Critical Role of the HMGI(Y) Proteins in Adipocytic Cell Growth and Differentiation

ROSA MARINA MELILLO,[1] GIOVANNA MARIA PIERANTONI,[1] STEFANIA SCALA,[1] SABRINA BATTISTA,[1]
MONICA FEDELE,[1] ANTONELLA STELLA,[2] MARIA CRISTINA DE BIASIO,[3] GENNARO CHIAPPETTA,[3]
VINCENZO FIDANZA,[4] GIANLUIGI CONDORELLI,[4] MASSIMO SANTORO,[1]
CARLO M. CROCE,[4] GIUSEPPE VIGLIETTO,[3] AND ALFREDO FUSCO[2]*

*Centro di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche, Dipartimento di Biologiae
Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli,[1] and Istituto
Nazionale dei Tumori Fondazione Senatore Pascale,[3] 80131 Naples, and Dipartimento di Medicina Sperimentale e
Clinica, Facoltà di Medicina e Chirurgia di Catanzaro, Università degli Studi di Catanzaro, 88100 Catanzaro,[2]
Italy, and Kimmel Cancer Center, Jefferson Medical College, Philadelphia, Pennsylvania 19107[4]*

The high-mobility group I (HMGI) nonhistone chromosomal proteins HMGI(Y) and HMGI-C have been implicated in defining chromatin structure and in regulating the transcription of several genes. These proteins have been implicated in adipocyte homeostasis: a severe deficiency of fat tissue is found in mice with targeted disruption of the HMGI-C locus, and lipomagenesis in humans is frequently associated with somatic mutations of HMGI genes. The aim of this study was to examine the role of HMGI(Y) proteins in adipocytic cell growth and differentiation. First, we found that differentiation of the preadipocytic 3T3-L1 cell line caused early induction of HMGI(Y) gene expression. Suppression of HMGI(Y) expression by antisense technology dramatically increased the growth rate and impaired adipocytic differentiation in these cells. The process of adipogenic differentiation involves the interplay of several transcription factors, among which is the CCAAT/enhancer-binding protein (C/EBP) family of proteins. These factors are required for the transcriptional activation of adipocyte-specific genes. We also tested the hypothesis that HMGI(Y) might participate in transcriptional control of adipocyte-specific promoters. We found that HMGI(Y) proteins bind C/EBPβ in vivo and in vitro. Furthermore, we show that HMGI(Y) strongly potentiates the capacity of C/EBPβ to transactivate the leptin promoter, an adipose-specific promoter. Taken together, these results indicate that the HMGI(Y) proteins play a critical role in adipocytic cell growth and differentiation.

The mammalian high-mobility group I (HMGI) family of chromosomal proteins includes HMG-I and HMG-Y, which are coded for by the same gene, *HMGI(Y)*, through alternative splicing (23), and the closely related HMGI-C protein (27). The HMGI proteins are involved in the regulation of chromatin structure and function (25). While not typical transcriptional activators, HMGI(Y) proteins are required for the expression of many eukaryotic genes. These proteins bind adenine- and thymine-containing sequences located in the minor groove of DNA. Their DNA-binding domain is located in the N-terminal region of the protein and contains three short basic repeats, the so-called AT hooks. HMGI(Y) DNA-binding sites have been identified in many promoters, e.g., interleukin-4 (13), interleukin-2 receptor α-chain (22), lymphotoxin (15), and the human papovavirus JC (24) genes. These sites are often close to the DNA-binding sites of known transcription factors like NF-κB (38) and Tst-1/Oct-6 (24) and appear critical for viral induction of the human beta interferon gene (14, 38, 39). HMGI(Y) also interacts directly with several transcription factors. In fact, it binds to the basic leucine zipper region of the activating transcription factor 2, thus promoting its dimerization and binding to the beta interferon promoter (14).

HMGI-C gene knockout mice show a pygmy phenotype with a reduction of the adult body weight, mainly affecting fat tissue (49). The fat index, a reliable indicator of the total fat content relative to body weight, is approximately eight times lower in pygmy mice than in the wild-type littermates. Furthermore, the regulation of HMGI-C in vivo modulates obesity in a mouse model, and rearrangements of the HMGI-C and the HMGI(Y) genes have been found in human lipomas carrying chromosomal translocations involving the regions 12q13-14 and 6p21, respectively (2, 34, 40). Our group has recently demonstrated that the *HMGI-C* rearrangement plays a critical role in the generation of lipomas. In fact, transgenic mice carrying a truncated *HMGI-C* gene, which contains only the three AT hook domains, develop a giant phenotype and predominantly abdominal and pelvic lipomatosis (4). These observations, taken together, implicated HMGI(Y) proteins in adipogenesis. To elucidate further the mechanism of action of HMGI(Y) in adipogenesis, we used mouse 3T3-L1 fibroblasts as a model system. These cells differentiate into adipocytes upon treatment with specific agents (35). Adipocyte differentiation involves a group of transcription factors, CCAAT/enhancer-binding proteins (C/EBPs) (5, 29, 33, 42, 46), which are expressed at specific stages of adipogenesis. Hormonal stimulation causes C/EBPβ and C/EBPδ levels to increase and induce the expression of the transcription factor peroxisome proliferator-activated receptor gamma (44). This factor, in turn, leads to an increase of C/EBPα, which promotes the

---

* Corresponding author. Mailing address: Dipartimento di Biologia e Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, Via Pansini, 5, 80131 Naples, Italy. Phone: 39 081 7463056. Fax: 39 081 7463037 or 7701016. E-mail: afusco@napoli.com.

induction of several adipocyte-specific genes, including that for the fatty acid-binding protein 422/aP2 (11, 12) and the *obese* gene, which encodes leptin (21). Here we report the following: (i) *HMGI(Y)* gene expression increases during adipocytic conversion of 3T3-L1 cells; (ii) blockage of HMGI(Y) synthesis stimulates cell growth and impairs 3T3-L1 differentiation; (iii) HMGI(Y) physically interacts with C/EBPβ in vivo and in vitro; and (iv) HMGI(Y) proteins greatly enhance the C/EBPβ-mediated transactivation of the leptin promoter.

MATERIALS AND METHODS

Cell culture, transfections, and plasmids. The mouse NIH 3T3-L1 cells used in this study were generously donated by E. Santos (National Cancer Institute, National Institutes of Health, Bethesda, Md.). Cell cultures were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (GIBCO BRL, Life Technologies, Gaithersburg, Md.). Induction of adipocytic differentiation in 3T3-L1 cells was performed essentially as described elsewhere (35). Briefly, confluent 3T3-L1 cells were grown in DMEM supplemented with 10% calf serum until confluency was reached. Two days later, they were grown in DMEM supplemented with 10% fetal calf serum, 0.5 mM 1-methyl-3-isobutylxanthine, 10^{-6} M dexamethasone, and 10 μg of insulin/ml for 48 h. Cells were further cultured in the same culture medium devoid of dexamethasone and methylisobutylxanthine for 6 days. The 3T3-L1 fibroblasts were transfected by the calcium phosphate technique (18). Transfected cells were subjected to G418 selection (400 μg/ml). 293 cells were maintained in DMEM medium containing 10% fetal calf serum (GIBCO BRL, Life Technologies) and transiently transfected for in vivo binding assays or luciferase assays as described above. A 1,500-bp cDNA including the *HMGI(Y)* gene was subcloned into the *Hin*dIII site of the expression vector pRc/CMV (Invitrogen). A 489-bp cDNA fragment corresponding to the entire coding sequence of the *HMGI(Y)* gene was subcloned into the *Hin*dIII and *Xba*I sites of the expression vector pRc/CMV (Invitrogen) in the antisense orientation. Expression of the sense and antisense *HMGI(Y)* RNA was achieved by reverse transcription (RT) PCR: the forward primer for the sense vector, designed on the ATG sequence of the *HMGI(Y)* gene, was as follows: 5′-AGGAGAATGAGCGAGTCG-3′. The reverse primer, designed on the Sp6 sequence of the cytomegalovirus (CMV) vector, was as follows: 5′-AGTCGAGGCTGATCAGCGAG-3′. The forward primer for the antisense vector, designed on the stop codon sequence of the *HMGI(Y)* gene, was as follows: 5′-CTGCGAGTGGTGATCACT-3′. The reverse primer, designed on the Sp6 sequence of the CMV vector, was as follows: 5′-AGTCGAGGCTGATCAGCGAG-3′. The p(−161)ob-luc plasmid, containing 161 bp of the *obese* gene promoter driving a luciferase gene, and the m52 mutant, in which the C/EBP binding motif has been disrupted, are also described elsewhere (20).

Growth curves. For standard growth curves, cells were seeded at a density of 10^5 in 60-mm-diameter plates (Falcon) and grown in DMEM supplemented with 10% calf serum (GIBCO). Medium was renewed every 2 days, and cells were counted (see Fig. 4A). The doubling time was measured when the cells were in the logarithmic phase of growth: for 3T3-L1 and 3T3-L1-HMGI(Y)s cells, it occurred between days 3 and 4, and for 3T3-L1-HMGI(Y)as cells, it occurred between days 4 and 5 of culture. For growth curves of differentiating cells, cells were seeded and grown as described above until confluent. Two days after reaching confluence, cells were induced to differentiate according to the standard protocol (see above). Starting from 1 day after induction of differentiation, cells were counted (see Fig. 4B).

Northern blot analysis. Total RNA was extracted with RNAzol (Tel-Test, Inc., Friendswood, Tex.) according to standard procedures (32). Northern blotting and hybridizations were carried out as previously described (32). The *HMGI* probe was derived from pHMGI(Y) (23). A 0.4-kb *Eco*RI-*Hin*dIII fragment corresponding to the cDNA of the constitutively expressed enzyme human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to control equal RNA loading. Quantification of the hybridization signal was performed using a Molecular Dynamics PhosphorImager. The images recorded by the PhosphorImager were analyzed by volume integration with the ImageQuant software.

RT-PCR analysis of the expression of the adipocyte differentiation markers. Total RNA, digested with DNase, was reverse transcribed using random exonucleotides as primers (100 mM) and 12 U of avian myeloblastosis virus reverse transcriptase (GIBCO). Subsequent PCR amplification was as follows: 200 ng of cDNA was amplified in a 25-μl reaction mixture containing *Taq* DNA polymerase buffer, 0.2 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.4 mM concentrations of each primer, and 1 U of *Taq* DNA polymerase (Perkin-Elmer-

Cetus, Branchburg, N.J.). The PCR amplification was performed for 30 cycles (94°C for 30 s, 55°C for 2 min, and 72°C for 2 min). The primers used for aP2 gene expression were 5′-GATGTCAGCAGGAAGTCACC-3′ and 3′-CGAAGGAGGTTTAGCAAGAG-5′, corresponding to nucleotides 109 to 138 and nucleotides 427 to 408 (41). For the *obese* gene expression the sequences of the primers used were 5′-CCTGCTCCAGCAGCTGCAAG-3′ and 5′-GAGGAAAATGTGCTGGAGACCC-3′, which map on exon 1 and exon 2, respectively, and give rise to a specific 195-bp product (20). In addition, a set of primers specific for *GAPDH* was added to each reaction after 20 cycles of PCR to serve as an internal control for the amount of cDNA tested. The *GAPDH*-specific primers were the following: 5′-ACATGTTCCAATATGATTCC-3′ (forward), corresponding to nucleotides 194 to 214, and 5′-TGGACTCCACGACGTACTCAG-3′ (reverse), corresponding to nucleotides 336 to 356. The reaction products were analyzed on a 2% agarose gel and then transferred by blotting to GeneScreen Plus nylon membranes (Dupont, Boston, Mass.). The membranes were hybridized with an HMGI(Y) cDNA probe (7, 23). The relative levels of aP2 and ob expression were assessed by comparison with the level of GAPDH in the same sample.

Immunoblotting and immunoprecipitation. Protein extracts were prepared from terminally differentiated or undifferentiated fibroblasts as previously described (6). The following antibodies were used for immunoprecipitation and Western blotting: anti-C/EBPβ (C-19) rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif.) and anti-HA 12CA5 mouse monoclonal antibodies (Boehringer, Mannheim, Germany). The rabbit polyclonal antibodies directed against the HMGI(Y) proteins already have been described (7, 8). For Western blot experiments, equal amounts of protein lysates were loaded, as demonstrated by staining of the membranes with Ponceau Red. To confirm equal loading, the same Western blots were incubated with antibodies to γ-tubulin (Sigma-Aldrich Corporation, St. Louis, Mo.). For coimmunoprecipitation experiments, antigens and antibodies were incubated for 1 h before the addition of protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). After another 1 h, the beads were collected and washed five times with lysis buffer. The beads were then boiled in sodium dodecyl sulfate (SDS) loading buffer for immunoblotting analysis. The protein extracts separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to Immobilon-P transfer membranes (Millipore). Membranes were blocked with 5% nonfat milk proteins and incubated with antibodies at the appropriate dilutions. Bound antibodies were detected by the appropriate horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham).

In vitro and in vivo binding assays. For in vitro binding assays, the *HMGI(Y)* cDNA was expressed as a glutathione *S*-transferase (GST) fusion protein in bacteria as described previously (10). Briefly, a 900-bp *Eco*RI-*Bam*HI fragment generated by PCR and including the complete coding sequence was subcloned in pGEX2T. The GST-*HMGI(Y)* construct was used to transform *Escherichia coli* strain BL21. Bacterially expressed GST and GST-HMGI(Y) proteins were bound to glutathione-agarose (Sigma-Aldrich Corporation). The beads were washed, and the size and purity of the bound protein were evaluated by Coomassie staining of an SDS-polyacrylamide gel. Equal amounts of GST and GST-HMGI(Y) proteins (5 μg) were used for binding assays. C/EBPβ cDNA was obtained by PCR amplification and cloned in the pBluescript vector (Stratagene, La Jolla, Calif.). Transcription and translation reactions were performed with the T7-rabbit reticulocyte lysate kit (Promega, Madison, Wis.) as suggested by the manufacturer. The in vitro-translated C/EBPβ was allowed to associate with glutathione-agarose-bound GST or GST-HMGI(Y) for 2 h in lysis buffer (6) at 4°C. The pellets were washed four times in lysis buffer, and the proteins were dissociated by boiling in loading buffer and electrophoresed on a 10% polyacrylamide–SDS gel. The proteins were transferred to Immobilon-P, and C/EBPβ was visualized as described above. For in vivo binding assays, 293 cells were transfected as described by Graham and van der Eb (18). Cells were transfected with 5 μg of each plasmid, and carrier DNA was added to a total of 10 μg. Cells were harvested 36 h after transfection, and protein extracts were prepared as described above. Extracts were immunoprecipitated and immunoblotted with the indicated antibodies.

Transient transfection and luciferase activity assay. Transfections into 293 cells were performed by calcium phosphate precipitation (18). Cells were transfected with 5 μg of p(−161)ob-luc, m52, or RSV-luc reporter plasmids together with 1 μg of pHMGI(Y)s. The hemagglutinin (HA)-tagged *HMGI(Y)* wild-type and deletion mutants were generated by PCR, sequenced, and subcloned into the pCEFL vector. One microgram of pSV2CAT plasmid was cotransfected to demonstrate equal transfection efficiency in the cell lines tested, and chloramphenicol acetyltransferase activity was measured by thin-layer chromatography with 95% chloroform–5% methanol. Cells were harvested 24 h after transfection, and luciferase activity was measured with a luminometer (Lumat LB9507; Berthold).
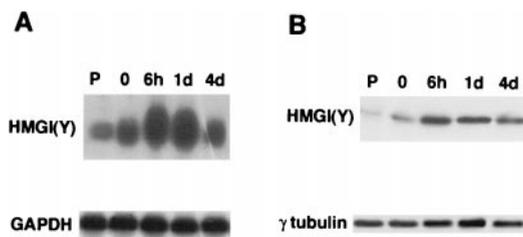
FIG. 1. (A) HMGI(Y) induction during NIH 3T3-L1 preadipocyte differentiation. Total RNA (20 μg/lane) extracted from proliferating and differentiated 3T3-L1 cells was hybridized with the *HMGI(Y)* cDNA and then with a rat *GAPDH* probe as a control for RNA loading. RNA was extracted from undifferentiated proliferating cells (P) at time zero and at 6 h, 1 day, and 4 days of differentiation, as indicated. (B) Nuclear proteins extracted from normal and induced 3T3-L1 cells were separated (20 μg/lane) by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blots were incubated first with antibodies specific for HMGI(Y) proteins and then with horseradish peroxidase-conjugated secondary antibodies; the immunocomplexes were detected by enhanced chemiluminescence. As a control for equal loading, the blotted proteins were stained with Ponceau Red. Moreover, the same Western blots were incubated with antibodies to the ubiquitous γ-tubulin protein. Proteins were extracted from the same cells as in panel A.

The relative activities were calculated by dividing the normalized activities by the activity of the m52 and the Rous sarcoma virus constructs, which were considered to be equal to 1. The data represent the average of results from three independent experiments, performed in duplicate, with standard deviations.

RESULTS

3T3-L1 adipocytic differentiation is associated with an increase in HMGI(Y) protein levels. We first investigated whether the expression of HMGI(Y) was regulated during adipocyte differentiation. As a model system, we used the 3T3-L1 preadipocytic cells, which have been extensively characterized. These cells undergo adipocytic conversion upon exposure to fetal bovine serum and differentiating agents (dexamethasone, methylisobutylxanthine, and insulin), as previously described (35). Cells were harvested in growing, undifferentiated conditions, at time zero (2 days postconfluence), and at different times during differentiation, and RNAs and proteins were prepared. Northern blot analysis showed that endogenous HMGI(Y) is expressed at low levels in growing cells, and it increases at time zero. It reaches its maximal level between 6 h and day 1 of treatment with differentiating agents and decreases again at day 4 (Fig. 1A), suggesting that the expression of HMGI(Y) is regulated, during differentiation, at the mRNA level. Western blot analysis showed a parallel increase of the *HMGI(Y)*-gene-specific protein product (Fig. 1B).

Inhibition of HMGI(Y) protein synthesis affects the differentiation and growth rate of 3T3-L1 cells. To investigate whether HMGI(Y) expression is a prerequisite for adipocytic differentiation, HMGI(Y) protein synthesis was suppressed by an antisense methodology. To this aim, 3T3-L1 cells were transfected with a plasmid carrying the *HMGI(Y)* gene in the antisense orientation (pCMV-HMGI-Yas) under the transcriptional control of the cytomegalovirus promoter (Fig. 2A). At the same time, to investigate the effect of the overexpression of HMGI(Y) in the 3T3-L1 cells, we generated the 3T3-L1-HMGI(Y)s cells, carrying the HMGI(Y) gene in the sense orientation [pCMV-HMGI(Y)s]. The empty vector

(pCMVneo) served as a control. Six 3T3-L1-HMGI(Y)as clones showing the lowest HMGI(Y) protein levels, four overexpressing HMGI(Y), and one mass population for each transfection were chosen for further analyses. Cells transfected with the empty vector (four clones and a mass population) were used as a control for our experiments. A strand-specific RT-PCR assay showed the expression of the sense and antisense HMGI(Y) mRNA in the transfected cells (Fig. 2B and B′). The HMGI(Y) protein levels were remarkably reduced in the 3T3-L1-HMGI(Y)as cells, whereas they were increased in the 3T3-L1-HMGI(Y)s cells (Fig. 2C), compared with the parental and the backbone-vector-transfected cells. As a positive control for HMGI(Y) expression, we used the PC MPSV cell line (rat thyroid cells transformed by the myeloproliferative sarcoma virus), which expresses high levels of the protein (9). The HMGI(Y)as cells failed to undergo adipocytic differentiation upon being given differentiating treatment. Adipocyte differentiation of 3T3-L1 cells in culture is similar to the in vivo process, i.e., enlarged cells filled with lipid droplets and expressing adipocyte-specific gene products appear. As shown in Fig. 3A, the typical fat droplets did not appear in the cytosol of antisense-expressing cells following differentiating treatment. We also analyzed the expression of two adipocyte-specific molecular markers, the adipocyte lipid-binding protein, aP2, and the product of the *obese* gene, leptin. To this aim, we used a semiquantitative RT-PCR assay with parental, HMGI(Y)as, and pCMVneo 3T3-L1 cells. Upon differentiating treatment, induction of the aP2 and leptin genes was suppressed in 3T3-L1-HMGI(Y)as cells but not in the parental and the pCMV-neo-transfected 3T3-L1 cells (Fig. 3B). When we analyzed the phenotype of the 3T3-L1-HMGI(Y)s cells, we did not observe any differences from the parental or the empty-vector-transfected 3T3-L1 cells (data not shown).

Since differentiation of 3T3-L1 cells requires arrest of growth in G_1, we investigated whether HMGI(Y) affected the 3T3-L1 growth rate. The 3T3-L1-HMGI(Y)as cells had a much shorter doubling time (15.4 h) and an increased growth rate compared with the parental cells (doubling time, 22.8 h) (Fig. 4A). Conversely, the 3T3-L1-HMGI(Y)s cells showed an opposite phenotype, with an increased doubling time (29.4 h) and a reduced growth rate (Fig. 4A). For 3T3-L1 cells undergoing adipocytic differentiation, there is a G_1/G_0 arrest at confluence, followed by a phase of clonal expansion initiated by the differentiating agents (35), and a subsequent arrest about 2 days later. The G_1/G_0 arrest was observed in parental and 3T3-L1-HMGI(Y)s cells but not in 3T3-L1-HMGI(Y)as clones (Fig. 4B). We also evaluated the effect of the HMGI(Y) protein on cell growth by performing a colony-forming assay. Exponentially growing 3T3-L1 cells were transfected with the HMGI(Y)as and HMGI(Y)s constructs and with the empty vector, selected with G418, and counted. Suppression of HMGI(Y) protein synthesis determined a remarkable increase in the number of colonies (387 colonies, compared to 46 with the empty vector), whereas its overexpression determined a reduction in the number of colonies (12 colonies). Therefore, these results indicate that HMGI(Y) exerts a negative effect on the growth rate of 3T3-L1 cells.

HMGI(Y) physically interacts with C/EBP proteins. Transcriptional regulation of adipocyte differentiation requires the concerted activity of several transcription factors that control

A

HMGI(Y) constructs

B   **B′**

C
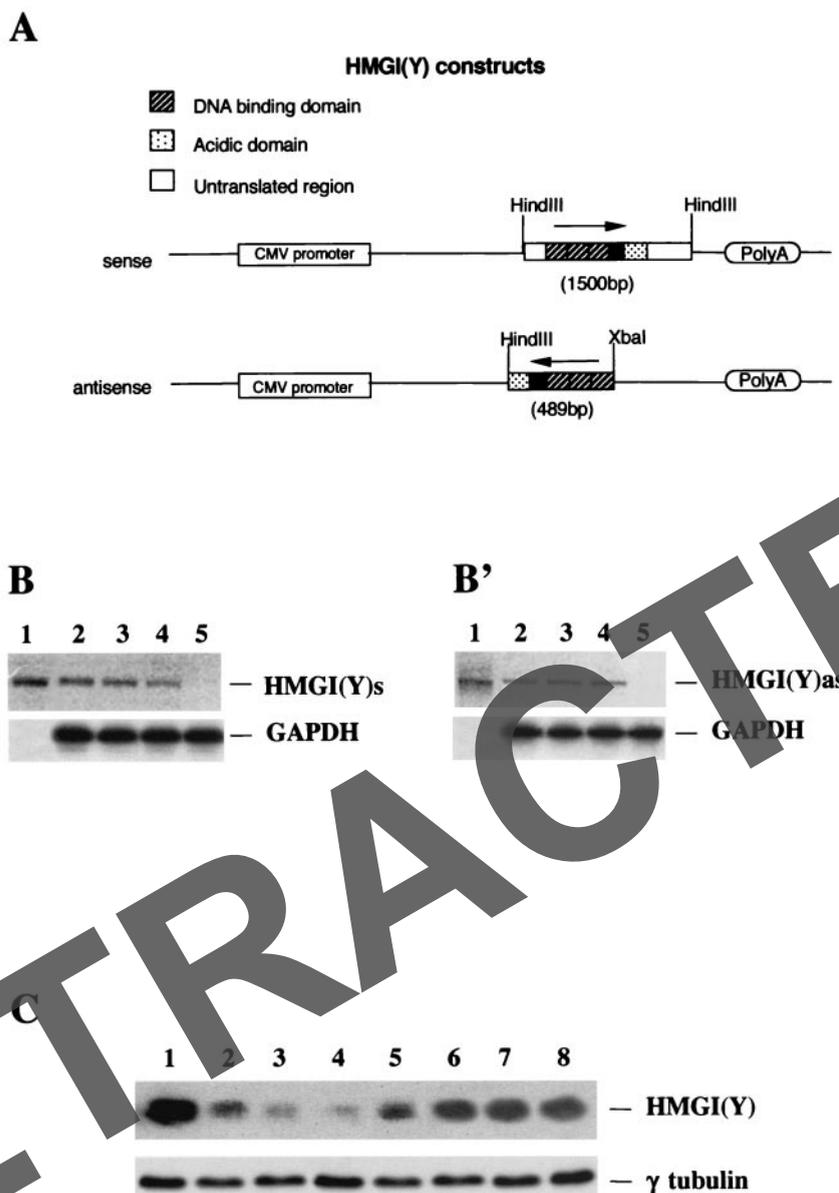
FIG. 2. (A) *HMGI(Y)* sense and antisense constructs were generated as described in Materials and Methods. The untranslated region, DNA-binding domain, and acidic domain of the HMGI(Y) protein are indicated. (B) RT-PCR analysis of pHMGI(Y)s expression in 3T3-L1-HMGI(Y)s cells. The sources of RNAs are the following: lane 1, PCR on the pCMV-HMGI(Y)s plasmid (positive control); lanes 2, 3, and 4, 3T3-L1-HMGI(Y)s (cell clones 1, 2, and 3); lane 5, 3T3-L1 pCMVneo clone 1. (B′) RT-PCR analysis of pHMGI(Y)as expression in 3T3-L1-HMGI (Y)as cells. The sources of RNAs are the following: lane 1, PCR on a pCMV-HMGI(Y)as plasmid (positive control); lanes 2, 3, and 4, 3T3-L1-HMGI(Y)as (cell clones 1, 2 and 3); lane 5, 3T3-L1 pCMVneo clone 1. All cDNAs were coamplified with GAPDH as an internal control. Bands of comparable intensity, obtained by the GAPDH sequence-specific primers, suggest comparable amplification of all samples. (C) 3T3-L1, 3T3-L1-HMGI(Y)as, 3T3-L1-HMGI(Y)s, and 3T3-L1 pCMVneo cell clones were treated with differentiating agents. Nuclear proteins were extracted and separated (20 μg/lane) by SDS–15% PAGE and transferred to polyvinylidene difluoride membranes. Western blots were incubated first with antibodies specific for the HMGI(Y) protein and then with horseradish peroxidase-conjugated secondary antibodies; the immunocomplexes were detected by enhanced chemiluminescence. As a control for equal loading, the blotted proteins were stained with Ponceau Red. The same Western blots were incubated with antibodies to the ubiquitous γ-tubulin protein. Sources of proteins were the following: lane 1, PC MPSV cells (positive control); lane 2, 3T3-L1 cells; lanes 3 and 4, 3T3-L1-HMGI(Y)as cells (clones 1 and 2); lane 5, 3T3-L1 pCMVneo clone 1; lanes 6, 7, and 8, 3T3-L1-HMGI(Y)s cells (clones 1, 2, and 3).

growth arrest and the coordinated expression of adipocyte-specific genes. Among these transcription factors, C/EBP proteins play a critical role in the development of the adipocyte differentiation program. Indeed, the levels of the C/EBP proteins increase during adipocyte differentiation. The increase of C/EBPβ and C/EBPδ occurs early during differentiation and is

followed by the increase of C/EBPα, which ultimately controls the expression of several genes, among which are the genes for aP2 and leptin (26). Our data indicated that HMGI(Y) proteins are also required for differentiation of 3T3-L1 preadipocytes. These observations suggested that HMGI(Y) might influence adipocytic differentiation through interactions with the
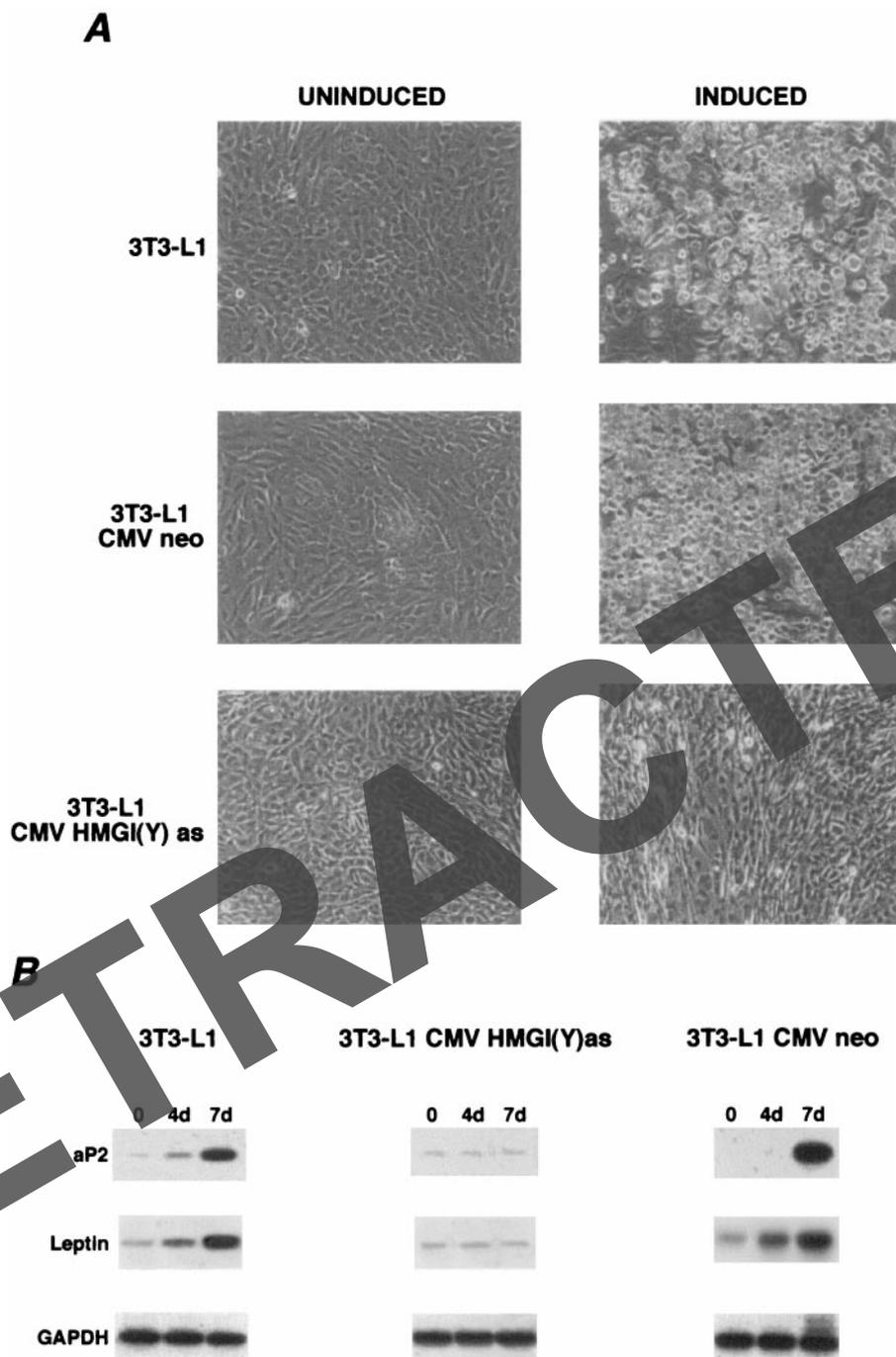
FIG. 3. (A) Inhibition of adipocytic differentiation induced by blockage of HMGI(Y) synthesis. Adipogenic differentiation of normal and pCMVneo- or pCMV-HMGI(Y)as-transfected 3T3-L1 cells is shown. Cell clones were cultured in the presence of standard differentiation induction medium containing 0.5 mM 1-methyl-3-isobutylxanthine, 1 mM dexamethasone, 5 μg of insulin/ml, and 10% fetal bovine serum. After 8 days of differentiation, cells were observed by light microscopy. Magnification, ×400. This experiment is representative of five independent assays. (B) mRNA levels of aP2 and leptin were determined by RT-PCR, gel electrophoresis, and Southern blot hybridization. For details, see Materials and Methods. The cDNAs were coamplified with *GAPDH*, as an internal control. No bands are seen in non-reverse-transcribed RNAs, thus excluding DNA contamination (data not shown). RNAs were extracted from these cells at days 0, 4, and 7 of differentiation, as indicated.

C/EBP transcription factors. To test this hypothesis, 3T3-L1 cells were synchronously differentiated into adipocytes by hormonal treatment. Cells were harvested at time zero and at various times during differentiation, and the interaction between C/EBPβ and HMGI(Y) was examined by coimmuno-

precipitation experiments. Protein extracts were immunoprecipitated with anti-C/EBPβ antisera and immunoblotted with anti-HMGI(Y) antibodies (Fig. 5A, upper panel). Interaction between C/EBPβ and HMGI(Y) was detected at time zero; it increased at 6 h and remained stable until day 6 of differenti-
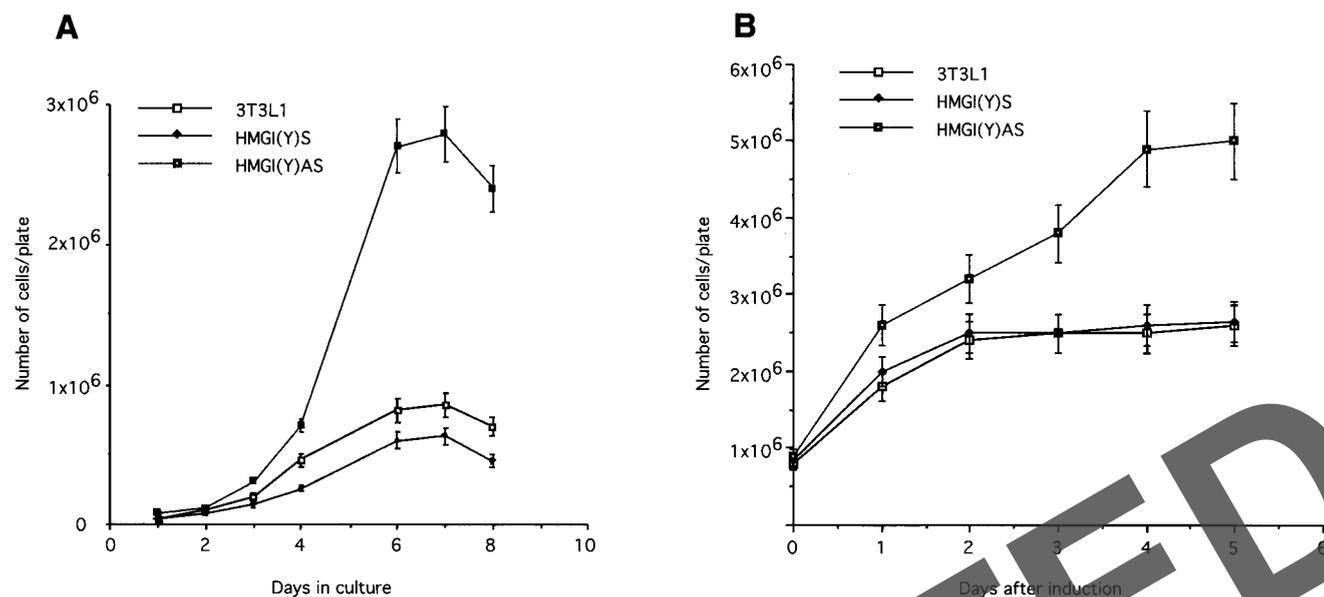
A

B

FIG. 4. (A) Cells were grown as described in Materials and Methods and counted daily. (B) Cells were grown until confluence and then induced to differentiate as described in Materials and Methods. Cell counts started 1 day after the addition of the differentiation cocktail, as indicated, and were performed daily. For each type of experiment, one representative clone is shown; the results were confirmed on two additional clones. The data reported are the average results of two independent experiments.

ation. This interaction was not detected when a preimmune serum was used for lysate extracts of 6 h (Fig. 5A). In agreement with previous observations, C/EBPβ levels increased during early differentiation of 3T3-L1 cells (Fig. 5A, lower panel). We also detected binding of HMGI(Y) and the other two C/EBP proteins (not shown); these interactions occurred with different kinetics.

To verify these interactions, we carried out in vitro and in vivo binding studies with C/EBPβ and HMGI(Y). C/EBPβ was synthesized in vitro by using rabbit reticulocyte lysates, and HMGI(Y) was produced as a GST fusion protein and bound to glutathione-agarose beads (GST-Y). A pull-down assay was performed by incubating the two proteins. GST-bound proteins were immunoblotted on Immobilon-P and detected with anti-C/EBPβ antibodies. As shown in Fig. 5B, GST-Y, but not GST, was able to coprecipitate with C/EBPβ. For the in vivo binding assays, the plasmids encoding C/EBPβ and HMGI(Y) were transiently transfected in 293 cells. The cDNA encoding C/EBPβ was expressed in the 293 cells alone and with HA-HMGI(Y). Cell extracts were immunoprecipitated with anti-C/EBPβ or with anti-HMGI(Y) antibodies and immunoblotted with the reciprocal antisera. Coexpression of C-EBPβ and HMGI(Y) resulted in reciprocal coimmunoprecipitation of the two proteins (Fig. 5C). Analogous results were obtained by using C/EBPα and C/EBPδ, which also bound to HMGI(Y) in vivo and in vitro (data not shown).

Mapping of the HMGI(Y) region responsible for binding to C/EBPβ. To map the HMGI(Y) region required for binding to C/EBPβ, we generated a series of progressive deletions of the HMGI(Y) gene in an area corresponding to the carboxy-terminal region of its product (Fig. 6A). The resulting cDNAs were tagged with the influenza virus HA epitope and cloned into the pCEFL expression vector. Immunoblotting analysis showed that approximately equal amounts of wild-type and

mutant proteins were produced. These mutants were tested for their interaction in vivo with C/EBPβ in coimmunoprecipitation experiments. Each *HMGI(Y)* plasmid was transfected in 293 cells together with a *C/EBP*β-expressing vector. Thirty-six hours after transfection, cells were harvested and protein extracts were immunoprecipitated with anti-C/EBPβ antibodies. As shown in Fig. 6B, deletion of the carboxy-terminal tail and of the third basic repeat did not impair the binding of the HMGI(Y) protein to C/EBPβ [compare wild-type HMGI(Y) with mutant 1-63]. Conversely, removal of the region between the middle and the last basic repeat (amino acids 54 to 63) and of the second repeat is detrimental to the interaction of HMGI (Y) with C/EBPβ [compare wild-type HMGI(Y) with mutants 1-53 and 1-43]. These results demonstrate that the carboxy-terminal tail and the third basic repeat are not essential for this interaction, whereas the region between the second and third repeats is required for the binding of HMGI(Y) and C/EBPβ.

HMGI(Y) cooperates with C/EBP in the regulation of the *obese* gene promoter, a C/EBP-regulated gene. The foregoing results suggested that C/EBPβ cooperates with HMGI(Y) in the activation and/or repression of adipocyte-specific gene promoters. We focused on the promoter of the leptin protein, encoded by the *obese* gene, since its expression during adipocytic induction seems to depend on HMGI(Y) synthesis. We first used the obese (ob) minimal promoter (−161), which contains C/EBP motifs and which is a natural target of C/EBP transcription factors (20, 28). A plasmid containing the ob minimal promoter fused to the luciferase reporter gene (−161 ob-luc) was transfected in 293 cells with or without C/EBPβ. As shown in Fig. 7A, C/EBPβ activated luciferase transcription. When C/EBPβ was cotransfected with HMGI(Y), activation of the ob promoter was significantly potentiated. Conversely, no activation was observed in the presence of HMGI (Y) alone or when the m52-ob-luc promoter, which is mutated
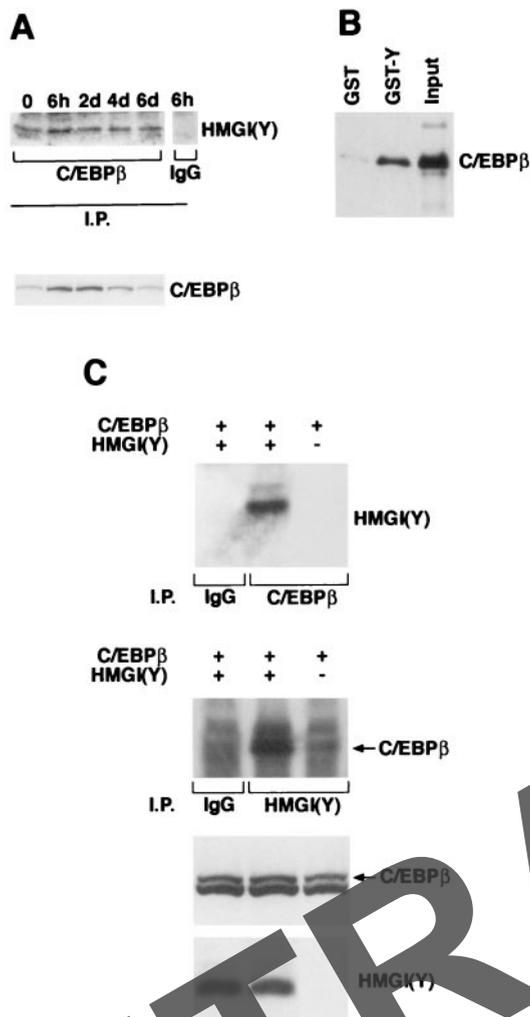
FIG. 5. (A) Interaction between C/EBPβ and HMGI(Y). Cell lysates were prepared from 3T3-L1 cells at the indicated times as described in Materials and Methods. Proteins were immunoprecipitated with antibodies directed against the protein C/EBPβ (Santa Cruz Biotechnology), as indicated. Immunoprecipitated (I.P.) proteins were immunoblotted with anti-HMGI(Y). Levels of C/EBPβ during differentiation are shown. IgG, immunoglobulin G. (B) Wild-type C/EBPβ protein was in vitro translated as described in Materials and Methods. Rabbit reticulocyte extracts were mixed with GST-HMG-Y recombinant protein for binding assays. Binding reaction products were washed, and proteins were separated on a polyacrylamide gel. Filters were probed with the anti-C/EBPβ antibody (Santa Cruz Biotechnology). (C) C/EBPβ and HMGI(Y) interaction in 293 cells. 293 cells were transfected with the indicated expression plasmids as described in Materials and Methods. Cell lysates were prepared, and equal amounts of proteins were immunoprecipitated with the indicated antibodies. In the top two panels, lane 1 shows a control immunoprecipitation. In lanes 2 and 3, the indicated cell lysates were immunoprecipitated either with the anti-C/EBPβ antibody (upper panel) or with the anti-HMGI(Y) antibody. The immunocomplexes were immunoblotted with the reciprocal antibodies, as indicated. In the bottom two panels, Western blot analysis shows the amounts of C/EBPβ and HMGI(Y) for each lysate used in the top two panels.

mediated transactivating activity, we used another reporter vector, RSV-luc. This vector contains a promoter which has a low basal activity in 293 cells and is insensitive to C/EBP. As shown in Fig. 7B, neither C/EBPβ or HMGI(Y) alone nor the combination of the two proteins was able to significantly stimulate this promoter. Moreover, we have demonstrated that HMGI(Y) is also able to cooperate with C/EBPα in the transactivation of the leptin promoter (data not shown).

We then asked whether physical interaction between HMGI(Y) and C/EBPβ was important for the potentiation of leptin transcription. We tested the HMGI(Y) deletion mutant 1-63, which is still able to bind C/EBPβ, and the mutants 1-53 and 1-43 (Fig. 7A), which are defective in binding C/EBPβ, for their ability to activate the ob-luc promoter in the presence of C/EBPβ. As shown in Fig. 7A, mutant 1-63 behaved like wild-type HMGI(Y) in the C/EBPβ-mediated transactivation assay. When mutant 1-53 was used, there was a significant (more than 50%) reduction of activity in the cooperativity assay. When we
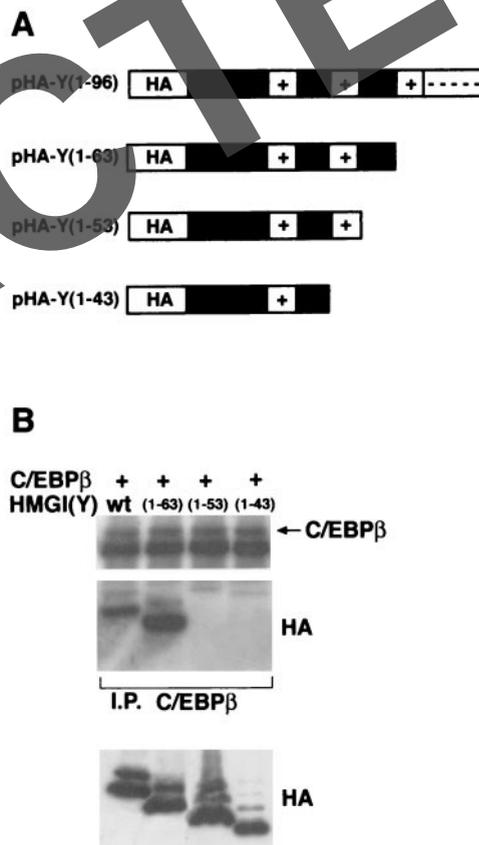

FIG. 6. The region between the second and the third AT hook is required for HMGI(Y)-C/EBPβ interaction. (A) Schematic diagram of plasmids expressing HA-tagged wild-type HMGI(Y), showing the 1-63, 1-53, and 1-43 deletion mutant proteins. The HA epitope tag, AT hooks (+), and C-terminal (----) domains are also indicated. (B) 293 cells were transiently cotransfected with C/EBPβ and the indicated HMGI(Y) mutant plasmids. Equal amounts of cell lysates (2 mg) were immunoprecipitated with anti-C/EBPβ antibodies, and the immunocomplexes were probed with either anti-C/EBPβ (upper panel) or anti-HA antibodies (lower panel). Aliquots of the same lysates (50 μg) were probed with anti-HA antibodies to evaluate the comparable expression of the transfected plasmids.
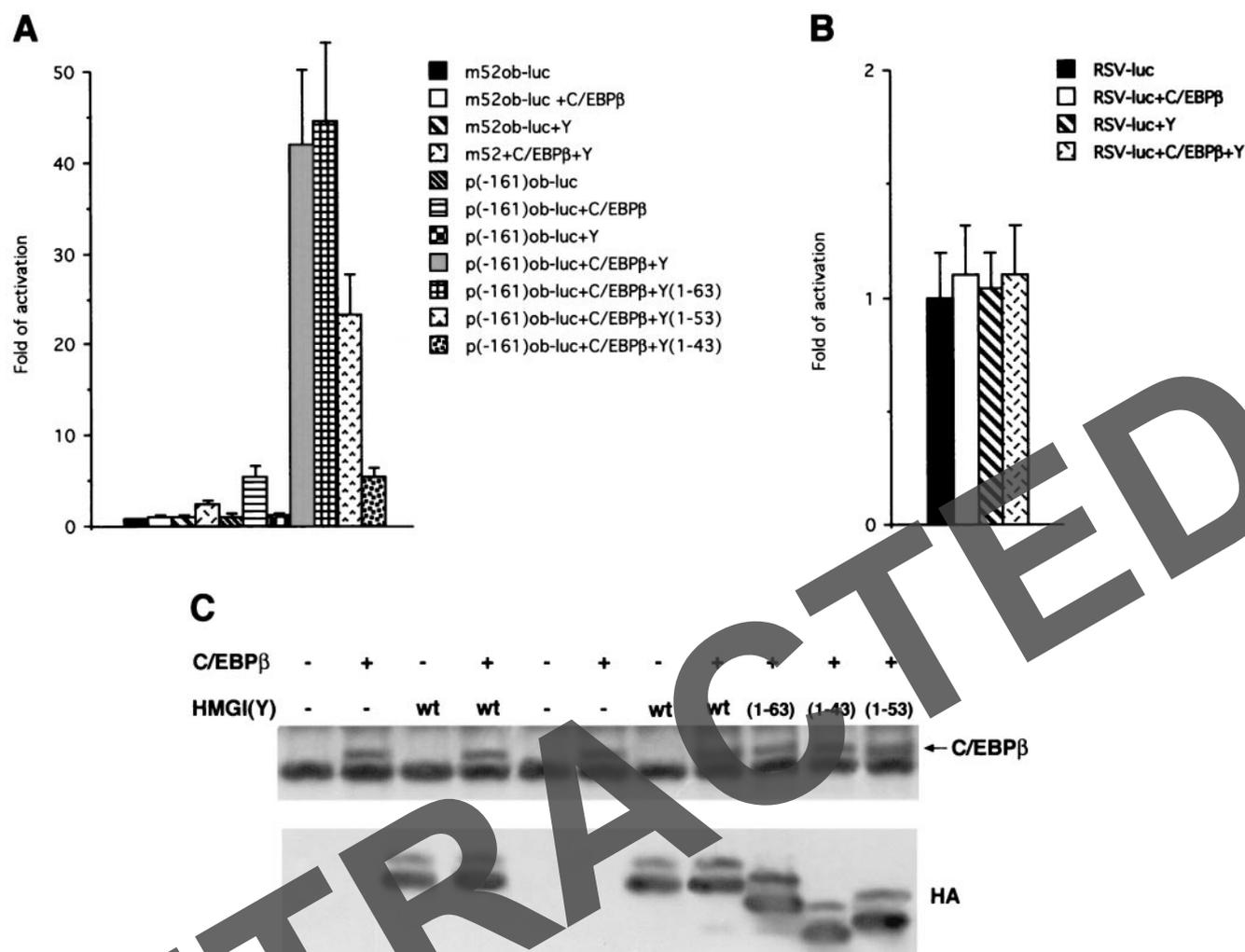
in the C/EBP-binding site, was used for the cooperativity assay. Analogous results were obtained when we used the ob-luc-762 long promoter (data not shown). As a further control for the specificity of the stimulatory effect of HMGI(Y) on C/EBP-

FIG. 7. (A) Leptin transactivation by C/EBPβ and cooperation with HMGI(Y). Histograms show the luciferase activities of extracts from 293 cells cotransfected with the p(−161)ob-luc reporter and the indicated *C/EBP*β and *HMGI(Y)* plasmids. The mutant m52 reporter plasmid was used as a negative control. (B) Histograms showing the luciferase activities of extracts from 293 cells cotransfected with the RSV-luc reporter and the *C/EBP*β and *HMGI(Y)* plasmids. The relative activities were calculated by dividing the normalized activities by the activity of the m52 and RSV-luc constructs, which has been considered equal to 1. The data represent the average of results of three independent experiments, performed in duplicate, with standard deviations. (C) After transfection, cell lysates were divided into two aliquots. One of these aliquots was used for transactivation assays, and the other was used for Western blot analysis as a control of protein expression. Protein extracts were separated by SDS-PAGE, transferred to Immobilon-P, and immunoblotted with the indicated antibodies.

used mutant 1-43, the effect was more dramatic, i.e., a sixfold loss of activity. These results suggest that deletion of residues 54 to 63, which are important for interaction between HMGI(Y) and C/EBPβ, partially impairs their functional cooperation. The more dramatic phenotype observed with the 1-43 mutant, which also lacks the second basic repeat of HMGI(Y), suggests that the HMGI(Y) 43-to-53 region mediates the activation of the leptin promoter independently from its ability to bind C/EBP. Western blot analysis showed that the transfected cells expressed adequate levels of the C/EBPβ and HMGI(Y) proteins (Fig. 7C).

DISCUSSION

Adipocytic differentiation requires HMGI(Y). HMGI(Y) and HMGI-C proteins are important architectural transcription factors (19), and a growing body of evidence suggests that

they are involved in adipocytic differentiation (2, 34, 40, 49). We have investigated the role of HMGI(Y) proteins in adipogenesis using 3T3-L1 preadipocytic cells as a model system. Northern and Western blot analyses demonstrated induction of the *HMGI(Y)* gene and protein when 3T3-L1 cells were induced to differentiate into adipocytes. HMGI(Y) expression is detectable at very low levels in growing, undifferentiated 3T3-L1 cells and increases during differentiation. These observations suggested that an increase in HMGI(Y) levels is necessary for 3T3-L1 differentiation. Indeed, suppression of HMGI(Y) protein synthesis through antisense methodology prevented terminal adipocytic differentiation. Not only did these cells not show the typical fat-laden phenotype, but they also lacked the expression of two adipocytic markers, aP2 and leptin. On the other hand, forced expression of the *HMGI(Y)* gene resulted in inhibition of growth, but it was not able to

induce differentiation. These data demonstrated that the wild-type HMGI(Y) protein is required, but not sufficient, for 3T3-L1 cells to differentiate into adipocytes. Three members of the C/EBP family of transcription factors (C/EBPα, -β, and -δ) have been implicated in the induction of adipocyte differentiation. In particular, overexpression of C/EBPα is sufficient to arrest growth and to start the adipocyte differentiation program in preadipocytic cell lines (26). Recent data obtained in our laboratory indicate that its forced expression in the 3T3-L1 HMGI(Y)as cells is not able to revert their phenotype, i.e., the block of growth arrest and adipocytic differentiation (data not shown). These data indicate that HMGI(Y) is indeed necessary for C/EBPα to induce its biological effects. This hypothesis is also supported by results of other experiments. The promoters of several adipocyte-specific genes contain C/EBP regulatory binding sites. For instance, C/EBPα was shown to bind and transactivate the aP2 promoter. Furthermore, the leptin promoter contains at least one functional C/EBP binding site: disruption of this consensus sequence by site-directed mutagenesis causes a remarkable decrease in promoter activity (20, 28). Based on these observations, we argued that HMGI(Y) modulates the transcriptional activity of the C/EBPs. This hypothesis was confirmed by the finding that HMGI(Y) physically interacts with C/EBP transcription factors.

HMGI(Y) proteins suppress 3T3-L1 cell proliferation. In 3T3-L1 cells undergoing adipocyte differentiation, there is a G_1/G_0 arrest at confluence, followed by a phase of clonal expansion initiated by the differentiating agents (26) and a subsequent arrest about 2 days later. We show that suppression of HMGI(Y) expression causes a blockage in the differentiation associated with an increased growth rate in 3T3-L1 cells. Furthermore, treatment of HMGI(Y) antisense-expressing cells with differentiating agents failed to induce the cell cycle arrest that precedes differentiation. Therefore, we suggest that HMGI(Y) plays a critical role in adipocytic cell growth. The levels of HMGI(Y) do not correlate with the cell cycle status of the cells during induction of differentiation, being highest in the phase of mitotic clonal expansion (6 h and day 1) and reduced in growth-arrested, differentiating cells (days 0 and 4). This paradox could be explained by the fact that HMGI(Y) is an accessory protein, with multiple functions, for a wide range of transcription factors. Its effect could enhance growth arrest or proliferation depending on the presence of different transcription factors.

Our data indicate that the role of HMGI(Y) in the control of adipocytic cell growth counteracts that of HMGI-C. In fact, while overexpression of HMGI(Y) negatively regulates adipocytic cell growth, HMGI-C expression seems to be necessary for physiological proliferation of adipocytes. Indeed, HMGI-C knockout mice display a pygmy phenotype with a remarkable reduction of the adipose tissue (49), and the suppression of the HMGI-C synthesis blocks proliferation of the 3T3-L1 cells (S. Battista et al., unpublished data). We suggest that the growth of adipocytic cells results from the balance between levels of HMGI-C and HMGI(Y). From the data presented here, the role of HMGI(Y) seems to be pleiotropic, depending on the cellular context. HMGI(Y) proteins often have been associated with cell proliferation: in fact, HMGI(Y) has been found overexpressed in several experimental and human malignant tumors (1, 3, 8, 9, 16, 17, 36), and overexpression of HMGI(Y)

causes transformation of Burkitt's lymphoma cells (43). Consistent with the hypothesis that the different cellular context may account for the different effects of overexpression of the *HMGI(Y)* gene, we have also recently demonstrated that overexpression of HMGI(Y) impairs the growth of normal PC Cl 3 rat thyroid cells by inducing apoptosis (M. Fedele et al., unpublished data). In our opinion, this hypothesis seems to be likely, since several genes involved in the control of cell proliferation can induce different biological effects, such as cell growth, differentiation, and apoptosis, depending on the cellular context.

The involvement of HMGI(Y) and HMGI-C in adipocytic cell growth has interesting implications for the pathogenesis of some human tumors. Indeed, the *HMGI-C* and *HMGI(Y)* genes are involved in chromosome translocations occurring in benign mesenchymal tumors, including lipomas (2, 12, 34, 40, 45). Consequent to the translocation, HMGI proteins fuse to heterologous genes, or they simply lose the carboxy-terminal domain and retain only the DNA-binding domain. Consistently, the expression of a truncated form of the protein (containing only DNA-binding domains) results in enhanced proliferation of 3T3-L1 cells (G. M. Pierantoni et al., unpublished data).

HMGI(Y) proteins physically interact with C/EBPβ and modulate C/EBP-mediated transcription. C/EBPs play a pivotal role in adipogenesis (6, 37). Here we demonstrate that HMGI(Y) binds to C/EBPβ in 3T3-L1 cells and in 293 cells by reciprocal coimmunoprecipitation. We also show that a GST-HMGI(Y) fusion protein binds C/EBP in a typical pull-down assay. Furthermore, we demonstrate that this binding requires the region between amino acids 53 and 63 of HMGI(Y). By binding to C/EBPs, HMGI(Y) may functionally cooperate with these proteins to activate and/or repress different promoters. Preliminary results obtained by using band shift analysis with a C/EBP-specific oligonucleotide show that high-molecular-weight complexes are formed at different times during 3T3-L1 differentiation (G. M. Pierantoni and R. M. Melillo, personal communication): HMGI(Y) may be involved in the assembly of higher-order complexes that are essential for both arrest of growth and the expression of the differentiated phenotype of adipocyte precursors. Consistent with the idea that HMGI(Y) serves as a general cofactor of adipocyte-specific transcription, we observed that HMGI(Y) expression modulates the transcription of a gene whose levels are regulated during adipocyte differentiation: the *obese* gene coding for leptin (48). Both C/EBPα and C/EBPβ bind to the C/EBP consensus site on the leptin promoter and are able to activate transcription of the *obese* gene (28). Here we show that HMGI(Y) functions as a specific cofactor for C/EBPβ. Indeed, we demonstrate that C/EBPβ-mediated activation of the leptin promoter was strongly potentiated by the presence of the HMGI(Y) protein. The same results were obtained with the C/EBPα transcription factor, suggesting that HMGI(Y) is an accessory factor for this family of proteins. This is consistent with the observation that HMGI(Y) is also able to bind to C/EBPα and -δ (data not shown). Analysis of the *obese* minimal promoter sequence failed to identify putative HMGI(Y)-DNA binding sites, which are represented by tracts of adenines and thymines arranged on the same face of the DNA helix. Furthermore, electrophoretic mobility shift analysis performed

in vitro with purified recombinant HMGI(Y) and the ob minimal promoter showed no high-affinity HMGI(Y) DNA-binding sites (Pierantoni and Melillo, personal communication). These observations, together with the ability of HMGI(Y) to bind C/EBP in solution in the absence of DNA, argue against the presence of HMGI(Y) in C/EBP-DNA complexes: it is possible that HMGI(Y) facilitates the binding of C/EBP to DNA by transiently associating with C/EBP.

We also mapped the domain of HMGI(Y) that is required for its functional cooperation with C/EBPβ. Deletion of the region between the third and the second AT hook impaired the ability of HMGI(Y) to cooperate with C/EBPβ. Interestingly, this region is also required for efficient HMGI(Y) and C/EBPβ binding, confirming that physical interaction between the two factors contributes to efficient functional cooperation. We show that a further deletion, which abrogates the second AT hook, completely abolishes the cooperation of HMGI(Y) and C/EBPβ. Consequently, the second AT hook also plays a role in the activation of the ob promoter. The cooperation between HMGI(Y) and C/EBP in the transactivation of the leptin promoter could be explained by several mechanisms, which are not mutually exclusive. One possibility is that the binding of HMGI(Y) to C/EBP could enhance the affinity of C/EBP for its target DNA. Such a mechanism has been demonstrated for other transcription factors, such as NF-κB (47). Alternatively, HMGI(Y) could recruit one or more components of the basal transcriptional machinery to the protein-DNA complexes, thus enhancing transcription. Whatever the mechanism, protein-protein interaction between HMGI(Y) and C/EBPβ might favor the activity of the C/EBP transcriptional complex. However, our data seem also to indicate that there is only a partial contribution of this interaction to the cooperation between HMGI(Y) and C/EBPβ in transactivating the leptin promoter. This suggests that other functions, dependent on HMGI(Y) residues 43 to 53, are important for this cooperation.

Furthermore, preliminary data obtained in our laboratory show that HMGI(Y) and C/EBPβ negatively regulate the promoter of the *Id1* gene, whose expression is down-regulated during adipogenesis and correlates with growth arrest that precedes differentiation (30, 31).

Conclusions. The data presented here show that HMGI(Y) exerts a negative effect on the proliferation of adipocyte precursors and a positive effect on differentiation. This dual role is consistent with the finding that HMGI proteins may positively and negatively affect gene expression. We also demonstrate that the HMGI(Y) protein physically interacts with C/EBP proteins and that it functionally cooperates in the transcriptional activity mediated by these proteins, whose function is required to trigger the expression of adipocyte-specific genes.

ACKNOWLEDGMENTS

REFERENCES

1. **Abe, N., T. Watanabe, M. Sugiyama, G. Chiappetta, A. Fusco, and Y. Atomi.** 1999. Analysis of high mobility group I(Y) protein expression in colorectal tumors. Cancer Res. **59:**1169–1174.
2. **Ashar, H. R., M. Schoenberg Fejzo, A. Tkachenko, X. Zhou, J. A. Fletcher, S. Weremowicz, C. C. Morton, and K. Chada.** 1995. Disruption of the architectural factor HMGI-C: DNA-binding AT hook motifs fused in lipomas to distinct transcriptional regulatory domains. Cell **82:**57–65.
3. **Bandiera, A., D. Bonifacio, G. Manfioletti, F. Mantovani, A. Rustighi, F. Zanconati, A. Fusco, L. D. Bonito, and V. Giancotti.** 1998. Expression of HMGI(Y) proteins in squamous intraepithelial and invasive lesions of the uterine cervix. Cancer Res. **58:**426–431.
4. **Battista, S., V. Fidanza, M. Fedele, A. J. Klein-Szanto, E. Outwater, H. Brunner, M. Santoro, C. M. Croce, and A. Fusco.** 1999. The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis. Cancer Res. **59:**4793–4797.
5. **Cao, Z., R. M. Umek, and S. L. McKnight.** 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3–L1 cells. Genes Dev. **5:**1538–1552.
6. **Chen, P.-L., D. J. Riley, Y. Chen, and W.-H. Lee.** 1996. Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev. **10:**2794–2804.
7. **Chiappetta, G., V. Avantaggiato, R. Visconti, M. Fedele, S. Battista, F. Trapasso, B. M. Merciai, V. Fidanza, V. Giancotti, M. Santoro, A. Simeone, and A. Fusco.** 1996. High level expression of the HMGI (Y) gene during embryonic development. Oncogene **13:**2439–2446.
8. **Chiappetta, G., A. Bandiera, M. T. Berlingieri, R. Visconti, G. Manfioletti, S. Battista, F. J. Martines-Tello, M. Santoro, V. Giancotti, and A. Fusco.** 1995. The expression of the high mobility group HMGI(Y) proteins correlates with malignant phenotype of human thyroid neoplasias. Oncogene **10:**1307–1314.
9. **Chiappetta, G., G. Tallini, M. C. De Biasio, G. Manfioletti, F. J. Martines-Tello, F. Pentimalli, F. De Nigris, A. Mastro, G. Botti, M. Fedele, N. Bergen, M. Santoro, V. Giancotti, and A. Fusco.** 1998. Detection of high mobility group I HMGI(Y) proteins in the diagnosis of thyroid tumors: HMGI(Y) expression represents a potential diagnostic indicator of carcinoma. Cancer Res. **58:**4193–4198.
10. **Chiariello, M., R. Visconti, F. Carlomagno, R. M. Melillo, C. Bucci, V. de Franciscis, G. M. Fox, S. Jing, O. A. Coso, J. S. Gutkind, A. Fusco, and M. Santoro.** 1998. Signalling of the Ret receptor tyrosine kinase through the c-Jun NH$_2$-terminal protein kinases (JNKs): evidence for a divergence of the ERKs and JNKs pathways induced by Ret. Oncogene **16:**2435–2445.
11. **Christy, R. J., K. H. Kaestner, D. E. Geiman, and M. D. Lane.** 1991. CCAAT/enhancer-binding protein gene promoter: binding of nuclear factors during differentiation of 3T3–L1 preadipocytes. Proc. Natl. Acad. Sci. USA **88:**2593–2597.
12. **Christy, R. J., V. W. Yang, J. M. Ntambi, D. E. Getman, W. H. Landschulz, A. D. Friedman, Y. Nakabeppu, T. T. Kelly, and M. D. Lane.** 1989. Differentiation-induced gene expression in 3T3–L1 preadipocytes: CCAAT/enhancer-binding protein interacts with and activates the promoters of two adipocyte specific genes. Genes Dev. **3:**1323–1335.
13. **Chuvpilo, S., C. Schoenberg, R. Gerwig, A. Heinfling, R. Reeves, F. Grummt, and E. Serfling.** 1993. Multiple closely-linked NFAT/octamer and HMGI(Y) binding sites are part of the interleukin-4 promoter. Nucleic Acids Res. **21:**5694–5704.
14. **Du, W., and T. Maniatis.** 1994. The high mobility group protein HMGI(Y) can stimulate or inhibit DNA binding of distinct transcription factor ATF-2 isoforms. Proc. Natl. Acad. Sci. USA **91:**11318–11322.
15. **Fashena, S. J., R. Reeves, and N. H. Ruddle.** 1992. A poly(dA-dT) upstream activating sequence binds high-mobility group I protein and contributes to lymphotoxin (tumor necrosis factor-β) gene regulation. Mol. Cell. Biol **12:**894–903.
16. **Fedele, M., A. Bandiera, G. Chiappetta, S. Battista, G. Viglietto, G. Manfioletti, A. Casamassimi, M. Santoro, V. Giancotti, and A. Fusco.** 1996. Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins. Cancer Res. **56:**1896–1901.
17. **Giancotti, V., E. Buratti, L. Perissin, S. Zorzet, A. Balmain, G. Portella, A. Fusco, and G. H. Goodwin.** 1989. Analysis of the HMGI nuclear proteins in mouse neoplastic cells induced by different procedures. Exp. Cell Res. **184:**538–545.
18. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of the infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
19. **Grosschedl, R., K. Giese, and J. Pagel.** 1994. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. Trends Genet. **10:**94–100.
20. **He, Y., H. Chen, M. J. Quon, and M. Reitman.** 1995. The mouse *obese* gene. Genetic organization, promoter activity, and activation by CCAAT/enhancer-binding protein alpha. J. Biol. Chem. **270:**28887–28891.
21. **Hwang, C. S., S. Mandrup, O. A. MacDougald, D. E. Geiman, and M. D. Lane.** 1996. Transcriptional activation of the mouse obese (ob) gene by CCAAT/enhancer binding protein alpha. Proc. Natl. Acad. Sci. USA **93:**873–877.
22. **John, S., R. B. Reeves, J.-X. Lin, R. Child, J. M. Leiden, C. B. Thompson,**

and W. J. Leonard. 1995. Regulation of cell-type-specific interleukin-2 receptor α-chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y), and NF-κB family proteins. Mol. Cell. Biol. **15:** 1786–1796.

23. **Johnson, K. R., D. A. Lehn, and R. Reeves.** 1989. Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y. Mol. Cell. Biol. **9:**2114–2123.

24. **Leger, H., E. Sock, K. Renner, F. Grummt, and M. Wegner.** 1995. Functional interaction between the POU domain protein Tst-1/Oct-6 and the high-mobility-group protein HMG-I/Y. Mol. Cell. Biol. **15:**3738–3747.

25. **Lovell-Badge, R.** 1995. Developmental genetics. Living with bad architecture. Nature **376:**725–726.

26. **Mandrup, S., and M. D. Lane.** 1997. Regulating adipogenesis. J. Biol. Chem. **272:**5367–5370.

27. **Manfioletti, G., V. Giancotti, A. Bandiera, E. Buratti, P. Sautiewre, P. Cary, C. Crane-Robinson, B. Coles, and G. H. Goodwin.** 1991. cDNA cloning of the HMGI-C phosphoprotein, a nuclear protein associated with neoplastic and undifferentiated phenotypes. Nucleic Acids Res. **19:**6793–6797.

28. **Mason, M. M., Y. He, H. Chen, M. J. Quon, and M. Reitman.** 1998. Regulation of leptin promoter function by Sp1, C/EBP, and a novel factor. Endocrinology **139:**1013–1022.

29. **McKnight, S. L., M. D. Lane, and S. Glueckson-Waelsch.** 1989. Is CCAAT/enhancer-binding protein a central regulator of energy metabolism? Genes Dev. **3:**2021–2024.

30. **Moldes, M., F. Lasnier, B. Feve, J. Pairault, and P. Djian.** 1997. Id3 prevents differentiation of preadipose cells. Mol. Cell. Biol. **17:**1796–1804.

31. **Saisanit, S., and X.-H. Sun.** 1997. Regulation of the pro-B-cell-specific enhancer of the *Id1* gene involves the C/EBP family of proteins. Mol. Cell. Biol. **17:**844–850.

32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

33. **Samuelsson, L., K. Stromberg, K. Vikma, G. Bjursell, and S. Enerback.** 1991. The CCAAT/enhancer-binding protein and its role in adipocyte differentiation: evidence for direct involvement in terminal adipocyte differentiation. EMBO J. **10:**3787–3793.

34. **Schoenmakers, E. F. P. M., S. Wanschura, R. Mols, J. Bullerdiek, H. Van den Berghe, and W. J. M. Van de Ven.** 1995. Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumours. Nat. Genet. **10:**436–443.

35. **Student, A. K., R. Y. Hsu, and M. D. Lane.** 1980. Induction of fatty acid synthetase synthesis in differentiating 3T3–L1 preadipocytes. J. Biol. Chem. **255:**4745–4750.

36. **Tamimi, Y., H. G. van der Poel, M. M. Denym, R. Umbas, H. F. M. Karthaus, F. M. J. Debruyne, and J. A. Schalken.** 1993. Increased expression of high mobility group protein I(Y) in high-grade prostate cancer determined by *in situ* hybridization. Cancer Res. **53:**5512–5516.

37. **Tanaka, T., N. Yoshida, T. Kishimoto, and S. Akira.** 1997. Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene. EMBO J. **24:**7432–7443.

38. **Thanos, D., and T. Maniatis.** 1992. The high mobility group protein HMG I (Y) is required for NF-κB dependent virus induction of the human IFN-β gene. Cell **71:**777–789.

39. **Thanos, D., and T. Maniatis.** 1995. Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. Cell **83:**1091–1100.

40. **Tkachenko, A., H. R. Ashar, A. M. Meloni, A. A. Sandberg, and K. K. Chada.** 1997. Misexpression of disrupted HMGI architectural factors activates alternative pathways of tumorigenesis. Cancer Res. **57:**2276–2280.

41. **Tontonoz, P., E. Hu, and B. M. Spiegelman.** 1994. Stimulation of adipogenesis in fibroblasts by PPARγ, a lipid-activated transcription factor. Cell **79:** 1147–1156.

42. **Umek, R. M., A. D. Friedman, and S. L. McKnight.** 1991. CCAAT/enhancer-binding protein: a component of a differentiation switch. Science **251:**288–292.

43. **Wood, L. J., M. Mukherjee, C. E. Dolde, Y. Xu, J. F. Maher, T. E. Bunton, J. B. Williams, and L. M. Resar.** 2000. *HMGI/Y*, a new c-Myc target gene and potential oncogene. Mol. Cell. Biol. **20:**5490–5502.

44. **Wu, Z., N. L. R. Bucher, and S. R. Farmer.** 1996. Induction of peroxisome proliferator-activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPβ, C/EBPδ, and glucocorticoids. Mol. Cell. Biol. **16:**4128–4136.

45. **Xiao, S., M. L. Lux, R. Reeves, T. J. Hudson, and J. A. Fletcher.** 1997. HMGI (Y) activation by chromosome 6p21 rearrangements in multilineage mesenchymal cells from pulmonary hamartoma. Am. J. Pathol. **150:**901–910.

46. **Yeh, W.-C., Z. Cao, M. Classon, and S. L. McKnight.** 1995. Cascade of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. **9:**168–181.

47. **Zhang, X. M., and G. L. Verdine.** 1999. A small region in HMGI(Y) is critical for cooperation with NF-κB on DNA. J. Biol. Chem. **274:**20235–20243.

48. **Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold, and J. L. Friedman.** 1994. Positional cloning of the mouse *obese* gene and its human homologue. Nature **372:**425–432.

49. **Zhou, X., K. F. Benson, H. R. Ashar, and K. K. Chada.** 1995. Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. Nature **376:**771–774.

AMERICAN SOCIETY FOR MICROBIOLOGY | **Molecular and Cellular Biology®**

Check for updates

Retraction for Melillo et al., "Critical Role of the HMGI(Y) Proteins in Adipocytic Cell Growth and Differentiation"

Rosa Marina Melillo,[1] Giovanna Maria Pierantoni,[1] Stefania Scala,[1] Sabrina Battista,[1] Monica Fedele,[1] Antonella Stella,[2] Maria Cristina De Biasio,[3] Gennaro Chiappetta,[3] Vincenzo Fidanza,[4] Gianluigi Condorelli,[4] Massimo Santoro,[1] Carlo M. Croce,[4] Giuseppe Viglietto,[3] and Alfredo Fusco[2]

Centro di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche, Dipartimento di Biologiae Patologia Cellulare e Molecolare, Facoltá di Medicina e Chirurgia, Universitá degli Studi di Napoli,[1] and Istituto Nazionale dei Tumori Fondazione Senatore Pascale,[3] 80131 Naples, and Dipartimento di Medicina Sperimentale e Clinica, Facoltá di Medicina e Chirurgia di Catanzaro, Universitá degli Studi di Catanzaro, 88100 Catanzaro,[2] Italy, and Kimmel Cancer Center, Jefferson Medical College, Philadelphia, Pennsylvania 19107[4]

    Volume 21, no. 7, p. 2485–2495, 2001, https://doi.org/10.1128/MCB.21.7.2485-2495 .2001. The publisher hereby retracts this article. Questions have been raised by concerned readers about the integrity of the data. The American Society for Microbiology has reviewed the figures and confirmed evidence of apparent manipulation and duplication. Since the integrity of the data as presented was compromised, this publication is retracted in its entirety. We apologize to the readers of *Molecular and Cellular Biology* and regret any inconvenience that this causes. The authors did not agree to this retraction.