMSA analysis comparing MIF-1-protein complexes between HL60 and HeLa cell extracts. Specific (sp) and non-specific (ns) MIF-1 oligonucleotide sequences are as described in the Materials and methods section. The MIF-1 radiolabelled probe (p) was incubated with HL60 protein extracts (lanes 1–3), HeLa extracts (lanes 4–6) or in the absence of protein extract (lane 7). The bracket depicts the faster-migrating 'smear' in untreated HL60 cells. Lower panel: EMSA analysis comparing MIF-1-protein complexes between undifferentiated and retinoic acid treated HL60 cells. Radiolabelled MIF-1 oligonucleotide probe was incubated with protein extracts isolated from undifferentiated (U) or retinoic acid-differentiated (D) HL60 cells.

that the steady-state levels of the MIBP1 and RFX1 polypeptides, as well as an active MIBP1/RFX1 protein-DNA complex, were induced during the retinoic acid-induced differentiation of HL60 cells.

MATERIALS AND METHODS

Cell culture

HL60 human leukaemia cells were propagated in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum supplemented with 2 mM glutamine and 100 µg/ml penicillin and streptomycin. Differentiation of HL60 cells was induced by the addition of 1 µM retinoic acid to cells growing ex-

ponentially. The cells were harvested 3 days after treatment with retinoic acid.

Preparation of nuclear and whole cell extracts, and electrophoretic mobility-shift assay (EMSA)

Nuclear protein extracts were prepared as described previously [23,24]. The extracts were incubated with 0.2 ng of ³²P end-labelled synthetic oligonucleotide probe corresponding to the MIF-1-binding sequence (5'-AGAGTAGTTATCGTAACTGG-3'), in the presence of 5 µg of poly(dI-dC). Competition experiments were run in the presence of either 100 ng of unlabelled MIF-1 recognition sequence (specific competition) or with the use of 100 ng of a non-specific competitor (5'-GATCAGCTCGCAAATTTAAAGGG-3'). The total volume was adjusted to 10 µl with buffer D [20% (v/v) glycerol/20 mM Hepes (pH 7.5)/0.2 mM EDTA/0.5 mM dithiothreitol/0.5 mM PMSF] to a final concentration of 100 mM KCl and incubated for 20 min at 25 °C. The reaction mixture was resolved on a 4% (w/v) polyacrylamide gel in 0.5% Tris/borate/EDTA (TBE) buffer and detected by autoradiography. For supershift analysis the reaction mixture was preincubated for 5 min at room temperature with 1 µl of antiserum before the addition of ³²P-labelled oligonucleotide probe. The reaction mixture [10 mM Hepes (pH 7.8)/75 mM KCl/2.5 mM MgCl₂/0.1 mM EDTA/1 mM dithiothreitol/3% (w/v) Ficoll and 1 µg of poly(dI-dC)] was incubated for 15 min at room temperature and analysed as above [17].

Purification of protein-DNA complexes

Nuclear extracts were prepared from 5 × 10⁹ undifferentiated HL60 cells and were diluted with buffer D to a final concentration of 180 mM KCl. Nuclear extracts (20 ml) were loaded on a 10 ml Bio-Rex 70 cation-exchange column that had been equilibrated with 180 mM KCl in buffer D. After the collection of the flow-through and extensive washing with buffer D at 180 mM KCl, proteins were eluted from the column with buffer D at 800 mM KCl, in a total of nine 4.5 ml fractions. The DNA-binding activity was assayed by EMSA, with the MIF-1-binding sequence as a probe. Fractions 3 and 4, containing MIF-1-binding activity, were pooled, diluted with buffer D to a final concentration of 180 mM KCl, and loaded on a 5 ml oligonucleotide-Sepharose affinity column. The oligonucleotide-Sepharose-4B affinity column coupled with an oligonucleotide containing the MIF-1-binding site was prepared as described [16] and equilibrated with buffer D at 180 mM KCl in buffer D. Proteins were eluted from the column with 1 M KCl in buffer D in 0.5 ml fractions; MIF-1-binding activity was assayed as described above. The total yield of this purification scheme was 174 µg of protein out of an initial load of 165 mg of protein in the nuclear extracts.

Cross-linking analysis

Oligonucleotide-affinity-purified proteins (500 ng) from undifferentiated HL60 cells (U-HL60) and HeLa cells were mixed with 2.5 ng of ³²P-labelled oligonucleotide probe (1.5 × 10⁶ c.p.m./ng) and 2.5 µg of poly(dI-dC), then adjusted to 100 mM KCl with buffer D in a total volume of 50–80 µl. A similar reaction with ³²P-labelled non-specific oligonucleotide was used as control. Both reactions were incubated at 25 °C for 15 min and cross-linked on ice for 15 min in the Stratagene 1800 UV Stratamaker[®]; proteins were resolved by SDS/PAGE [10%

(w/v) gel]. In addition, the cross-linked reaction mixtures were run on a 4% (w/v) polyacrylamide gel in 0.5×TBE buffer and the wet gel was autoradiographed for 2 h. The specific bands and parallel areas from the non-specific reactions were excised, mixed with 5×loading buffer, boiled for 3 min and subjected to SDS/PAGE [10% (w/v) gel] and autoradiography.

Immunoblot analysis

Whole-cell extracts (200 µg of protein per lane) from undifferentiated and retinoic acid-differentiated HL60 cells were subjected to 7.5% (w/v) PAGE and immunoblotting with the MIBP1 antiserum (1:250 dilution) or RFX1 antiserum (1:1000 dilution) and then detected with ¹²⁵I-Protein A as described previously [25].

RNA isolation and Northern blot analysis

RNA was isolated at intervals from controls and from 1 µM retinoic acid-treated HL60 cells by the guanidine isothiocyanate method [26]. Total RNA (15 µg) was fractionated on a 1% (w/v) agarose/2% formaldehyde gel and RNA blot analysis was performed [26]. The *c-myc* probe (5PP) consisted of the *Pst*I–*Pst*I fragment spanning exon I, intron I and part of exon II [10]. Probes were ³²P-labelled by nick translation and hybridized; the filter was washed with 0.5×SSC (1×SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1% SDS at 65 °C and subjected to autoradiography [26].

RESULTS

Induction of MIF-1-binding activity during retinoic acid-induced differentiation of haemopoietic cells

MIF-1 activity, identified initially as protein-binding activity to an intron I element within the *c-myc* gene, was shown to consist

of a heterodimer between the *myc* intron-binding polypeptide, MIBP1, and the MHC-II-promoter-binding protein RFX1 (also referred to as the HBV-enhancer-binding protein) [17]. Recent studies indicate that MIBP1/RFX1–DNA complexes from the HeLa epithelial carcinoma tumour cell line consist of a 160 kDa MIBP1 and a 130 kDa RFX1 product [17] that can be detected in the 'A' complex by EMSA (Figure 1, upper panel, lanes 4–6). In contrast, the MIBP1/RFX1–DNA complex was absent from undifferentiated HL60 haemopoietic cells; however, a faster migrating smear was detected by EMSA with the same 20 bp MIF-1-binding sequence (Figure 1, upper panel, lane 1). The specificities of these complexes were tested by the addition of 100 ng of the unlabelled MIF-1-binding sequence, which completely blocked the protein-binding signal (Figure 1, upper panel, lanes 2 and 5), whereas no competition was observed with 100 ng of a non-specific sequence (Figure 1, upper panel, lanes 3 and 6). In addition, we observed the formation of complex 'B' by using HeLa cell extracts with MIF-1 oligonucleotide sequences. We have previously shown that this complex does not contain the 160 kDa MIBP1 or 130 kDa RFX1 product; however, the exact composition of complex 'B' remains to be established [17].

Treatment of HL60 cells with 1 µM retinoic acid, which induces cellular differentiation along a granulocytic lineage [7,10,27], resulted in the appearance of a slower-migrating protein–DNA complex 'A', which was absent from the undifferentiated cells and was similar to the pattern observed in HeLa cells (Figure 1, upper panel, lane 4). To compare the protein–DNA complexes between undifferentiated and differentiated HL60 cells, nuclear protein extracts were incubated with a MIF-1-radiolabelled oligonucleotide probe and the retarded protein–DNA complexes were resolved by EMSA. We observed that after the differentiation of HL60 cells, the slower-migrating complex was induced (Figure 1, lower panel, lane 2) and that the migration of this complex resembled that of the protein–DNA complex observed with a HeLa nuclear extract (Figure 1, lower panel, lane 2; Figure 1, upper panel, lane 4).

Figure 2 Time-course analysis of *c-myc* expression and the MIF-1 binding activity during the differentiation of HL60 cells

(A) RNA blot analysis of total RNA isolated at the indicated time points (in days) from untreated HL60 cells used as controls and retinoic acid-treated cells. Day 1 for *c-myc*-specific probe is defined as 24 h after treatment with retinoic acid. Staining with ethidium bromide (EtBr) was used to demonstrate equal loading of RNA in each lane. (B) EMSA analysis of MIF-1–protein complexes after treatment with retinoic acid as described above. RNA blotting and EMSA were performed with *c-myc*-specific probe and MIF-1 oligonucleotide respectively, as described in the Materials and methods section.

To study whether the observed changes in protein–DNA complexes during the retinoic acid-induced differentiation of HL60 cells were related to changes in *c-myc* expression, we performed a time course experiment in which *c-myc* expression and MIF-1–protein-binding activity were examined after the treatment of HL60 cells with 1 μ M retinoic acid. Cells were harvested for both RNA and protein extracts on days 1, 2 and 3 after treatment with retinoic acid, and RNA blotting and EMSA analysis were performed (Figure 2). We observed that the time course of *c-myc* down-regulation as measured by RNA analysis corresponded to the appearance of MIF-1-binding activity as determined by EMSA (Figure 2).

Purification and characterization of MIF-1-binding peptides from undifferentiated HL60 cells

To define the molecular basis for the difference between MIF–DNA complexes in undifferentiated and differentiated cells, we purified the protein–DNA complexes from undifferentiated HL60. We employed cation-exchange and oligonucleotide-affinity chromatography under conditions reported previously [16] to purify MIF-1-binding proteins from HeLa cells. We compared the chromatographic profile of proteins purified from HeLa cells with the proteins purified from undifferentiated HL60 cells (Figure 3). HeLa nuclear proteins fractionated on a cation-exchange Bio-Rex 70 column in 180 mM KCl were eluted in the flow-through of the column (Figure 3B, lane 2). In contrast, when nuclear extracts from undifferentiated HL60 cells were fractionated under identical conditions, MIF-1-binding activity was retained on the cation-exchange column. These proteins were eluted from the column in 0.8 mM KCl and each fraction was tested for MIF-1 binding activity by EMSA (Figure 3C). Further purification of proteins from undifferentiated HL60 cells was achieved by the passage of the fractions containing binding activity through an affinity column coupled with the MIF-1 oligonucleotide-binding sequence (Figure 4). Fractions 3 and 4 from the Bio-Rex 70 cation-exchange column (Figure 3C, lanes 3 and 4) were diluted to 180 mM KCl in buffer D, loaded on the MIF-1 oligonucleotide-affinity column and eluted in 1 M KCl. Purified proteins from undifferentiated HL60 cells retained the distinctive DNA-binding pattern on EMSA (Figure 4B, lanes 3–5). Affinity-purified proteins from HeLa cells are shown for comparison (Figure 4A, lanes 5–12). These results suggest that polypeptides with distinct properties interact with the *c-myc* intron I MIF-1 sequences from undifferentiated HL60 cells and from HeLa cells.

To identify the polypeptides present in the oligonucleotide-affinity-purified fractions from undifferentiated HL60 cells, we cross-linked purified proteins from undifferentiated HL60 cells to the radiolabelled MIF-1 recognition sequence and then resolved the cross-linked product by SDS/PAGE (Figure 5A). Two specific proteins of 97 and 30 kDa were detected in the undifferentiated HL60 fraction (Figure 5A, lane 1). These proteins were not present in the cross-linked products of undifferentiated HL60 proteins with a labelled non-specific sequence (Figure 5A, lane 2). To confirm that the bands seen on EMSA corresponded to the cross-linked bands seen on SDS/PAGE, the cross-linked products were first separated by EMSA and the specific retarded bands were excised and fractionated by SDS/PAGE [16]. Both the 97 kDa and the 30 kDa proteins were detected in the undifferentiated HL60 oligonucleotide-affinity-purified protein fraction (Figure 5B, lane 1). Comparison of the cross-linked proteins from undifferentiated HL60 cells and HeLa cells demonstrated the presence of a high-molecular-mass protein

Figure 3 Comparison of chromatographic profiles between HeLa and HL60 nuclear extracts by cation-exchange chromatography

(A) Purification scheme for MIF-1-binding proteins. Nuclear extracts from HeLa or undifferentiated HL60 cells were applied to a Bio-Rex 70 column as described in the Materials and methods section. (B, C) MIF-1 binding activity was detected in the flow-through (FT) from the HeLa nuclear extract (B), whereas MIF-1 binding activity from HL60 nuclear extract was retained in the column (C).

complex (Figure 5B, lane 2) in HeLa cells that was absent from the undifferentiated HL60 purified protein fractions (Figure 5B).

Induction of MIBP1 and RFX1 during the retinoic acid-induced differentiation of HL60 cells

Treatment of HL60 cells with 1 μ M retinoic acid resulted in the induction of the slower-migrating protein–DNA complex, which was absent from the undifferentiated cells (Figure 1, lower panel). Because this slower-migrating complex from HeLa cells consists of two polypeptides, MIBP1 and RFX1 [17], we examined whether MIBP1 and RFX1 were also induced during the differentiation of HL60 cells. Protein extracts from undifferentiated and retinoic acid-treated HL60 cells were resolved by SDS/PAGE and immunoblotted with antiserum raised against either oligonucleotide-affinity-purified MIBP1 from HeLa cells or peptide sequences derived from RFX1 cDNA [17,18]. We observed that both the 160 kDa MIBP1 and the 130 kDa RFX1 were induced during the treatment of HL60 cells with retinoic acid (Figures 6A and 6B, lanes 2). Because MIBP1 and RFX1 co-precipitate *in vivo* [17,18], we examined whether MIBP1 and

Figure 4 Distinct DNA–protein complexes after oligonucleotide-affinity purification of HL60 and HeLa proteins

EMSA of protein fraction from MIF-1 oligonucleotide-affinity column assay with the HeLa flow-through fraction from the Bio-Rex 70 column (**A**) and HL60 fractions 3 and 4 from the Bio-Rex 70 column (**B**).

Figure 5 p30 and p97 bind to MIF-1 oligonucleotide in undifferentiated HL60 cells

(**A**) Cross-linking of purified HL60 proteins from oligonucleotide-affinity chromatography (fractions 3–5 from Figure 4B) to the MIF-1 probe (lane 1) or to a non-specific (NS) probe (lane 2) followed by SDS/PAGE. (**B**) Cross-linked proteins were resolved by EMSA with the MIF-1 probe, and specific bands from the purified HL60 and HeLa fractions were excised and the proteins were resolved by SDS/PAGE. The arrows depict the p30 and p97 species from HL60 cells and p160 from HeLa cells. The positions of molecular mass markers are indicated (in kDa) at the right.

RFX1 polypeptides were both present in the protein–DNA complexes formed with cell extracts from differentiated HL60 cells. We performed a supershift analysis to test whether the MIBP1 and RFX1 antisera shifted the migration of these complexes. The MIF-1 oligonucleotide was incubated with nuclear extracts isolated from differentiated HL60 or the same extracts supplemented either with the preimmune serum or with the antisera directed against either MIBP1 or RFX1 (Figure 7, lanes 12–16). We observed that both the MIBP1 and the RFX1 antisera specifically retarded the migration of the upper band ‘A’ (Figure 7, lanes 14 and 16), whereas preimmune sera showed no effect (Figure 7, lanes 13 and 15). Moreover, the protein–DNA

complexes induced during the treatment of HL60 cells with retinoic acid (Figure 7, lanes 12–16) were identical with those from HeLa cells (lanes 2–6). In addition, the antisera against neither MIBP1 nor RFX1 could supershift the protein–DNA complex from undifferentiated HL60 cells (Figure 7, lanes 7–11).

DISCUSSION

MIBP1 and RFX1 are nuclear proteins that bind to related consensus sequences located in the regulatory region of several different cellular and viral genes [17,20,21,28–31]. MIBP1 was previously identified as a component of a MIF-1-binding complex

Figure 6 Induction of MIBP1 and RFX1 proteins after treatment of HL60 cells with retinoic acid

Protein extracts from undifferentiated (U) and differentiated (D) HL60 cells were resolved by SDS/PAGE and immunoblotted with anti-MIBP1 (A) and anti-RFX1 (B). The arrow depicts 160 kDa MIBP1 and 130 kDa RFX1, which were detected only in retinoic acid-treated cells. The positions of molecular mass markers are indicated (in kDa) at the left.

Figure 7 MIBP1 and RFX1 are present in the MIF-1-DNA complexes after retinoic acid-induced differentiation of HL60 cells

Supershift analysis of MIF-1 protein-DNA complexes generated with nuclear extracts from HeLa (lanes 2–6), undifferentiated (U-HL60; lanes 7–11), and retinoic acid-differentiated HL60 cells (D-HL60; lanes 12–16). Extracts were preincubated with the MIF-1 oligonucleotide probe in the absence (–) of serum or in the presence of either preimmune serum (P) or MIBP1-specific or RFX1-specific immune serum (I). Lane 1, MIF-1 probe in the absence of nuclear extract. The arrows depict the supershift with the MIBP1 and RFX1 antisera.

that interacts with a recognition element within intron I of the *c-myc* gene [12,17]. The gene for RFX1 encodes a protein that binds and regulates the MHC class II promoter and the EP site of HBV enhancer I [19]. With the use of polyclonal sera raised against either oligonucleotide-purified MIBP1 or a peptide derived from RFX1 [17], we have demonstrated previously that the 160 kDa MIBP1 and the 130 kDa RFX1 interact *in vivo* and that both bind to the MHC class II (X-box), HBV (EP) and *c-*

myc (MIF-1) sites. We have now shown that MIBP1 and RFX1 are absent from undifferentiated HL60 leukaemia cells and that both proteins can be subsequently induced during the differentiation of these cells by retinoic acid.

The binding of MIBP1/RFX1 to its recognition sequences resulted in a slowly migrating complex on EMSA analysis [17,22]. This slowly migrating protein-DNA complex, previously designated the 'A' complex [17], was not detected in undifferentiated HL60 cells, where, in contrast, a faster-migrating smear was observed. Purification of proteins from undifferentiated cell extracts combined with protein-DNA cross-linking analysis revealed the presence of two polypeptides, p30 and p97, that can interact with the MIF-1 recognition sequences. Neither the MIBP1 antiserum nor the RFX1 antiserum could supershift these faster-migrating complexes, confirming that MIBP1 and RFX1 do not interact with the MIF-1 recognition sequence in undifferentiated HL60 cells. In addition, differences in the chromatographic profiles between proteins purified from the faster-migrating complex and the slower-migrating complex 'A' suggest that MIBP1 and RFX1 do not interact with this binding sequence in undifferentiated cells. Our results therefore demonstrate that two other polypeptides, p30 and p97, interact with MIF-1 sequences from undifferentiated HL60 cells, whereas the binding of p160 MIBP1 and p130 RFX1 is observed only after the induction of both proteins in retinoic acid-treated cells.

The MIBP1/RFX1 binding sequence was originally identified as the MIF-1-binding site in the *c-myc* gene, which is frequently targeted for mutation in Burkitt's lymphoma [12]. In addition, it was shown that the binding of MIF-1 activity to its recognition sequence was blocked due to point mutations found in DNA from Burkitt's lymphoma cells. It was therefore previously suggested that this region could function to regulate *c-myc* transcription negatively [12]. In addition, when multimers of the MIF-1-binding site were placed under the control of a heterologous simian virus 40 promoter, they acted as a silencer element [17,22]. We have now shown that the induction of MIBP1 and RFX1 during the differentiation of the HL60 cells coincides with the down-regulation of *c-myc* gene expression during the differentiation of these cells. In addition, the observation that a DNase-I-hypersensitive site appears during the retinoic acid-induced differentiation of HL60 cells in the vicinity of the MIF-1-binding sequences suggests that the opening of chromatin structure *in vivo* might allow MIBP1 and RFX1 to interact with their binding site and might participate in the down-regulation of *c-myc* transcription. However, future experiments will be needed to determine whether a causal relationship exists between MIBP1/RFX1 activity, *c-myc* mRNA levels and the differentiation of HL60 cells.

In contrast, the HBV (EP) and the MHC class II (X-box) sequences that serve as a DNA recognition site for the MIBP1/RFX1 complex are positive elements that are required for the promoter and enhancer activities of HBV and MHC class II genes respectively [29,32–34]. The dual nature of these regulatory elements can be explained by the observation that all three binding sites, HBV (EP), MHC class II (X-box) and *c-myc* (MIF-1), are located adjacent to other functional elements that can act co-operatively either to activate or to repress transcription [13,34–37]. For example, within a 50 nt region of the HBV enhancer core there are three *cis* elements, which bind seven different protein factors [22,38]. Adjacent to the EP sequence which binds MIBP1/RFX1 is the GB/RXR α element, which binds liver-enriched nuclear proteins HNF-4 and RXR α [39–42]. Adjacent to the GB site is another element that binds liver-specific factor HNF-3 [43,44]. Thus, two liver-specific sites co-operate with the EP site to confer liver-specific enhancer function

[42]. In contrast, five tandem repeats of the EP element down-regulate the simian virus 40 promoter in all hepatocarcinoma cell lines tested [17,22], suggesting a complex interaction between MIBP1/RFX1 and liver-specific factors. The MIF-1-binding site, similarly to the EP site of the HBV enhancer, is located adjacent to three other *cis* elements in the intron I region of the *c-myc* gene, designated MIF-2 to MIF-4 [13,37]. All four sites are targeted for somatic mutations in Burkitt's lymphoma cells and act co-operatively in the regulation of *c-myc* transcription [13,37,45]. Cloning of the gene that encodes MIBP1 would facilitate the further characterization of the RFX1–MIBP1 interaction with the adjacent binding factors and would allow a more direct examination of their role in the regulation of *c-myc* expression during the differentiation of HL60 cells.

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