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Subnuclear Localization of C/EBPβ Is Regulated by Growth Hormone and Dependent on MAPK*

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Localization of transcription regulatory proteins in the nucleus is dynamically regulated, and may alter nucleoplasmic concentrations and/or assembly of multimolecular transcription regulatory complexes, which ultimately regulate gene expression. Since growth hormone (GH) regulates multiple transcription factors including C/EBPβ, the effect of GH on the subcellular localization of C/EBPβ was examined in 3T3-F442A preadipocytes. Indirect immunofluorescence shows that C/EBPβ is diffusely distributed in nuclei of quiescent cells. Within 5 min of GH treatment, the diffuse pattern dramatically becomes punctate. The relocalization of C/EBPβ coincides with DAPI staining of heterochromatin. Further, C/EBPβ and heterochromatin protein (HP)-1α colocalize in the nucleus, consistent with localization of C/EBPβ to pericentromeric heterochromatin. In contrast, C/EBPδ exhibits a diffuse distribution in the nucleus that is not modified by GH treatment. C/EBPβ is rapidly and transiently phosphorylated on a conserved MAPK consensus site in response to GH (Piwien-Pilipuk, G., MacDougald, O. A., and Schwartz, J. (2002) *J. Biol. Chem.* 277, 44557–44565). Indirect immunofluorescence using antibodies specific for C/EBPβ phosphorylated on the conserved MAPK site shows that GH also rapidly induces a punctate pattern of staining for the phosphorylated C/EBPβ. In addition, phosphorylated C/EBPβ colocalizes to pericentromeric heterochromatin. The satellite DNA present in heterochromatin contains multiple C/EBP binding sites. DNA binding analysis shows that C/EBPβ, C/EBPδ, and C/EBPα (p42 and p30 forms) can bind to satellite DNA as homo- or heterocomplexes *in vitro*. Importantly, GH rapidly and transiently increases binding of endogenous C/EBPβ from 3T3-F442A cells to satellite DNA. Further, the GH-promoted nuclear relocalization of C/EBPβ to pericentromeric heterochromatin was prevented by the MEK inhibitor U0126. This observation suggests that GH-dependent MAPK activation plays a role in the regulation of nuclear relocalization of C/EBPβ. Nuclear redistribution introduces a new level of transcriptional regulation in GH action, since GH-mediated phosphorylation and nuclear redistribution of C/EBPβ may be coordinated to achieve spatial-temporal control of gene expression.

Chromatin is organized into higher order fibers in interphase chromosomes, which localize into discrete territories, providing an excellent scenario for establishing spatio-temporal regulation of gene transcription (1, 2). Transcriptional regulatory proteins are generally restricted to several hundred discrete foci throughout the nucleoplasm (3, 4). Interestingly, foci rich in some transcription factors such as E2F-1 or Oct1 show only minor overlap with sites of transcription or RNA polymerase II (3, 4), whereas steroid hormone receptors and RNA polymerase II show significant colocalization (5, 6). Thus, the function of the transcription factor-rich foci in the nucleus that do not contain RNA polymerase II or nascent RNA is unclear. A simple explanation is that foci represent nonchromatin structures that function in the regulation of nucleoplasmic concentrations of gene regulatory factors and/or the assembly of multimolecular transcription factor complexes. This subnuclear organization of multimolecular complexes may dynamically participate in regulating the patterns of gene expression that are established and maintained during cellular differentiation. This level of regulation of gene expression may work coordinately in conjunction with changes in chromatin organization, establishing "open" or "closed" states for transcription.

Among the nuclear proteins that participate in the regulation of gene expression is CCAAT/enhancer-binding protein (C/EBP)[1] β, a transcription factor that belongs to the bZIP family of transcription factors (7). C/EBPβ has three alternate translation products: 35- and 32-kDa proteins in murine cells known as LAP (Liver-enriched-activating protein) and a 20-kDa protein known as LIP (liver-enriched inhibitory protein) (8). The N-terminal half of LAP contains the transactivation domain, whereas LIP lacks this transactivation domain and acts as an inhibitor of transcription (8). C/EBPβ plays an important role during differentiation of a number of cell types (9–14), and is also implicated in mammary gland development (15) and in ovulation (16, 17). The participation of C/EBPβ in a wide variety of physiological events suggests that multiple features of its regulation may contribute to diverse biological outcomes. One mode of regulation of C/EBPβ is by phosphorylation. It has recently been shown that C/EBPβ is present in multiple forms that exhibit different degrees of phosphorylation in 3T3-F442A preadipocytes (18). Further, MAPK-dependent phosphorylation of murine (m)LAP on Thr^{188} and mLIP on Thr^{37} is rapidly and transiently (5–15 min) induced by GH, as well as other agonists such as IGF-1 and PDGF. Phosphoryla-

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[1] The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; DAPI, 4′,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SFC, splicing factor compartment; HP, heterochromatic protein; GH, growth hormone; LAP, liver-enriched-activating protein; LIP, liver-enriched inhibitory protein.

tion of LAP at the MAPK site is required for LAP to be transcriptionally active in the context of the c-*fos* promoter (18). Subsequently, GH induces dephosphorylation of C/EBPβ, which leads to an increase in DNA binding of LAP complexes (19, 20). The GH-induced dephosphorylation of C/EBPβ is mediated, at least in part, by GH-stimulated phosphatidylinositol 3-kinase (PI3K)/Akt signaling, resulting in inhibition of GSK-3 (20).

C/EBPs play a central role in adipocyte differentiation (11, 21) during which their nuclear localization changes. C/EBPβ and δ, which are diffusely distributed in the nuclei of 3T3-L1 preadipocytes, acquire a punctate pattern of nuclear distribution 12–24 h after induction of differentiation, which corresponds to localization to pericentromeric heterochromatin (22). Further, C/EBPα also localizes in areas of pericentromeric heterochromatin concomitant with cessation of mitotic clonal expansion of the cells (22). These observations suggest that the subnuclear distribution of C/EBPs may be associated with their roles during differentiation. However, little is known about the molecular mechanisms that regulate the nuclear redistribution of C/EBPs.

3T3-F442A fibroblasts undergo GH-dependent adipocyte differentiation (23–25). In 3T3-F442A preadipocytes GH priming is required before induction of terminal differentiation (26, 27). In addition, GH accounts for ~60% of the differentiation-promoting activity in the serum (24). The present study examines the nuclear distribution of C/EBPβ and C/EBPδ, and how their distribution is regulated in 3T3-F442A preadipocytes in response to GH. C/EBPβ was found to exhibit a primarily diffuse and minutely speckled pattern of nuclear distribution in quiescent 3T3-F442A cells. Upon treatment with GH the diffuse pattern of C/EBPβ rapidly and dramatically changes to a punctate one. C/EBPβ relocalizes to pericentromeric heterochromatin, consistent with colocalization of C/EBPβ with markers for heterochromatin. On the other hand, C/EBPδ exhibits a diffuse nuclear distribution both in the presence and absence of GH. The rapid nuclear relocalization of C/EBPβ is blocked by MAPK inhibitors, suggesting that MAPK activation is required for GH-induced nuclear redistribution of C/EBPβ. Thus, tight regulation of transcription factor phosphorylation and subcellular redistribution may be coordinated to achieve spatial-temporal control of gene expression in response to a physiological stimulus such as GH.

EXPERIMENTAL PROCEDURES

Materials—Murine 3T3-F442A preadipocyte fibroblasts, which are highly responsive to GH through endogenous GH receptors (25), were provided by Dr. H. Green (Harvard University) and Dr. M. Sonenberg (Sloan-Kettering). Human embryonic kidney 293T cells were provided by Dr. M. Lazar (University of Pennsylvania). Recombinant human GH was provided by Eli Lilly, Inc. Culture medium, calf serum, L-glutamine, and antibiotic-antimicotic solution were purchased from Invitrogen. Bovine serum albumin (BSA, CRG7) was purchased from Intergen. DAPI nucleic acid stain (4′,6-diamino-2-phenylindol) was purchased from Molecular Probes, Inc. The MEK inhibitor U0126 was purchased from Promega. Wortmannin was purchased from Calbiochem, and Complete® protease inhibitor mixture (EDTA-free), leupeptin, aprotinin, and pepstatin were purchased from Roche Applied Science. [α-^{32}P]dATP was purchased from PerkinElmer Life Sciences. ECL detection system was purchased from Amersham Biosciences.

Cell Culture and Hormone Treatment—3T3-F442A fibroblasts and 293T cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/liter of glucose and 8% calf serum (DMEM complete medium) in an atmosphere of 10% CO_2, 90% air at 37 °C. All media were supplemented with 1 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin. Prior to treatment, cells were deprived of serum by incubation overnight in DMEM containing 1% BSA. Then cells were incubated with or without GH at 500 ng/ml (23 nM).

Antibodies and Plasmids—Specific rabbit polyclonal antibodies against a synthetic phospho-Thr^{235} peptide corresponding to residues surrounding Thr^{235} of human C/EBPβ (identical to Thr^{188} in rat C/EBPβ) (anti-P-C/EBPβ) were provided by Cell Signaling Technology, Inc. (Beverly, MA). Specific rabbit polyclonal antibodies against a peptide corresponding to amino acids 278–295 at the C terminus of C/EBPβ (anti-C/EBPβ), amino acids 115–130 of C/EBPδ, an internal amino acid sequence of C/EBPα, specific polyclonal antibodies that recognize HP-1α, and anti-ELK-1 were purchased from Santa Cruz Biotechnology, Inc. Monoclonal anti-Splicing Factor SC35 that recognizes a phosphoepitope of the non-snRNP (small nuclear ribonucleoprotein particles) factor SC-35 (spliceosome component of 35 kDa) (Sigma) was kindly provided by Drs. K. Sitwala and D. Markovitz (University of Michigan). Anti-kinetochore antibodies were purchased from CORTEXBiochem (San Leandro, CA). Antibodies against CREB were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-rabbit, anti-human, or anti-goat IgG labeled with rhodamine or fluorescein were purchased from Jackson Immunochemicals, Inc. (West Grove, PA).

Plasmids encoding rat LAP or LIP driven by the CMV promoter were kindly provided by U. Schibler (University of Geneva) courtesy of Dr. L. Sealy (Vanderbilt University). Plasmids encoding human (h)LAP (also known as NF-IL6) and a mutant where Thr^{235} (phosphorylation site for ERK1/2) was mutated to Ala (hLAP-T235A) were kindly provided by Dr. S. Akira (Osaka University) courtesy of Dr. L. Sealy (Vanderbilt University). Plasmids encoding p42-C/EBPα and p30-C/EBPα were provided by Dr. O. A. MacDougald (University of Michigan). Plasmid encoding CREB was kindly provided by Dr. R. Kwok (University of Michigan). The DNA for Elk-1 has been previously described (28).

Indirect Immunofluorescence—3T3-F442A cells were grown on coverslips in DMEM complete medium for 24 h, prior to 18 h of serum deprivation in DMEM with 1% BSA instead of serum. Cells were then treated with or without GH 500 ng/ml (23 nM) for the indicated periods of time. The coverslips were rinsed twice with ice-cold PBS and simultaneously fixed and permeabilized by immersion in cold methanol (−20 °C) for 2 h. Coverslips were rinsed with PBS containing 1% BSA, and inverted onto a 50-μl drop of PBS 1% BSA with the corresponding antibodies (1:100), as indicated in the figure legends. After overnight incubation with antibody at 4 °C and subsequent washing with PBS containing 1% BSA, coverslips were inverted again onto 50-μl drops of PBS 1% BSA containing the corresponding rhodamine- or fluorescein isothiocyanate-conjugated secondary antibody (1:100) and incubated 2 h at room temperature. Coverslips were washed in PBS 1% BSA and counterstained with DAPI (1 μg/ml). Dual-colored staining employed appropriate species-specific reagents, in which specificity was verified in control stainings. Laser-scanning confocal microscopy was performed on a Zeiss LSM510 META.

Cell Fractionation and Immunoblotting—3T3-F442A cells were rinsed twice with ice-cold PBS and once with ice-cold hypotonic buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonylfluoride (PMSF),1 μg/ml each of aprotinin, leupeptin, and pepstatin) and scraped into hypotonic buffer. Cells were transferred to tubes and Nonidet P-40 was added to a final concentration of 0.2%. Samples were homogenized (10 times) using a Dounce homogenizer with a loose pestle and were centrifuged 30 s at 13,000 rpm. The supernatant corresponded to the cytosolic fraction and the pelleted nuclei were resuspended in 50 μl of high ionic strength buffer (hypotonic buffer with 420 mM NaCl and 30% glycerol). The cytosolic and nuclear fractions were stored at −70 °C. Immunoblotting for C/EBPβ in nuclear and cytosolic fractions was performed as previously described (19, 20). The apparent M_r are based on prestained molecular weight standards (Invitrogen).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from 3T3-F442A fibroblasts were prepared as previously described (20). Cell extracts enriched in LAP, LIP, hLAP, hLAPT235A, CREB, or Elk-1 were obtained by transfection of 293T cells with 1 μg of the corresponding DNA as previously described (20). Binding reactions proceeded for 30 min at room temperature as previously described (20), using a ^{32}P-labeled oligonucleotide containing a sequence of mouse satellite DNA ^{182}TGA AAA ATG ACG AAA TCA CTA^{202} (C/EBP-satellite), (22) encompassing a putative C/EBP site, or an oligonucleotide containing a mutation in the C/EBP site of the satellite DNA (underlined nucleotides were replaced by A). For competition experiments, increasing concentrations of unlabeled wild-type c-*fos* C/EBP probe (previously referred as C/EBP-SRE, Ref. 19) or the c-*fos* C/EBP probe mutated at the C/EBP site (19) were used. DNA binding of CREB was tested using a probe encoding the CRE sequence of the somatostatin promoter (5′-CGAGC-CTTGGCTGACGTCAGAGAGGGCG). In some experiments, cell extracts were incubated for 20 min at room temperature with 1 μl of anti-CEBPβ (1:10 dilution), anti-P-C/EBPβ (1:10 dilution), anti-CREB (1:10 dilution), or anti ELK-1 (1:10 dilution) prior to EMSA, as indicated

in figure legends. Complexes were separated by nondenaturing 7% PAGE followed by autoradiography.

RESULTS

GH Promotes Nuclear Redistribution of C/EBPβ—The nucleus is highly organized and nuclear molecules exhibit a high mobility throughout the nucleoplasm to find their targets (29). Nuclear compartments are therefore determined by the functional status of exchanging proteins which determines the composition, morphological appearance, and possibly function of a compartment. Since C/EBPβ is a ubiquitously distributed transcription factor regulated by GH by changes in its phosphorylation state (18–20), the subcellular distribution of C/EBPβ was investigated in GH-treated cells. Indirect immunofluorescence reveals that C/EBPβ is localized in the nucleus of quiescent 3T3-F442A fibroblasts (Fig. 1*a*, *panel A*) showing a predominantly diffuse, minutely speckled distribution. GH treatment of the cells for as little as 5 min rapidly changes the nuclear pattern from diffuse to punctate (Fig. 1*a*, *panel B versus A*). Foci of intense staining (20–26 per nucleus) were seen within the nuclei of all cells examined. The nucleoli of cells were excluded from staining. The punctate pattern persists for 1–6 h of GH treatment (data not shown). In addition, the same punctate pattern of nuclear staining is observed when 3T3-F442A cells were treated with IGF-1 or PDGF (data not shown). Cells were counterstained with DAPI (Fig. 1*a*, *panels E* and *F*), whose bright fluorescent condensations in murine nucleus correspond to heterochromatic DNA located at centromeric regions of interphase chromosomes (30–32). Merging of immunofluorescense for C/EBPβ (*panel A*) and DAPI (*panel E*) in control cells shows no coincidence (*panel I*). However, overlay of nuclear images from cells treated with GH (*panels B* and *F*) shows that C/EBPβ and DAPI staining do coincide (Fig. 1*a*, *panel J*), suggesting that GH promotes the relocalization of C/EBPβ to areas of heterochromatin. To examine further the relocalization of C/EBPβ to pericentromeric heterochromatin, heterochromatin protein 1-α (HP-1α), a constitutive non-histone heterochromatin protein (33, 34) was labeled with anti-HP-1α antibodies. HP-1α exhibits the same pattern of distribution as C/EBPβ in cells treated with GH (Fig. 1*a*, *panel D versus B*), but not in control cells (*panel C versus A*). Merging of nuclear images shows coincidence of C/EBPβ and HP-1α only in GH-treated cells (Fig. 1*a*, *panel H*) but not in control cells (*panel G*), reinforcing that GH promotes the redistribution of C/EBPβ to areas of pericentric heterochromatin. C/EBPβ also colocalizes with kinetocore proteins known to be associated with pericentromeric heterochromatin as observed by labeling with a serum containing human autoantibodies against centromeric kinetocore proteins (data not shown).

The intracellular localization of C/EBPβ was also confirmed by cell fractionation. C/EBPβ is a nuclear but not cytoplasmic protein in the absence (Fig. 1*b*, *lane 5*) or presence of GH (60 min) (Fig. 1*b*, *lane 6*), in agreement with indirect immunofluorescence. The immunoblot shows a GH-induced shift in the migration (*bands a* to *b*) of each of the three forms of C/EBPβ, consistent with GH-promoted dephosphorylation after 60 min in 3T3-F442A cells, as previously reported (18–20). Taken together these results demonstrate that in 3T3-F442A preadipocytes C/EBPβ is a nuclear protein, which undergoes a rapid nuclear relocalization to pericentric heterochromatin upon GH treatment.

C/EBPβ Foci Are Not Associated with Splicing Factor Compartments (SFCs)—Numerous studies have localized sites of active transcription, measured as [^{3}H]uridine or Br-UTP incorporation into nascent RNA transcripts, at the periphery of nuclear domains enriched in splicing factors (35), called SFCs (36, 37). SFCs may serve as storage sites from which splicing factors are recruited to adjacent active transcription sites (38). Since splicing can take place as a co-transcriptional event coordinating splicing and transcription (37), the spatial relationship between C/EBPβ and SC-35, a non-snRNP, which is a component of SFCs (36, 39), was examined. Double immunostaining for C/EBPβ (Fig. 2, *panels A* and *B*) and SC-35 (*panels C* and *D*) shows no colocalization of these two nuclear proteins,

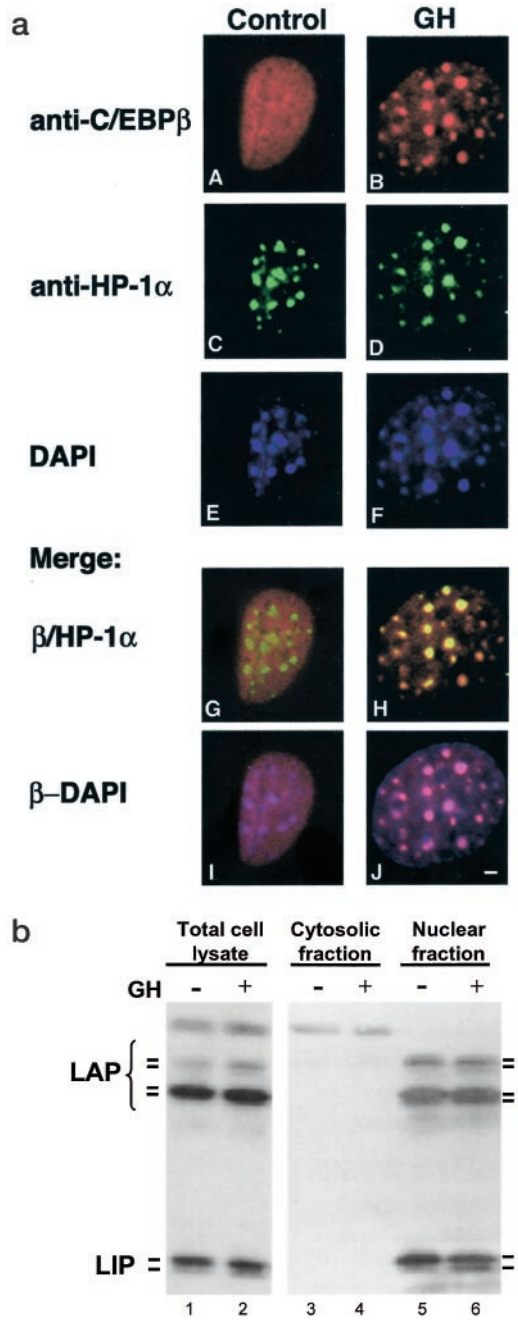

FIG. 1. **GH promotes nuclear redistribution of C/EBPβ in 3T3F442A preadipocytes.** *a*, cell monolayers were grown on coverslips, deprived of serum, and treated without (*panels A*, *C*, and *E*) or with GH for 15 min (*panels B*, *D*, and *F*). Cells were fixed and subjected to indirect immunofluorescence using anti-C/EBPβ (1:100) and anti-HP-1α (1:100). Cells were counterstained with DAPI. Merge of C/EBPβ and HP-1α immunostaining is shown in *panels G* and *H*. Merge of C/EBPβ immunostaining and DAPI is shown in *panels I* and *J*. Images are representative of five independent experiments. *Bar* is equal to 1 μm. *b*, 3T3-F442A cells treated without (−) or with GH (+) for 60 min were lysed and resolved into nuclear and cytosolic fractions. The total lysate, cytoplasmic and nuclear fractions were subjected to SDS-PAGE and then immunoblotted with anti-C/EBPβ (1:1000).

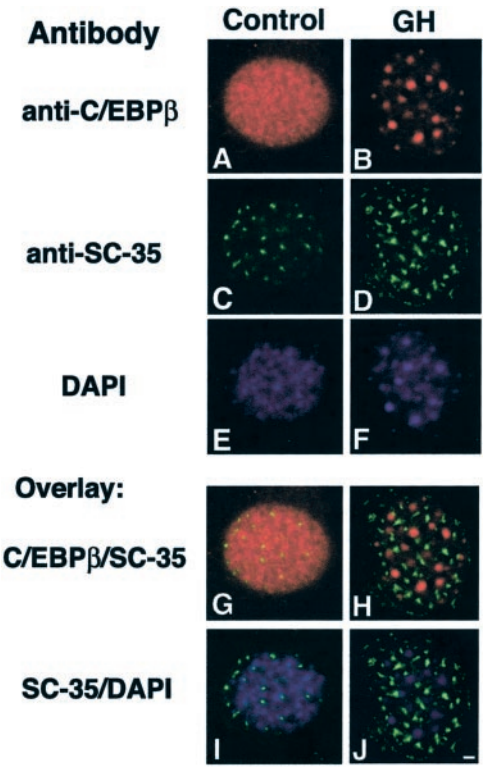

FIG. 2. **C/EBPβ does not localize in SFCs.** Indirect immunofluorescence was performed in 3T3-F442A cells using anti-C/EBPβ (*A* and *B*) (1:100) and anti-SC-35 (*C* and *D*) (1:100), followed by DAPI (*E* and *F*) counterstaining. *Panels G* and *H* show the merge of the images obtained with anti-C/EBPβ and anti-SC-35. *Panels I* and *J* show the merge of the images obtained with anti-SC-35 and DAPI staining. *Bar* is equal to 1 μm. Images are representative of two independent experiments.

as observed when the nuclear images are merged (Fig. 2, *panels G* and *H*), indicating that neither in quiescent nor GH-treated 3T3-F442A cells does C/EBPβ colocalize with SFCs. In addition, DAPI staining (*panels E* and *F*) coincides with C/EBPβ only in GH-treated cells, as observed above, but does not coincide with SC-35 staining under any condition (Fig. 2, *panels I* and *J*) consistent with SC-35 localization in spliceosomes adjacent to areas of active transcription and not in areas of heterochromatin. Thus, C/EBPβ concentrates in areas of pericentric heterochromatin and not in areas of active transcription upon GH treatment of the cells.

Phosphorylated C/EBPβ Localizes to Pericentromeric Heterochromatin—Since GH promotes a rapid and transient phosphorylation of C/EBPβ at the MAPK consensus site (18), the possibility that phosphorylated C/EBPβ also localizes in areas of pericentromeric heterochromatin was examined. Indirect immunofluorescence using antibodies that specifically recognize phosphorylated Thr in the MAPK consensus site of C/EBPβ (anti-P-C/EBPβ) shows that no phosphorylated C/EBPβ at this site is present in the nuclei of untreated cells (Fig. 3, *panel A*). GH treatment rapidly (5 min) induces the appearance of phosphorylated C/EBPβ (*panel B*). Further, phosphorylated C/EBPβ assumes a punctate pattern of staining in the nuclei of GH-treated cells (Fig. 3, *panels B versus A*). This pattern of staining is evident within 5–15 min of GH treatment, subsiding within 30–60 min of GH treatment of the cells (data not shown). These results are consistent with our previous studies showing transient GH-induced phosphorylation of C/EBPβ by immunoblotting and isoelectric focusing analysis (18). Incubation of the antiserum containing anti-P-C/EBPβ with the phosphopeptide used to generate the antibodies, but not with the unphosphorylated peptide, completely blocked the nuclear staining (data not shown), verifying the specificity of the immunostaining. Furthermore, phosphorylated C/EBPβ and DAPI counterstaining coincide as observed when images are merged (Fig. 3, *panel E*), supporting that phosphorylated C/EBPβ colocalizes with pericentromeric heterochromatin. In addition, when cells are doubled stained with anti-P-C/EBPβ (Fig. 3, *panel F*) and anti-C/EBPβ (Fig. 3, *panel G*) the pattern of staining coincides in GH-treated cells, as further demonstrated when the images are overlaid (Fig. 3, *panel H*). Taken together these results demonstrate that C/EBPβ that relocalizes to areas of pericentric heterochromatin is transiently phosphorylated at the MAPK site.

C/EBPδ and C/EBPβ Are Differentially Distributed in GH-treated 3T3-F442A Preadipocytes—It has been previously shown that GH differentially regulates C/EBPβ and C/EBPδ in 3T3-F442A preadipocytes (19, 40). C/EBPβ is regulated posttranslationally by changes in its phosphorylation state, while the expression of C/EBPδ is increased as demonstrated by Northern blot and immunoblotting. Indirect immunofluorescence shows that C/EBPδ like C/EBPβ localizes in the nucleus and exhibits a diffuse pattern of staining in tiny speckles in untreated 3T3-F442A cells (Fig. 4, *panel A*). However, in contrast to C/EBPβ, GH treatment does not cause accumulation of C/EBPδ in a punctate pattern (Fig. 4, *panel B*). DAPI staining demonstrates nuclear integrity and the areas enriched in heterochromatin, which do not coincide with C/EBPδ (Fig. 4, *panels C* and *D*). Further, double staining for C/EBPδ and C/EBPβ in GH-treated cells shows a differential pattern of distribution, in which C/EBPδ remains diffuse in minute speckles (*panel E*) while C/EBPβ forms larger punctate structures (*panel F*). Interestingly, C/EBPδ and C/EBPβ appear mainly to concentrate in different foci in the nucleus (Fig. 4, *panel G*). However, it is possible the existence of a few areas of colocalization between C/EBPδ and -β as illustrated in the magnified nuclear area inside the box (*panel H*, *arrows*). Thus, different members of the C/EBP family may localize in different nuclear foci, and their nuclear localization appears to be differentially regulated, as demonstrated for GH.

C/EBPβ Binds to Pericentromeric Satellite DNA—Mouse satellite DNA present in heterochromatin is located primarily in centromeres and contains DNA sequence repeats (32). The nucleotide sequences of the major species of mouse satellite DNA contain eight repeats of a consensus C/EBP binding site (**TT/G*XX*G*X*AAT/G**) to which C/EBPβ and C/EBPα from 3T3-L1 adipocytes can bind as multiple DNA-protein complexes (22). To characterize C/EBPβ-satellite DNA complexes, 293T cell extracts enriched in overexpressed LAP or LIP were subjected to EMSA to detect C/EBPβ complexes bound to a probe encoding the C/EBP site of mouse satellite DNA. The binding of LAP appears as a band (Fig. 5*A*, *lane 1*), which is supershifted by anti-C/EBPβ, indicating that complexes contained C/EBPβ (Fig. 5*A*, *lane 2*, *arrowheads*). LIP appears to form at least two complexes with satellite DNA (Fig. 5*A*, *lane 3*), which supershift in the presence of antibodies against C/EBPβ (Fig. 5*A*, *lane 4*, *arrowheads*). To examine the specificity of C/EBPβ binding to satellite DNA, binding of the transcription factors Elk-1 or CREB was tested using 293T cell extracts enriched in Elk-1 or CREB. No band is detected for Elk-1 (Fig. 5*A*, *lanes 5* and *6*) or CREB (*lanes 7* and *8*). Nevertheless, Elk-1 forms 2 complexes with the c-*fos* SRE (Fig. 5*A*, *lane 9*, *triangles*) (41) that supershift with anti-Elk (*lane 10*, *asterisk*) and that are competed with unlabeled probe (*lane 11*), as expected. In addition, CREB binds to a consensus CRE probe as at least two complexes (*lane 12*, *stars*) that supershift with anti-CREB (*lane 13*) and are competed with unlabeled CRE probe (*lane 14*). Thus, binding of C/EBPβ to satellite DNA is specific.

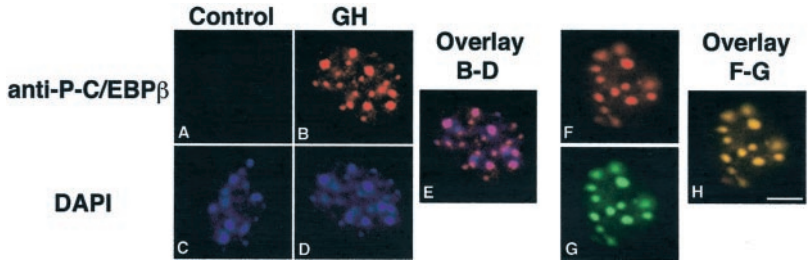

FIG. 3. **Phosphorylated C/EBPβ localizes to pericentric heterochromatin.** 3T3-F442A cells grown on coverslips were deprived of serum and treated without (*panels A* and *C*) or with GH for 5 min (*panels B, D, F,* and *G*). Cells were fixed and subjected to indirect immunofluorescense using anti-P-C/EBPβ (1:100) (*panels A*, *B*, and *F*) or anti-C/EBPβ (*panel G*) and counterstained with DAPI (*panels C* and *D*). *Panel E* corresponds to the merge of images obtained in GH-treated cells with anti-P-C/EBPβ (*panel B*) and DAPI staining (*panel D*). *Panel H* corresponds to the merge of images obtained with anti-P-C/EBPβ (*panel F*) and anti-C/EBPβ (*panel G*). *Bar* is equal to 5 μm.

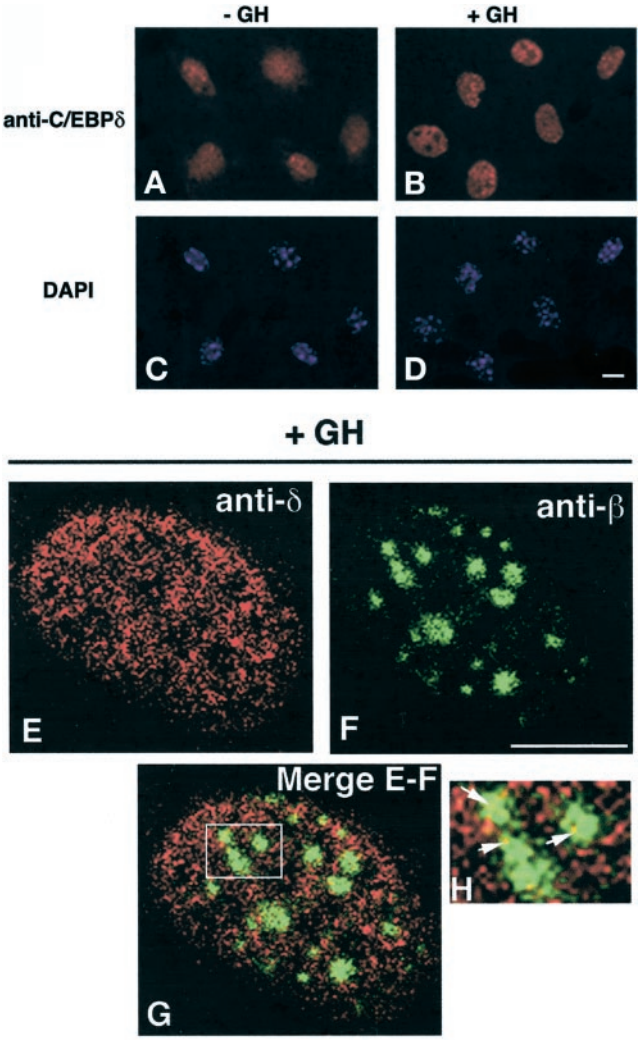

FIG. 4. **C/EBPδ is a nuclear protein and GH does not modify its nuclear distribution.** 3T3-F442A fibroblasts grown on coverslips were deprived of serum and treated without (*panels A* and *C*) or with GH (*panels B*, *D*, *E*, and *F*) for 45 min. Cells were fixed and labeled with anti-C/EBPδ (*panels A* and *B*) anti-C/EBPβ (*panel F*) and counterstained with DAPI (*panels C* and *D*). *Bar* is equal to 10 μm. *Panels E* and *F* show nuclear images of GH-treated cells obtained with anti-C/EBPδ or anti-C/EBPβ, respectively. *Panel G* corresponds to the merge of the nuclear images shown in panels *E* and *F*. *Panel H* shows a magnification of the nuclear region in the *white box* in *panel G*. *Bar* in *panel F* is equal to 5 μm.

Further, binding of LAP to the C/EBP site of satellite DNA is efficiently competed with increasing concentrations of unlabeled DNA based on the C/EBP site of the c-*fos* promoter (wt-C/EBP) (Fig. 5*B*, *lanes 3–5 versus 1*). Binding of LIP is also competed by unlabeled wt-C/EBP probe (Fig. 5*B*, *lanes 11–13 versus 9*). As expected, when the c-*fos* C/EBP site is mutated (mut-C/EBP), it fails to compete for LAP (Fig. 5*B*, *lanes 6–8 versus 1*) or LIP (Fig. 5*B*, *lanes 14–16 versus 9*). Further, in satellite DNA mutation of the C/EBP binding site (mut- *versus* wt sat-C/EBP) also abolishes the binding of LAP (Fig. 5*C*, *lane 2 versus 1*) and LIP (*lane 4 versus 3*). When cell extracts enriched in LAP and LIP are combined, binding of LAP·LAP, LAP·LIP, and LIP·LIP dimers to satellite DNA are detected (Fig. 5*C*, *lane 5*), and binding of homo- and heterodimers is also prevented when the C/EBP site of the satellite DNA is mutated (Fig. 5*C*, *lane 6 versus 5*). Taken together, these observations indicate that C/EBPβ can bind as homo- or heterodimers to satellite DNA.

Since C/EBPβ phosphorylated at the MAPK site localizes in areas of pericentric heterochromatin, the importance of phosphorylation on binding to DNA was examined using extracts enriched in human (h)LAP or hLAP where the conserved MAPK consensus site Thr^{235} was mutated to Ala (hLAPT235A). Both wild-type hLAP and mutant hLAPT235A bind to the wild-type sat-C/EBP probe (Fig. 5*C*, *lanes 7* and *9*, respectively). The migration of hLAP complexes appears as 2–3 bands, which migrate more slowly than LAP, as previously shown (18). Binding of hLAP and hLAPT235A is abrogated when the C/EBP site in satellite DNA is mutated (Fig. 5*C*, *lanes 8* and *10*, respectively). Thus, rat and human LAP, as well as LIP are able to bind specifically to C/EBP sites in satellite DNA. Further, phosphorylation of LAP at the conserved MAPK site does not appear to be required for binding of LAP complexes to C/EBP sites in satellite DNA, as previously shown for binding of LAP to the c-*fos* C/EBP site (18).

C/EBPδ and C/EBPα Also Bind to Satellite DNA as Homo- or Heterodimers—The binding of other members of the C/EBP family of transcription factors to the C/EBP site of satellite DNA was also examined. Extracts from 293T cells overexpressing C/EBPδ were tested alone or in combination with LAP or LIP in EMSA using the C/EBP-satellite probe. C/EBPδ binds as at least 5 different complexes (Fig. 6*A*, *lane 5*, *asterisks*) all of which supershift in the presence of antibodies that specifically recognize C/EBPδ (*lane 6*). When extracts enriched in C/EBPδ and LAP are combined, a different pattern of 3 complexes is detected (Fig. 6*A*, *lane 7*, *triangles*). All bands partially supershift in the presence of anti-C/EBPβ (*lane 8*), and anti-C/EBPδ (*lane 9*), suggesting the presence of C/EBPδ and LAP homo- and/or heterodimers. When a combination of extracts from 293T cells enriched in C/EBPδ and LIP is analyzed, 3 complexes are detected (Fig. 6*A*, *lane 10*, *dots*). All 3 bands supershift in the presence of anti-C/EBPβ (Fig. 6*A*, *lane 11*), indicating the presence of LIP in all complexes. In contrast, in the presence of anti-C/EBPδ, only the uppermost band supershifts (Fig. 6*A*, *lane 12*), suggesting that only the upper band corresponds to C/EBPδ·LIP heterodimers. Taken together these re-

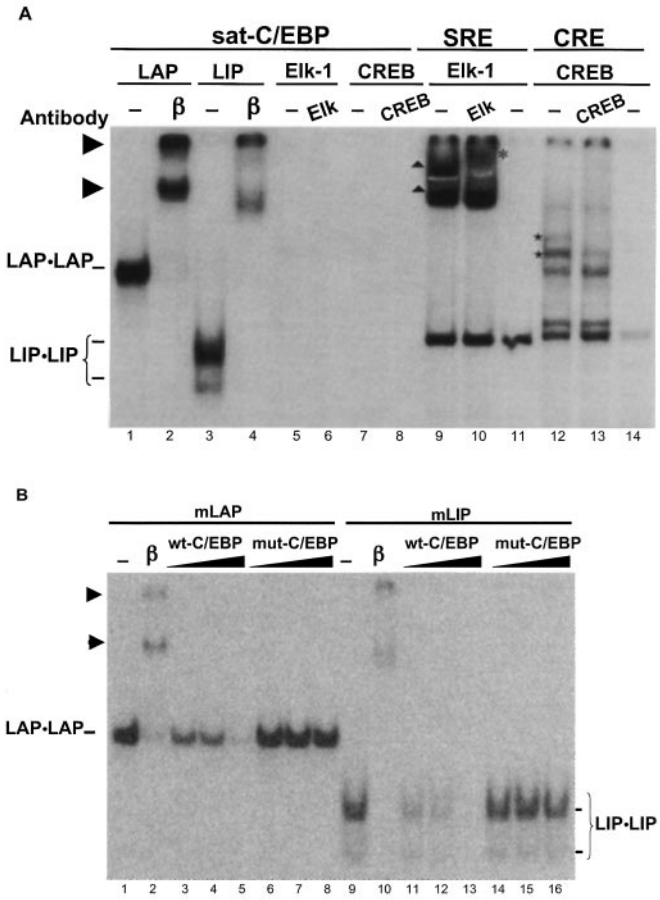

FIG. 5. **C/EBPβ binds to C/EBP sites from satellite DNA.** *A*, extracts from 293T cells overexpressing LAP, LIP, Elk-1, or CREB were incubated in the absence (*lanes 1*, *3*, *5*, and *7*) or presence (*lanes 2*, *4*, *6*, and *8*) of the indicated antibody and subjected to EMSA using a probe that encodes the C/EBP site of satellite DNA. Complexes containing LAP (*lanes 1* and *2*) and LIP (*lanes 3* and *4*) and the corresponding supershift (*arrowheads*) are indicated on the *left*. Binding of Elk-1 was tested using a c-*fos* SRE probe (*lanes 9–11*, *triangles*), supershifted with anti-Elk (*lane 10*, *asterisk*), and competed with with unlabeled probe (*lane 11*). Binding of CREB was tested using a probe containing a CRE consensus sequence (*lanes 12–14*, *stars*), bands were supershifted with anti-CREB (*lane13*), and competed with unlabeled probe (*lane14*). *B*, binding of LAP and LIP to the C/EBP site in satellite DNA was competed with increasing concentrations of unlabeled probe based on the wild type c-*fos* C/EBP site (wt-C/EBP, *lanes 3–5* and *11–13*), or with increasing concentrations of the same probe mutated at the C/EBP site (mut-C/EBP, *lanes 6–8* and *14–16*). LAP and LIP complexes were supershifted with anti-C/EBPβ (*lanes 2* and *10*, *arrowheads*) *C*, EMSA was performed with extracts from 293T cells overexpressing LAP, LIP, hLAP, and hLAPT235A using wild-type sat-C/EBP probe (*wt*, *lanes 1*, *3*, *5*, *7*, and *9*) or the same probe mutated at the C/EBP site (*mut*, *lanes 2*, *4*, *6*, *8*, and *10*).

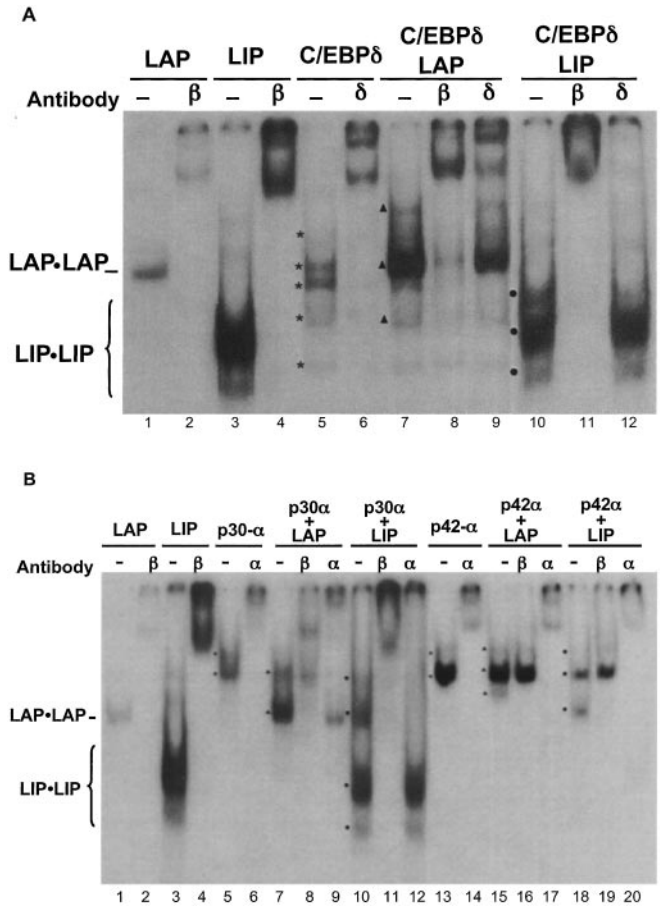

FIG. 6. **C/EBPβ, C/EBPδ and C/EBPα homo- and heterodimers bind to the C/EBP site in satellite DNA.** *A*, extracts from 293T cells overexpressing LAP, LIP, or C/EBPδ were incubated in the absence (*lanes 1*, *3*, *5*, *7*, and *10*) or presence of specific antiserum for C/EBPβ (β, *lanes 2*, *4*, *8*, and *11*) or C/EBPδ (δ, *lanes 6*, *9*, and *12*). Complexes containing LAP (*lanes 1* and *2*), LIP (*lanes 3* and *4*), or C/EBPδ (*lanes 5* and *6*, *asterisks*) were identified by EMSA using the sat-C/EBP probe. Cell extracts enriched in LAP and C/EBPδ (*lanes 7–9*, *triangles*) or LIP and C/EBPδ (*lanes 10–12*, *dots*) were combined to detect the formation of homo- and heterocomplexes. *Asterisks*, *triangles*, and *dots* highlight relevant complexes, as discussed in the text. *B*, cell extracts enriched in LAP, LIP, p30-C/EBPα, or p42-C/EBPα were analyzed separately or in combination in EMSA using the sat-C/EBP probe. Bands were supershifted in the presence of the indicated antibody. *Asterisks*, *triangles*, and *dots* indicate complexes discussed in the text. The autoradiograms shown in *parts A* and *B* are representative of three independent experiments.

sults show that C/EBPδ can bind to the C/EBP site of satellite DNA in homo- or heterocomplexes with LAP or LIP.

During induction of adipogenesis, C/EBPα expression is followed by transcriptional activation of genes encoding proteins that establish the adipocyte phenotype (11). C/EBPα has been shown to localize in areas of pericentric heterochromatin in 3T3L1-cells upon adipocyte differentiation (22). Therefore, the binding of full-length p42-C/EBPα and the truncated form p30-C/EBPα to satellite DNA was examined. When extracts enriched in p30-C/EBPα are tested, 2 bands are detected (Fig. 6*B*, *lane 5*, *asterisks*), and the bands supershift in the presence of anti-C/EBPα (Fig. 6*B*, *lane 6*). When extracts enriched in p30-C/EBPα and LAP are combined, 2 bands are detected (*lane 7*, *triangles*). The upper band is partially supershifted by anti-C/EBPβ (*lane 8*), and completely supershifted with anti-C/EBPα (*lane 9*), indicating that the upper band corresponds to p30-C/EBPα homodimers and to p30α·LAP heterodimers. Conversely, the lower band (*lane 7*) completely supershifts in the presence of anti-C/EBPβ (*lane 8*) and partially supershifts with anti-C/

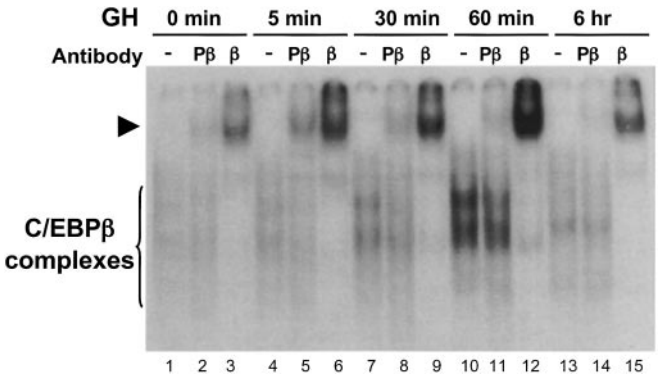

FIG. 7. **Endogenous C/EBPβ from 3T3-F442A preadipocytes bind to the C/EBP site in satellite DNA.** Nuclear extracts from 3T3-F442A cells treated without (*lanes 1–3*) or with GH for 5 min (*lanes 4–6*), 30 min (*lanes 7–9*), 60 min (*lanes 10–12*), or 6 h (*lanes 13–15*) were subjected to EMSA using the sat-C/EBP probe. Nuclear extracts were preincubated with the indicated antisera specific for phosphorylated C/EBPβ (Pβ) or C/EBPβ (β) prior to EMSA. Similar results were obtained in two independent experiments.

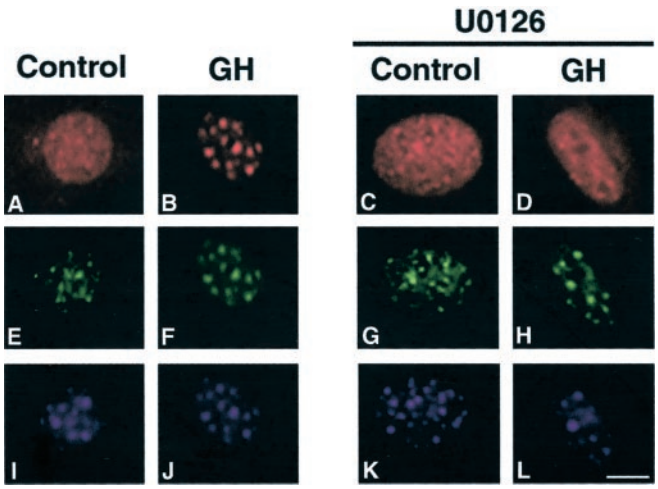

FIG. 8. **MAPK activation is required for C/EBPβ nuclear relocalization.** 3T3-F442A cells were grown on coverslips, deprived of serum and incubated with vehicle (*panels A*, *B*, *E*, and *F*) or the MEK inhibitor U0126 (10 μM) 30 min prior GH treatment (10 min). Cells were fixed and subjected to indirect immunofluorescence using anti-C/EBPβ, HP-1α, and DAPI counterstaining. *Bar* is equal to 5 μm. Images were taken with a Zeiss Aristoplan microscope and are representative of three independent experiments.

EBPα (*lane 9*), indicating that the lower band corresponds to LAP homodimers and p30α·LAP heterodimers. Combining LIP with extracts enriched in p30-C/EBPα leads to the formation of 4 complexes on satellite DNA (Fig. 6*B*, *lane 10*, *dots*). All bands supershift in the presence of anti-C/EBPβ, except the uppermost which supershifts partially (*lane 11*). Thus LIP appears to be present in the complexes. In the presence of anti-C/EBPα only the 2 upper bands supershift (*lane 12*). These results indicate that the 2 upper bands correspond to p30-C/EBPα homodimers and p30α·LIP complexes, while the 2 bands with fastest migration correspond to LIP·LIP complexes. The full-length form of C/EPBα, p42-C/EBPα, forms 2 complexes with satellite DNA (Fig. 6*B*, *lane 13*, *asterisks*), which both supershift in the presence of anti-C/EPBα (*lane 14*). When LAP is combined with p42-C/EBPα, 3 bands are observed (Fig. 6*B*, *lane 15*, *triangles*). In the presence of anti-C/EBPβ, only the lower band supershifts (*lane 16*), while in the presence of anti-C/EBPα all 3 bands supershift (*lane 17*). These results suggest that the upper and middle bands in *lane 15* correspond to p42-C/EBPα homodimers and the lower band corresponds to p42α·LAP heterocomplexes. Note that LAP·LAP complexes have a faster migration than p42·LAP complexes(Fig. 6*B*, *lane 15 versus 1*). When LIP is combined with p42-C/EBPα, at least 3 bands are observed (Fig. 6*B*, *lane 18, dots*). The upper and middle bands supershift only in the presence of anti-C/EBPα (*lane 20*), and their migration coincides with the migration of the p42-C/EBPα homodimers (*lane 18 versus 13*). The lower band supershifts in the presence of anti-C/EBPβ (*lane 19*) and anti-C/EBPα (*lane 20*), indicating that the band corresponds to p42·LIP heterodimers. LIP homocomplexes are not evident since they exhibit a faster migration under these experimental conditions (Fig. 6*B*, *lane 18 versus 3*). Thus, p42-C/EBPα binds to satellite DNA as homo- or heterocomplexes with LAP or LIP. Taken together, all the members of the C/EBP family tested bind to the C/EBP site present in satellite DNA as homo- or heterocomplexes.

Endogenous Phosphorylated C/EBPβ from 3T3-F442A Preadipocytes Binds to Satellite DNA—To examine whether GH regulates the binding of endogenous C/EBPβ to the C/EBP site of satellite DNA, nuclear extracts from 3T3-F442A cells treated with GH for different periods of time were analyzed by EMSA using the sat-C/EBP probe. In untreated cells, relatively low but detectable binding of endogenous C/EBPβ complexes is observed (Fig. 7, *lane 1*), as bands, which supershift in the presence of anti-C/EBPβ (*lane 3*). GH treatment for 5, 30, or 60 min increases binding of C/EBPβ complexes to satellite DNA (Fig. 7, *lanes 4*, *7*, and *10*). Binding of C/EBPβ decreases after 6 h of GH treatment (*lane 13*). The presence of C/EBPβ complexes on satellite DNA is verified by the fact that they supershift in the presence of anti-C/EBPβ (Fig. 7, *lanes 6*, *9*, *12*, *15*, *arrowhead*). These results suggest that GH induces the binding of endogenous C/EBPβ complexes to satellite DNA.

C/EBPβ phosphorylated at its conserved MAPK consensus site is found in complexes bound to the c-*fos* C/EBP site (18). The presence of phosphorylated C/EBPβ in complexes bound to the C/EBP site in satellite DNA was analyzed by supershift with anti-P-C/EBPβ, an antibody that specifically recognizes C/EBPβ phosphorylated at its MAPK consensus site (18). In nuclear extracts from untreated cells, C/EBPβ complexes supershift slightly with anti-P-C/EBPβ (Fig. 7, *lane 2*, *arrowhead*). In contrast, within 5 min of GH treatment the increased C/EBPβ complexes associated with satellite DNA supershift with anti-P-C/EBPβ (Fig. 7, *lane 5*, *arrowhead*). The presence of phosphorylated C/EBPβ in complexes bound to sat-C/EBP probe then decreases 30–60 min after GH (*lanes 8* and *11*, *arrowhead*) and returns to basal levels 6 h after GH treatment (*lane 14*). Taken together, these results indicate that GH rapidly and transiently promotes the binding of endogenous C/EBPβ complexes to the C/EBP site of satellite DNA. Further, phosphorylated C/EBPβ at the MAPK site is present in complexes bound to satellite DNA. The presence of phosphorylated C/EBPβ in complexes bound to DNA within 5 min and then subsiding by 30–60 min is consistent with the timing of GH-dependent phosphorylation of C/EBPβ at the MAPK site, as previously reported (18).

MAPK Activation Is Required for Nuclear Relocalization of C/EBPβ—C/EBPβ is rapidly and transiently phosphorylated at the conserved MAPK consensus site in response to GH (18), and relocalizes to pericentric heterochromatin (Fig. 3). The importance of MAPK activation for nuclear relocalization of C/EBPβ was examined in 3T3-F442A cells using the MEK inhibitor U0126 in combination with GH. In quiescent cells C/EBPβ exhibits a predominantly diffuse pattern of nuclear distribution (Fig. 8, *panel A*, Fig. 1*a*) that rapidly changes to a punctate pattern of staining upon GH treatment (Fig. 8, *panel B*, Fig. 1*a*). In quiescent cells incubated in the presence of the

inhibitor U0126, C/EBPβ also exhibits a diffuse nuclear distribution (Fig. 8, *panel C*). It is notable that C/EBPβ remains diffusely distributed in GH-treated cells in the presence of the MEK inhibitor, indicating that the GH-induced nuclear pericentromeric relocalization of C/EBPβ is prevented (Fig. 8, *panel D*). HP-1α (Fig. 8, *panels E*, *F versus G*, *H*) and DAPI (Fig. 8, *panels I*, *J versus K*, *L*) staining demonstrates that organization of heterochromatin itself is not affected in the presence of U0126. This observation suggests that GH-dependent MAPK activation is required to regulate the nuclear redistribution of C/EBPβ. In the presence of U0126, GH treatment for up to 1h failed to induce C/EBPβ redistribution in areas of pericentric heterochromatin (data not shown), suggesting that inhibition of MAPK does not simply delay the nuclear relocalization of C/EBPβ. In addition, GH-promoted nuclear distribution of C/EBPβ retains its punctate pattern in the presence of inhibitors of PI-3K (wortmannin) or GSK-3 (Li^{2+})(data not shown), suggesting that MAPK signaling, but not PI-3K, is a key signaling pathway to promote nuclear redistribution of C/EBPβ in areas of heterochromatin. Taken together these results indicate that GH-induced MAPK activation is required for relocalization of C/EBPβ to areas of pericentric heterochromatin.

DISCUSSION

C/EBPβ Relocalizes to Areas of Heterochromatin—The current studies describe for the first time that nuclear localization of C/EBPβ can be regulated upon hormonal stimulus, and further show that such regulation utilizes a MAPK-mediated pathway. In quiescent 3T3-F442A fibroblasts C/EBPβ and C/EBPδ exhibit a diffuse, minutely speckled nuclear pattern of distribution. Upon GH treatment, C/EBPβ, but not C/EBPδ, relocalizes to areas of pericentric heterochromatin. Further, C/EBPβ co-localizes with proteins constitutively associated with heterochromatin, including HP-1α and kinetochore protein. In contrast, C/EBPδ localizes in different nuclear foci, exhibiting only a minor colocalization with C/EBPβ.

The nucleus is highly organized and recent studies have brought insight into the concept of nuclear compartmentalization (1, 29). The existence of numerous intranuclear compartments suggests that particular processes occur in specific locations within the nucleus. These compartments include the nucleolus, the splicing factor compartments, the large family of small nuclear foci, including Cajal bodies and promyelocytic leukemia bodies (PMLs), and a variety of nuclear inclusions that are often associated with degenerative diseases (1, 29, 42). Nuclear compartments are stable yet extremely dynamic structures, whose morphology represents the equilibrium between the release and binding of proteins. C/EBPβ and C/EBPδ, as well as many other transcription factors including the glucocorticoid receptor, the mineralocorticoid receptor, Oct1, and the basal transcription factor TFIIH, are found in discrete nuclear domains (3, 43). However, how these domains enriched in transcription factors are related to the regulation of target gene expression remains elusive. Analysis of nuclear localization of transcription factors shows that a relatively small fraction of the molecules of a given transcription factor is located at transcriptional sites, as shown in asynchronous HeLa cell culture (3). Thus, the domains enriched in transcription factors elsewhere in the nucleus may represent incomplete transcription initiation complexes, inhibitory complexes and/or storage sites. C/EBPβ, which is distributed in tiny speckles throughout the nucleus of quiescent cells, rapidly redistributes to areas of pericentric heterochromatin upon GH stimulation of the cells. This testifies to the dynamic equilibrium that nuclear foci exhibit, and the rapid movement of nuclear factors such as C/EBPβ. Further, C/EBPβ does not overlap with SFCs, which are adjacent to areas of active transcription (35). Therefore, it is relevant to examine the mechanism(s) through which C/EBPβ is redistributed in the nucleus to areas of heterochromatin.

C/EBPβ Binds to Satellite DNA—Differences between heterochromatin and euchromatin are well established (44–47). Most genes are contained in the transcriptionally active euchromatic compartment. In contrast to euchromatin, constitutive heterochromatin contains relatively few transcribed genes, remains condensed during interphase, replicates late, and is rich in repetitive sequences. At the sequence level, the repeats that are found in constitutive pericentromeric heterochromatin range from 5–7 bp satellite repeats in Drosophila centromeres, to 171 bp satellite repeats in human pericentric heterochromatin and 234 bp γ-satellite repeats that form the bulk of pericentric heterochromatin in murine cells. Putative C/EBP consensus binding sites are present in satellite DNA. Different C/EBPβ containing complexes bind to these sequences as shown in this and another study (22). Importantly, GH increases the binding of C/EBPβ complexes to the C/EBP site in satellite DNA. C/EBPβ, but not other transcription factors such as Elk-1 or CREB, binds to the C/EBP site of satellite DNA *in vitro*. It is tempting to speculate that redistribution of C/EBPβ in areas of heterochromatin is mediated, at least in part, through binding of C/EBPβ complexes to satellite DNA. However, C/EBPδ, which also binds as homo- or heterodimers to the C/EBP site in satellite DNA, shows a minor localization to areas of heterochromatin (Fig. 4). The differential nuclear localization of C/EBPβ and C/EBPδ raises the possibility that differential recruitment of members of the C/EBP family in areas of heterochromatin is achieved through specific protein-protein interaction(s). In this scenario, the interaction of C/EBPβ and not C/EBPδ with other protein(s) present in heterochromatin, such as HP-1α, may lead only C/EBPβ to concentrate in areas of heterochromatin. It is possible that cell type specific nuclear architecture may also contribute to the differential distribution of a transcription factor, since in 3T3L1 cells undergoing adipocyte differentiation C/EBPδ, as well as C/EBPβ and C/EBPα, localize in areas of pericentric heterochromatin (22).[2] In contrast, in 3T3-F442A cells, C/EBPδ does not localize in areas of heterochromatin even when the cells undergo adipocyte differentiation.[2] This observation reinforces the existence of differences in spatial organization in the nucleus between different cell lines which may account for differential regulation of localization of nuclear factors and differential transcriptional regulation.

Nuclear Relocalization of C/EBPβ Depends on MAPK Signaling—GH-dependent activation of MAPK (extracellular signal-regulated kinases 1 and 2) appears to be required for nuclear redistribution of C/EBPβ, since in the presence of MAPK inhibitors, the GH-dependent relocalization of C/EBPβ to pericentric heterochromatin is blocked. The possibility that redistribution of C/EBPβ to areas of heterochromatin could depend on binding of C/EBPβ to C/EBP sites present in satellite DNA has been discussed. However, mutation of the Thr that corresponds to the MAPK site does not interfere with the binding of LAP to satellite DNA (Fig. 5*C*). Therefore it is tempting to speculate that binding of C/EBPβ to satellite DNA does not require MAPK-mediated phosphorylation but relocalization of C/EBPβ to areas of heterochromatin does require MAPK-dependent phosphorylation of C/EBPβ and/or other protein(s) involved in the nuclear movement of C/EBPβ. Further, although phosphorylation at the MAPK site is rapid (5 min of GH treatment) and transient (subsides within 30–60 min), C/EBPβ remains concentrated in areas of heterochromatin for

[2] G. Piwien-Pilipuk and J. Schwartz, unpublished results.

6 h after GH treatment. It is possible that MAPK signaling may be required for phosphorylation of other nuclear factor(s) that maintain the nuclear redistribution of C/EBPβ upon GH stimulation.

What Is the Functional Importance of Nuclear Redistribution of C/EBPβ?—Pericentromeric heterochromatin appears to be critical for regulating transcription in a number of cell systems (42). In developing B and T lymphocytes, genes that are transcriptionally silenced undergo a dynamic repositioning in the nucleus and become localized at pericentric heterochromatin (48, 49). It is thought that the cell cycle-related or developmentally controlled placement of a gene from transcriptionally active euchromatin to transcriptionally inactive pericentromeric heterochromatin may silence genes. Progressive heterochromatinization of most of the genome is also associated with terminal differentiation of diverse cell types, which include glial cells, plasma cells, and reticulocytes. C/EBPβ plays a key role during differentiation of a number of cell types, including adipocytes (9–11), hepatocytes (12), and cells of the hematopoietic system (13, 14). C/EBPβ also plays a role in mammary gland development (15) and in ovulation (16, 17). C/EBPβ has been shown to localize in areas of pericentric heterochromatin in 3T3-F442A predipocytes upon GH treatment, as well as during the induction of adipocyte differentiation of 3T3-F442A[2] and 3T3-L1 cells (22). These observations raise the possibility that relocalization of C/EBPβ to areas of heterochromatin may contribute to silencing of genes during the programming of the subset of genes required to achieve the final differentiated cellular phenotype.

On the other hand, it has been proposed that even when a gene remains associated with heterochromatin, the juxtaposition with repetitive DNA is not incompatible with expression of the gene. However, transcription of a gene in a heterochromatic environment is thought to require a strong activator to overcome the repressed state (50, 51). An example is provided by the gene λ5, which is expressed by pre-B cells and silenced as cells differentiate into immunoglobulin secreting mature B cells (52). When a λ5 transgene is placed in a heterochromatic environment, it is totally inactive and inaccessible to DNase. Binding of transcription factors to the heterochromatinized λ5 promoter can induce the translocation of the λ5 locus to the surface of the centromeric heterochromatin, but still does not induce the gene to be actively transcribed. However in a cellular context (only in lymphoid cells) and in the presence of the appropriately elevated concentration of its activator, the λ5 gene could be transcribed even when it remained on the surface of the heterochromatic domain (51). This suggests that juxtaposition of a gene to heterochromatin is not incompatible with its expression when a strong activator is present to overcome the repressed state. Intriguingly, C/EBPβ concentrates in areas of pericentric heterochromatin when it is rapidly and transiently phosphorylated upon GH-dependent MAPK activation. Further, such phosphorylation is required for LAP to be transcriptionally active in the context of the c-*fos* promoter (18). Thus, it is tempting to speculate that concentration of transcriptionally active C/EBPβ in areas of heterochromatin allows it to function as a strong activator for genes located in a heterochromatic environment. Another possibility is that C/EBPβ recruits a co-activator that facilitates transcription. It has been shown that overexpressed C/EBPα also concentrates in pericentromeric heterochromatin in a pituitary cell line, and that it recruits the co-activator CBP, which possesses histone acetylase activity (53). Heterochromatin is globally deficient in acetylated histone H3, and the expression of C/EBPα appears to overcome this deficit by recruitment of CBP (54). It has been proposed that recruitment of CBP to heterochromatin by C/EBPα may mediate histone acetylation, and in consequence modulate transcription (53, 54). C/EBPβ also interacts with p300/CBP (55, 56). Further, we have observed not only an interaction between C/EBPβ and p300/CBP but also an enhancement of C/EBPβ-mediated transcription by p300/CBP.[3] It will be of interest to determine whether C/EBPβ, like C/EBPα, recruits and concentrates p300 in areas of heterochromatin, and whether such an interaction would mediate changes in histone acetylation. On the other hand, since the LIP form of C/EBPβ is a well known inhibitor of transcription (8, 19) localization of LIP in heterochromatin may be correlated with silencing of genes. LIP heterodimerizes with LAP and LAP·LIP complexes are thought to be transcriptionally inactive. Inasmuch as C/EBPβ plays a role in the differentiation of various cell types, its localization to heterochromatic foci raises the possibilities that C/EBPβ may participate in the silencing of some genes, and/or the activation of other genes, potentially by overcoming a threshold in the concentration of C/EBPβ or by recruiting nuclear factor(s).

In summary, GH promotes relocalization of C/EBPβ to foci of heterochromatin, in association with the activation of MAPK signaling. Such rapid relocalization introduces a new level of transcriptional regulation in GH-dependent gene expression, since GH-mediated phosphorylation and nuclear redistribution of C/EBPβ may be coordinated to achieve spatial-temporal control of gene expression.

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