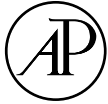

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The *mif* gene is transcriptionally regulated by glucose in insulin-secreting cells

Valérie Plaisance, Nancy Thompson, Guy Niederhauser, Jacques-Antoine Haefliger, Pascal Nicod, Gérard Waeber,* and Amar Abderrahmani

Department of Internal Medicine and Institute of Cellular Biology and Morphology, University of Lausanne, Lausanne CH-1011, Switzerland

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Abstract

Macrophage migration inhibitory factor (MIF) is an important regulator of glucose homeostasis. In pancreatic β-cells, MIF expression is regulated by glucose and its secretion potentiates the glucose-induced insulin secretion. The molecular mechanisms by which glucose mediates its effect on MIF expression are not elucidated. Herein, we report that incubating the differentiated insulin-secreting cell line INS-1 in high glucose concentration increases MIF transcriptional activity as well as the reporter gene activity driven by the −1033 to +63 bp fragment of the MIF promoter. A minimal region located between −187 and −98 bp of this promoter sequence contributes both to basal activity and glucose-responsiveness of the gene. Within this promoter region, two *cis*-binding sequences were identified by mobility shift assays and footprinting experiments. Both *cis*-elements interact with nuclear proteins expressed specifically in insulin-secreting cells. In conclusion, we identified a minimal region of the MIF promoter which contributes to the glucose stimulation of the *mif* gene in insulin-secreting cells. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Macrophage migration inhibitory factor; MIF; Glucose; Transcription; Gene regulation; Insulin; Secreting cells; *cis*-Elements

The protein mediator known as macrophage migration inhibitory factor (MIF) was originally identified as a T-cell product involved in the host response to stress and inflammation [1]. Although generally described as a proinflammatory cytokine and glucocorticoid-induced immunoregulator [2–5], MIF was also recognized as an important factor involved in glucose homeostasis [6]. In muscle, MIF induces glucose metabolism and contributes to glucose disposal. In pancreatic-β cells, MIF acts as an autocrine regulator of insulin secretion [7,8]. The inactivation of MIF by immunoneutralization of secreted MIF or the inhibition of endogenous MIF production by RNA antisense reduced the glucose-induced insulin secretion [8]. Since the glucose-induced insulin secretion is one of the main altered processes implicated in the pathogenesis of type 2 diabetes, it has been hypothesized that altered expression, secretion or action of MIF could contribute to abnormal β-cell function [6]. Thus, the understanding of the transcriptional mechanism involved in the control of MIF expression in insulin-secreting cells is potentially important for integrating the role of MIF in glucose homeostasis. Since it has also been reported that the MIF mRNA levels were positively regulated in a time- and a dose-dependent manner by the glucose in β-cells [8], the goal of this study was to clarify the molecular mechanism by which glucose mediates its effect on the MIF gene expression in these cells.

In pancreatic-β cells, it is known that glucose triggers pleiotropic effects that are associated with transcriptional activation of several secretory and metabolic genes, which in turn modulate insulin biosynthesis and secretion [9–13]. The mechanisms by which glucose regulates the transcription of several genes are only partially elucidated. Elevated glucose concentration can target its effect through specific *cis*-acting elements located in the promoter region of glucose-responsive genes [14,15]. Some of these elements have been identified such as the palindromic repeat of sequence CANNTG termed E elements (or E boxes) [16,17], the A elements (also termed CT or A boxes), which are defined by the core

* Corresponding author. Fax: +41-21-314-09-28.
E-mail address: gwaeber@chuv.hospvd.ch (G. Waeber).

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sequence TAAT [18], the Z element, and the carbohydrate response element (ChoRE) [19–21]. The E elements are bound by several members of the basic helix-loop-helix (bHLH) class of transcription factors such as USF, BETA2, E47, E2A, and E12 [22–28]. The A element is the binding site for homeodomain class of transcription factors such as ISL-1, CDX3, LMX-1, HNF-1, and PDX-1 (also termed IPF-1, STF-1, and IDX-1) whereas the Z and ChoRE elements bind unidentified transcription factors [29–32].

Herein, we report that the glucose induction of MIF mRNA levels in insulin-secreting INS-1 cells occurs at transcriptional levels. This study also provides evidence that both basal and glucose-stimulated transcriptional activities are mediated by the regulatory region between −187 and −98 bp of the MIF promoter. Two *cis*-binding sequences termed ME1 and ME2 were identified within this region that are different from known glucose-responsive *cis*-elements and bind nuclear factors expressed specifically in β-cells.

Materials and methods

Plasmid construction. To generate a series of 5′ deletion mutants of the MIF promoter, the −1033/+63 bp of MIF promoter sequence cloned upstream of the luciferase gene reporter (MIFluc) [33] was protected at the *Nhe*I site and subsequently digested with exonuclease III as recommended by the supplier (Promega). The selected plasmids were blunted, religated, and sequenced to determine the exact 5′-end of the deletion. Sequences of all plasmid constructs were verified by DNA sequencing.

Cell lines and transient transfection assays. INS-1 cells (passages 39–55) were maintained as previously described [34]. Two days before transfection, 4×10^5 cells were plated in each well of the 12-well plates. The day before transfection, the medium was changed to 2 mM glucose. On the day of transfection, 1 μg total DNA (1 μg reporter construction plus 0.1 μg pRLSV40 renilla were transiently transfected using the liposome-mediated TRANSFAST reagent (Promega). Transfected cells were then replaced in media containing either 2 or 12 mM glucose concentration 2 h after transfection. Cells were then harvested and subjected to luciferase analysis 48 h after transfection.

RNA extraction and Northern blot analysis. Total RNA from INS-1 cells cultured in 2 or 12 mM glucose concentration with the presence or absence of 5 μg/ml RNA synthesis inhibitor, actinomycin D, was extracted by the method of Chomczynski and Sacchi [35]. Total isolated RNA was analyzed by Northern blotting as previously described [34].

Preparation of nuclear extracts and DNAse I footprinting experiments. Nuclear extracts from cell lines were prepared as previously described [34]. The probe for the DNAse I protection experiments was generated by PCR using a set of oligonucleotides encompassing the MIF promoter region −187 to −98 bp. Dnase I protection experiments were performed by incubating the radiolabeled probe with 25 μg nuclear extract exactly as described [36]. After 20 min of incubation on ice, 1 and 2 U DNAse I (Pharmacia) were added and digestion was performed for exactly 1 min at room temperature. Finally, 200 μl stop solution containing 60 μg proteinase K/ml in 0.33 M Na-acetate, 10 mM EDTA, and 1% sodium dodecyl sulfate was added and samples were mixed thoroughly before being incubated for 15 min at 55 °C. After phenol chloroform extraction, samples were precipated, resuspended in 90% formamide, heated 3 min at 95 °C, and loaded onto an 8% sequencing polyacrylamide gel [36].

Electrophoretic mobility shift assays (*EMSA*). Oligonucleotides used for EMSA are described in Table 1. Complementary sense and antisense oligonucleotides were hybridized, filled-in by the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]deoxycytosine triphosphate, and purified as described [34]. Approximately 1 ng end-labeled double-stranded oligonucleotide probe was incubated with 10 g nuclear extract and 2 μg poly dI/dC (Boehringer–Mannheim) for 10 min on ice and next for 30 min at room temperature in 20 μl buffer consisting of 15 mM HEPES, pH 7.8, 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 10% glycerol. Competition experiments were performed by adding 100 ng double-stranded cold oligonucleotides with the labeled probes in the binding reaction. For supershift assays, 10 μg nuclear protein was preincubated on ice with or without polyclonal antibodies for 30 min before addition of the labeled probe. Antibodies used were as follows: anti-PDX1 (a gift from B. Thorens, Institute of Pharmacology, Lausanne, Switzerland), anti-USF (Santa-Cruz Biotechnology), anti-E47 (Santa-Cruz Biotechnology), and anti-NeuroD (a gift from P. Huang, Baylor College of Medecine, Texas, USA). Samples were then separated on a 5% nondenaturating polyacrylamide gel. The gels were fixed in a solution of 10% acetic acid and 10% methanol, dried, and exposed to hyperfilm-MP (Amersham).

Statistics. Data are expressed as means ± SEM and compared by the Newman–Keuls test.

Results

The transcription of the mif gene is increased by high glucose concentration in INS-1 cells

To investigate whether the induction of the *mif* gene by glucose [8] is transcriptionally regulated, total RNA was isolated from insulin-secreting INS-1 cells incubated in 2 or 12 mM glucose in the presence or absence of the RNA synthesis inhibitor, actinomycin D. In the absence

Table 1
Oligonucleotide sequences

Name	Sequence (sense 5′–3′)
ME 1	GTTTCCATCTTAGGAAACAA
ME 2	GAGCCCATGTAATACTTCC
ME1/ME2	CCTCCGTTTCCATCTTAGGAAACAAAGAGCCCATGTAATACTTCC
mME1/mME2	CCTCCGTTgagcggccatGAAACAAAGAGCCCgcagcgcgaTTCC
ME1/mME2	CCTCCGTTTCCATCTTAG GAAACAAAGAGCCCgcagcgcgaTTCC
mME1/ME2	CCTCCGTTgagcggccaGAAACAAAGAGCCCATGTAATACTTCC
MLTF	TAGGTGTAGGCCACGTGACCGGGTGTTC
OCT1	TGTCGAATGCAAATCACCTAGAA

of actinomycin D, the mRNA level is increased by 6-fold when cells incubated from 2 to 12 mM glucose, whereas the glucose effect is blunted in the presence of actinomycin D (Figs. 1A and B). We then evaluated whether the MIF promoter activity was glucose-responsive. The promoter construct tested consisted of a −1033/+63 bp fragment of the mouse MIF promoter which was linked to the luciferase reporter gene (MIFluc) [33]. INS-1 cells were transfected with the MIF promoter construct together with the pRLSV40 renilla promoter as internal control. The transfected cells were grown in 2 or 12 mM glucose concentration. As shown in Fig. 2B, the MIFluc drove a high transcriptional activity in insulin-secreting cells compared to the promoterless pGL3 basic vector activity and this activity was increased fourfold when cells were changed from low to high glucose concentration. To verify whether this glucose-induced promoter activity was not due to a change in the osmolarity state, the MIF promoter construct was transfected into INS-1 cells cultured in 12 mM mannitol. No increase of the MIF promoter activity was observed in the presence of mannitol, indicating that the glucose-stimulated activity is not the result of an osmolarity change in the medium (data not shown). In an attempt to evaluate the minimal region responsible for the glucose-responsiveness, 5′ deleted fragments containing regions of the −1033/+63, −650/+63, −496/+63, −297/+63, −187/+63,

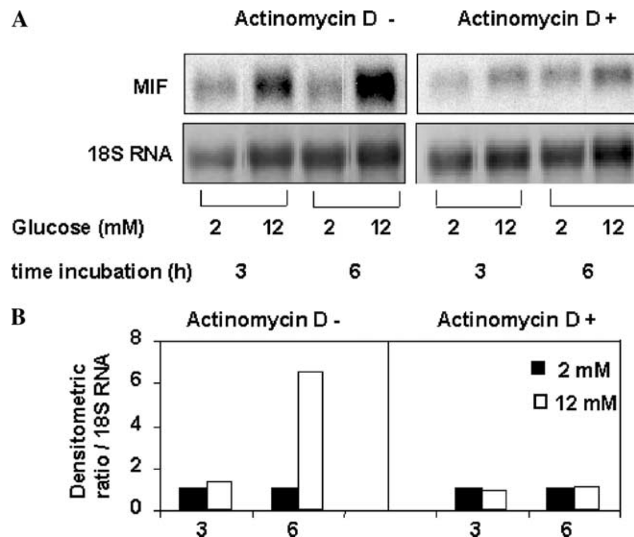

Fig. 1. Effect of actinomycin D on glucose-stimulated MIF expression in INS-1 cells. After a 24-h preincubation period at 2 mM glucose, insulin-secreting INS-1 cells were incubated at 2 or 12 mM glucose concentration for 3 or 6 h in the presence or absence of 5 μg/ml actinomycin D. (A) Representative Northern blot of MIF. Total RNA (15 μg) was hybridized with the mouse MIF cDNA probe as previously described [34]. (B) The MIF mRNA expression level was measured by densitometric scanning. Normalized to rRNA 18S, MIF mRNA level increased by 6-fold at 12 mM versus 2 mM glucose for 6 h. This glucose stimulation of MIF mRNA expression was totally inhibited upon when cells were treated with actinomycin D.

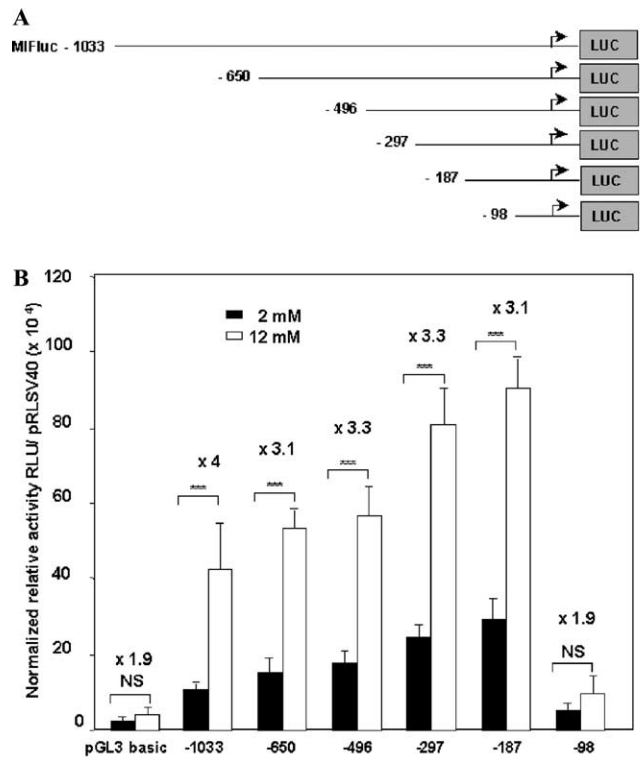

Fig. 2. Structure and glucose-stimulated expression of the 5′-flanking MIF promoter deletion mutants. (A) Fragments of the 5′-flanking region of MIFluc (a −1033 to +63 bp of the mouse MIF promoter linked upstream to a luciferase gene reporter) were generated by digestion with exonuclease III. Each construct is named according to the 5′ end point of deletion. (B) After a 24-h preincubation of 2 mM glucose, INS-1 cells were then transiently transfected with generated constructs and grown in either 2 or 12 mM glucose for 48 h. Luciferase activities of −1033, −650, −496, −297, and −187 constructs were glucose-induced by 4–3-fold whereas the −98 activity did not significantly increase at 12 mM glucose. Luciferase activities were normalized using pRLSV40 renilla and results are expressed as means ± SEM (triple asterisk, $P < 0.001$). Each experiment was repeated at least four times in triplicate. NS, not significative. RLU, relative light unit.

and −98/+63 bp of the MIF promoter (MIFluc) were then generated by exonuclease III deletions (Fig. 2A). These constructs were transiently transfected together with the pRLSV40 renilla into INS-1 cells which were incubated in 2 or 12 mM glucose concentration for 48 h. As shown in Fig. 2B, these constructs drove high basal transcriptional activity in 12 mM glucose with the exception of the −98/+63 bp construct. Furthermore, when cells were incubated from 2 to 12 mM glucose concentration, the luciferase activities of these constructs including −650, −497, −297, and −187 were increased by 3–4-fold, whereas the activity of the −98 construct was not significantly modulated by glucose. Thus, these data indicate that the MIF expression is transcriptionally regulated by the glucose and that the −187 and −98 bp sequence of the promoter may contain required *cis*-element(s) for both basal and glucose-stimulated regulation of the MIF promoter.

The −187/−98 bp region of the MIF promoter contains two distinct cis-binding sequences

We further investigated whether this glucose-regulatory region within the −187 to −98 bp of the MIF promoter could interact with nuclear protein extracts. DNAse I footprinting experiment was performed using protein nuclear extracts from INS-1 cells cultured in 12 mM glucose using the −187 to −98 bp sequence. Two protected regions were observed as shown in Fig. 3A. The sequence analysis identified two *cis*-sequences termed MIF elements (ME) ME1 (located between −164 and −155 bp) and ME2 (−140 to −132 bp) (Fig. 3B). ME1 and ME2 sequences were then analyzed by computer analysis for sequence homologies (http://bioinformatics.weizmann.ac.il/transfac/). The program failed to identify any sequence homologies with known consensus glucose-responsive element for ME1 whereas ME2 contains one motif related to A box sequence TAAT (Fig. 3B). ME1 and ME2 were then radiolabeled to be used as probes for EMSA (Table 1). Slow migrating complexes were formed using ME1 (Fig. 4A, **Ia**) or ME2 (Fig. 4B, **IIa** and **IIb**) incubated with protein nuclear extracts from INS-1 cells. Specific interactions were assessed by competition using excess of cold competitors that were either wild type or mutated in the ME1 or ME2 sequences as described in Table 1. The **Ia** binding pattern was fully competed with a 100-fold excess of cold competitors ME1, ME1/mM2, and ME1/ME2 but was not competed with ME2, mME1/ME2, mME1/mME2, and the unrelated oct-1 sequences (Fig. 4A). Conversely, **IIa** and **IIb** binding complexes were competed with ME2, mME1/ME2, and ME1/ME2 but not with ME1, ME1/mME2, mME1/mME2, and also oct-1 (Fig. 4B). These results show that ME1 and ME2 are able to bind specifically nuclear proteins present in insulin-secreting cells.

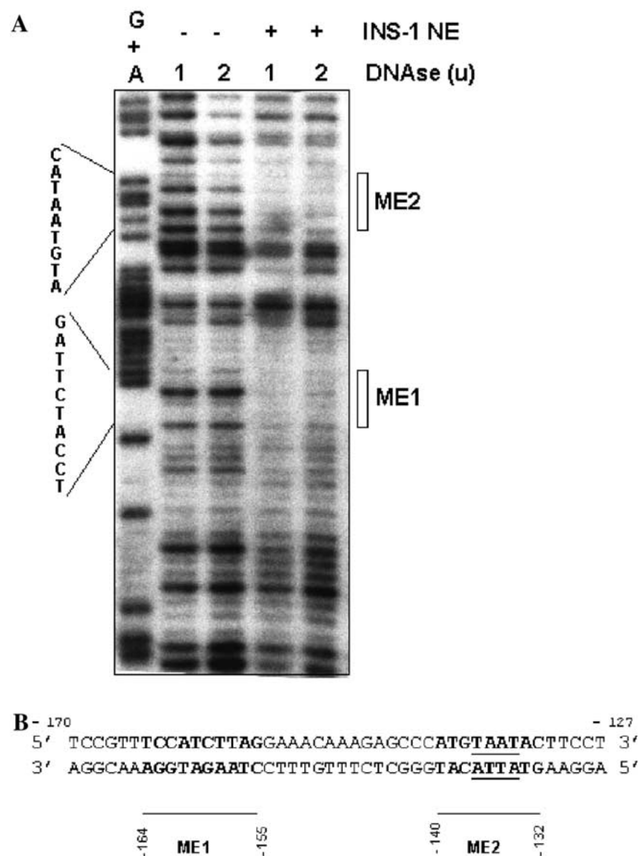

Fig. 3. In vitro DNAse I footprinting of the proximal MIF promoter region. (A) The 5′-flanking sequence −187 to −98 bp of the MIF promoter was used as the labeled probe. ME1 and ME2 alongside the lane indicate the corresponding protected regions. The line G + A is the sequencing Maxam and Gilbert ladder obtained with the respective probes. NE, Nuclear extracts, u, units. (B) Nucleic acid sequences of the mouse MIF proximal promoter containing protected regions ME1 and ME2. Protected ME1 and ME2 sequences from DNAse I cleavage are indicated in bold in the region −170 to −127 bp of the MIF promoter. A sequence homology to TAAT motif in ME2 is underlined.

We also investigated whether ME1 and ME2 binding activities are only detected in pancreatic-β cells. EMSA were performed using nuclear extracts obtained from several cell lines known to express high levels of MIF (adipocyte-differentiated 3T3-L1, macrophage-like RAW, lymphoma-Jurkat, and adeno-corticotrope AtT-20 cells). EMSA using the ubiquitous MLTF (adenovirus-5 major late transcription factor) radiolabeled oligoprobe were also performed to control the quality of the nuclear extracts (Fig. 5A). As a result, similar ME1 and ME2 binding activities were observed using nuclear extracts from rat INS-1 and the murine βTC3 cells (data not shown) whereas no binding complexes were detected with nuclear extracts from the unrelated cell lines, 3T3-L1, RAW, Jurkat, and AtT-20 cells (Figs. 5A and B). These results indicate that ME1 and ME2 binding activities are only detectable in insulin-secreting cells and may therefore contribute to the β-cell specific regulation of MIF. Using antibodies directed against known β-cell transcription factors involved in glucose-responsiveness, such as PDX1, E47, USF, and NeuroD/BETA2, we attempted to identify proteins contained in ME1 and ME2 complexes. EMSA showed that PDX1, E47, USF, and NeuroD antibodies failed to supershift the ME1 and ME2 binding complexes using nuclear extracts from INS-1 cells (data not shown). Taken together, these results suggest that unidentified transcription factors present in insulin-secreting cells bind to ME1 and ME2.

Discussion

In pancreatic β-cells, MIF potentiates insulin secretion in a positive autocrine fashion and its mRNA levels were shown to be regulated by glucose [8]. In this report, we establish that the glucose regulation of the *mif* gene occurs at transcriptional level and is recapitulated using

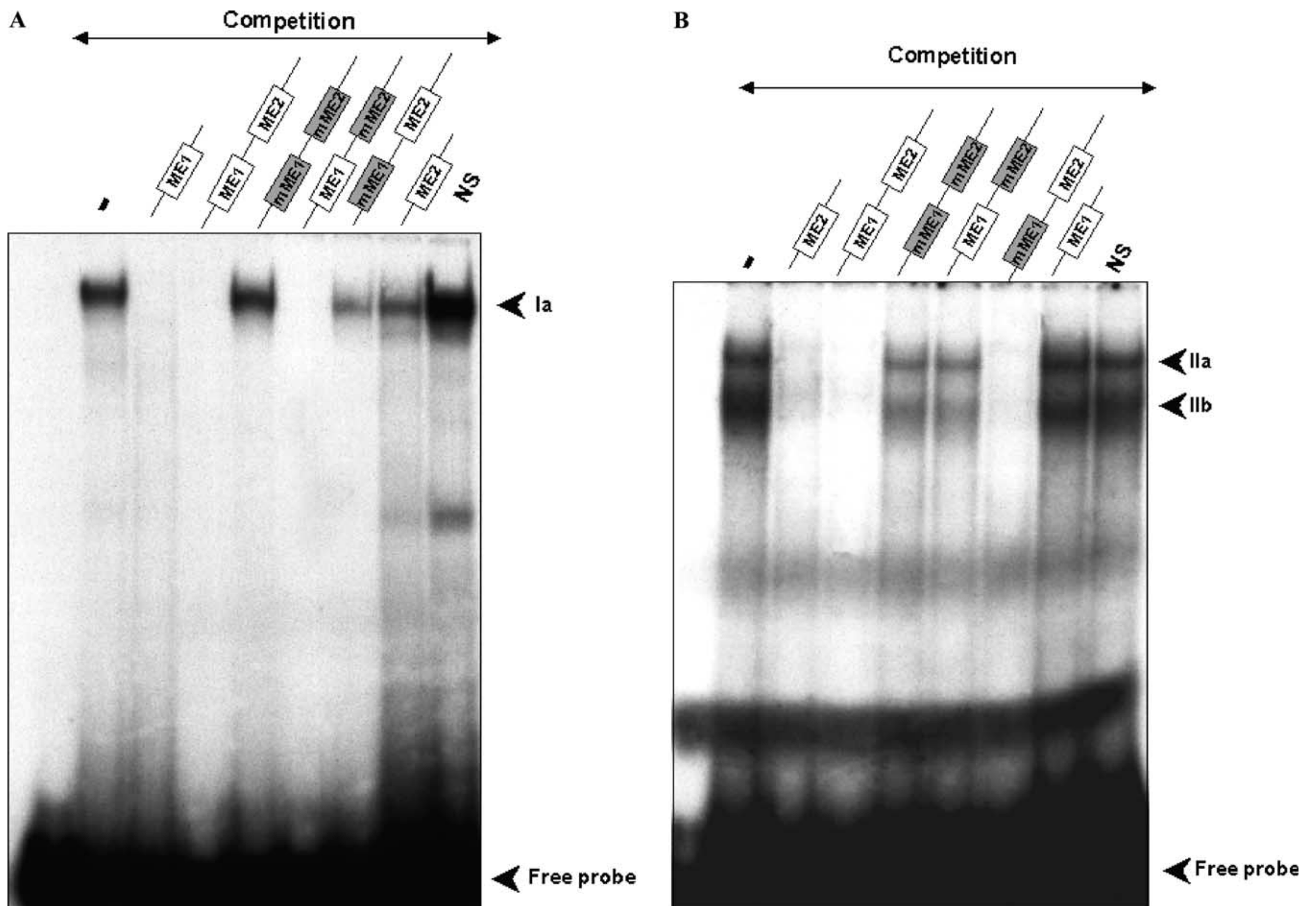

Fig. 4. Sequence-specific binding activity of ME1 and ME2. (A) EMSA with labeled ME1 probe using nuclear extracts from INS-1 cells cultured in 12 mM glucose. A slow migrated complex (arrow **Ia**) was detected compared to the free probe. This pattern was totally competed by using a 100-fold molar excess of cold wild type, ME1, ME1/ME2, and ME1/mME2, while no competition is observed using unlabeled mutated mME1, ME2, mME1/ME2, mME1/mME2, and a non-specific oct1 oligonucleotide (NS). The shaded boxes refer to mutated elements. (B) A binding pattern (arrows **IIa** and **IIb**) is detected using the labeled ME2 probe incubated with nuclear extracts from INS-1 cells cultured in 12 mM glucose. This pattern was fully competed with 100-fold excess molar of unlabeled ME2, ME1/ME2, and mME1/ME2 but not with unlabeled ME1, ME1/mME2, mME1/mME2, and the non-specific oct1 (NS). NE, nuclear extract.

a fragment of the murine promoter in transiently transfected INS-1 cells. This result is consistent with previous studies which formally demonstrated the direct transcriptional effect of glucose in β-cells for many genes such as those encoding insulin, glucokinase or the glucose transporter GLUT2 [37].

A detailed deletion study of the promoter region identified a crucial sequence (−187 to −98 bp), which is responsible for both the basal and glucose responsiveness of the promoter activity. Two *cis*-binding sequences termed ME1 and 2 (ME for MIF elements), were located within this minimal regulatory region. Since most of the glucose-induced promoter activities in insulin-secreting cells are mediated by transcription factors which are preferentially expressed in these cell types, we assessed whether ME1 and ME2 binding activities were detected only in β-cells. ME1 and ME2 binding patterns were detected only in rat and mouse pancreatic-β cell extracts and not in non-β-cells, known to highly express MIF. Thus, this result suggests that MIF is transcriptionally regulated by glucose through ME1 and ME2 in a β-cell-specific manner. In support of this hypothesis, a previous work has shown that *mif* gene expression was regulated by both glucose and insulin in differentiated adipocytes [38]. Since ME1 and ME2 binding activities were not detected in adipose cells, it can be hypothesized that the transcriptional regulation of MIF by insulin and glucose in these cells is mediated through different *cis*-elements.

Some well-known transcription factors such as PDX1, NEUROD/BETA2 or E47 [29–32] are responsible for the glucose-responsiveness of the preproinsulin promoter and are predominantly expressed in β-cells. We tested whether they could be involved in ME1 and ME2 binding activities. Supershift experiments using specific antibodies showed that ME1 and ME2 binding activities did not contain any of the tested factors. These results were consistent with the

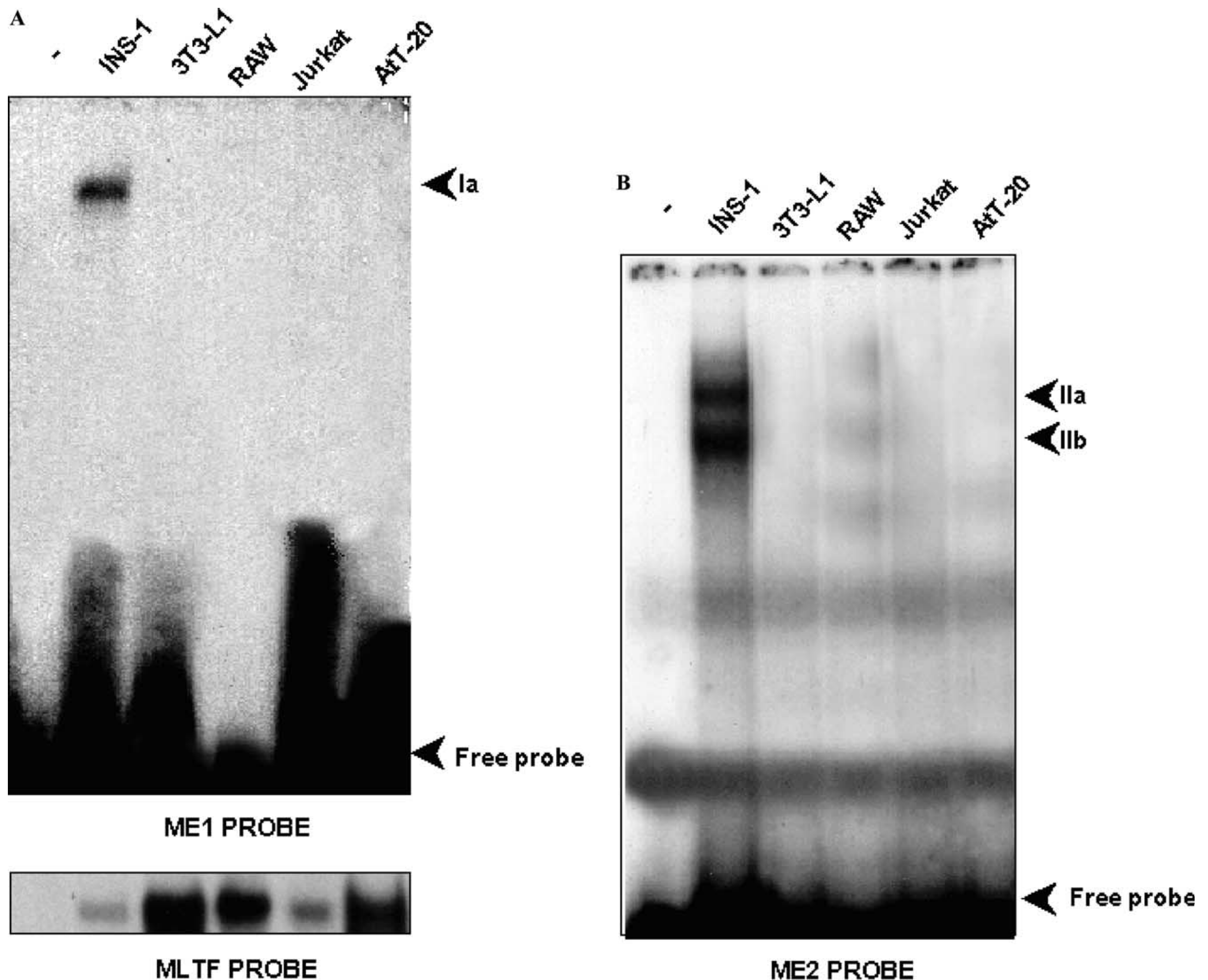

Fig. 5. Cell line distribution of ME1 and ME2 binding activities. EMSA with labeled ME1 (A) and ME2 (B) were performed using protein nuclear extracts from the rat insulin-secreting INS-1 cells, the mouse adipocyte-differentiated 3T3-L1, the mouse macrophage-like RAW, the human lymphoma-Jurkat, and mouse pituitary corticotrope AtT-20 cells which are all known to express MIF. The ME1 and ME2 binding patterns were only detected in INS-1 cells but not in unrelated cell lines. In (A) EMSA with the labeled ubiquitous MLTF probe were performed to control the quality of protein nuclear extraction from different cell lines.

sequence comparison analysis of ME1 and ME2 which failed to identify any homologies with known consensus *cis*-regulatory sequences with the exception of a TAAT sequence related to an A box in ME2, a consensus binding motif for homeodomain-transcription factors. Taken together, our data suggest the implication of a novel β-cell transcriptional factor complex that could be crucial for both the basal and glucose stimulation of the *mif* gene transcription. This hypothesis is consistent with the fact that glucose mediates its transcriptional effect through different transcriptional factor complexes such as those implicated in A, E, or Z element activities within the preproinsulin promoter. Moreover, glucose response elements can also vary between genes such as the glucose-response element of the L-pyruvate kinase which differ from elements of the preproinsulin promoter [37]. Thus, further analysis will be required to identify binding proteins implicated in the regulation of *mif* gene transcription through ME1 and ME2.

By modulating the glucose-induced secretion of insulin in β-cells, MIF expression level is crucial to maintain a normal glucose homeostasis. Thus, transcriptional regulators implicated in the glucose-stimulated expression of MIF become of importance to a better understanding in the pathogenesis of type 2 diabetes, a disease at least partially caused by a failure of the β-cell to secrete insulin in sufficient amount to maintain glucose homeostasis. Similarly, transcription factors, which regulate the preproinsulin promoter such as HNF-1, PDX-1, HNF-4α, and NEUROD/BETA2,

have been associated with monogenic forms of early onset-type 2 diabetes [39], characterized by a severe deficiency of insulin secretion.

In conclusion, the identification and characterization of transcriptional regulators modulating the glucose-stimulated MIF expression through ME1 and ME2 is therefore one of the steps required to elucidate the mechanism regulating the insulin secretion in pancreatic β-cells.

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