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Okadaic Acid Stimulates H Ferritin Transcription in HeLa Cells by Increasing the Interaction between the p300 CO-Activator Molecule and the Transcription Factor Bbf

M. A. Bevilacqua,* M. C. Faniello,* F. Cimino,*[,1] and F. Costanzo*[,†]

**Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli 'Federico II', Via S. Pansini 5, I-80131 Napoli, Italy; and †Dipartimento di Medicina Sperimentale e Clinica, Università degli Studi di Reggio Calabria, Via T. Campanella, I-88100 Catanzaro, Italy*

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The transcription of the human H ferritin gene is regulated by a transcription factor, called Bbf, which binds an enhancer element located in the −100/+1 region of the H promoter. To evaluate a possible role of Bbf phopshorylation on the promoter efficiency, we exposed HeLa cells to the phosphatase inhibitor okadaic acid (OA). The okadaic acid treatment increased about 4-fold the transcription driven by the −100/+1 region of the H promoter. However, the DNA binding activity of Bbf was not modified by OA, as assessed by EMSA. Immunoprecipitation experiments demonstrated that the OA-treatment stimulates and/or stabilizes the complex between Bbf and the nuclear protein p300, most probably by inducing the phosphorylation state of the complex. Bbf depends on the p300 molecule to trigger RNA polymerase II and thus transcription of the H ferritin gene.

Phosphorylation of nuclear transacting factors generally represents the final event of a signal transduction pathway. The phosphorylation of specific residues could either increase the DNA binding activity of the transcription factor (1), or may allow the interaction of the transcription factor with cognate proteins in the nucleus (2). This activation process is mediated by protein kinases (PKs) and is reversibly counteracted by protein phosphatases (PPs). Thus, the extent of transcriptional activation of a given gene could be controlled -via specific transcription factors- by activation of one or more PK(s) or by changes in the activity of the related PPs (for a review, see 3).

Ferritin is an ubiquitary iron storage protein. The ferritin shell, in which the free iron is sequestered, is composed, in humans, by 24 subunits of the heavy (H) and light (L) types (4). The coordinate expression of the H and L genes (5, 6) is responsible for the type of isoferritins expressed within a specific cell type (7). Ferritin gene expression varies during cell differentiation (8, 9) and neoplastic transformation (10) as well as in response to a variety of stimuli (11, 12, 13). Moreover, the H gene promoter appears to be positively regulated by different hormones (14, 15) and by cAMP (16, 17, 18).

The DNA control elements of the H gene transcription are included in 170 bp upstream the transcription start site. They include a canonical Sp1 site in position −132 (A-site) and an inverse CCAAT box, in position −62 (B-site) (19). In basal conditions the B-site accounts for about 50% of the total strength of the promoter, while its role is critical in stimulatory conditions. Trans-activation of the H promoter is due either to an increased binding of the transcription factor Bbf (B-box-binding factor) to the B-site (9, 11), either to an increased binding of Bbf to the nuclear protein p300 (18).

Here we show that okadaic acid (OA), a tumor promoting agent that also acts as a potent phosphatase inhibitor, increases the efficiency of the H ferritin promoter in HeLa cells. The B-element is the target site of OA stimulation. EMSA and immunoprecipitation experiments revealed that the binding efficiency of Bbf to the DNA is not modified by the phosphatase inhibitor, while the Bbf/p300 complex is more abundant in the treated cells. p300 is an essential component of the transcriptional complex on H ferritin promoter (18); the results on OA-treated HeLa cells suggest that phosphorylation plays an important role in the formation and/or stabilization of this complex.

MATERIALS AND METHODS

Enzymes and chemicals. DNA polymerase large fragment, T4 polynucleotide kinase, ^{32}P-labelled compounds and ^{14}C- labelled chloro-

[1] To whom correspondence should be addressed. Fax: +39.81.7463650.

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amphenicol were supplied by Amersham. Acrylamide and agarose were from Bio-Rad Laboratories. The acetyl-coenzyme A, the okadaic acid and the fetal calf serum were supplied by Sigma. Dulbecco's modified Eagle's medium was supplied from Life Technologies. Penicillin was supplied from Hyclone.

Cell culture. HeLa cells were cultured as monolayers in DMEM medium supplemented with 10% (v/v) fetal calf serum. Okadaic acid was added directly to the culture medium at a final concentration of 100 nM for the indicated times.

DNA transfection, CAT and luciferase assays. The H promoter/CAT construct 5′ H H/A has been previously described (19). The CRE-CAT construct was a gift of Dr. P. Sassone-Corsi (20). Transfections were carried out with the calcium-phosphate technique (21), using 10 pmoles of total transfected DNA/dish. Variations in transfection efficiency were corrected by including in each experiment the luciferase gene under the control of the CMV promoter (CMV-luc construct) (22). CAT assays were performed as described (23). The luciferase activity was assayed as described (21) with a luminometer (Berthold, model LB9501). The data reported in Fig. 1 are the mean of the results of three independent transfection experiments.

EMSA and immunoprecipitations. Nuclear extracts and EMSAs were performed as previously described (9, 11). To study DNA/protein interaction on the B-site, it was used the −100/+1 region of the H promoter, terminally labelled. Whole-cell extracts and deoxycholate elutions were performed as described (18, 24), using an anti-p300 antibody (05-222 UBI).

RESULTS AND DISCUSSION

H Ferritin Transcription in Okadaic Acid-Treated HeLa Cells

The H ferritin gene expression in cells exposed to the phosphatase inhibitor Okadaic acid (OA) was first evaluated at the mRNA level by northern blot hybridization. Total RNA, extracted from untreated HeLa cells and from 2 and 4 hours-treated cells was probed with an H ferritin cDNA (5). The results demonstrated a significant increase in the steady-state amount of the H mRNA in the cells exposed to the drug compared to the untreated ones (data not shown).

The amount of the H and L ferritin mRNAs is controlled by nuclear and cytoplasmic mechanisms; thus, the transcripts level could be determined by variations in transcriptional efficiency of the genes (9, 13, 14) or by modifications in the mRNAs half-life (25). To evaluate if the OA-treatment acts at the transcriptional or at the post-transcriptional level, we performed transient transfection assays with H promoter/CAT fusions in treated and untreated HeLa cells. Specifically, we have used a construct, named 5′ H H/A (19), in which the transcription of the CAT reporter gene is driven by the region of the H promoter from position −100 to position +1. This promoter segment harbours, along with the TATAA box, a positive regulatory element, called B-site, from position −62 to −45. The B-site binds a factor (Bbf for B-box-binding factor), required for both basal and induced transcription of the H gene (9, 11, 18, 19).

The 5′ H H/A plasmid was transiently transfected in untreated HeLa cells and in cells exposed for 4 hours to OA, together with a CMV-luciferase construct, and the relative efficiency in promotion of transcription of the CAT gene was normalized to the luciferase activity. As a positive control, HeLa cells were also transfected with a CRE-CAT construct, in which the CAT gene is under the control of a cAMP-responsive element (CRE) derived from the somatostatin promoter (20). The CRE sequence binds the transcription factor CREB, whose activity is positively modulated by phosphorylation. The negative control of the experiment is represented by the RSV/CAT construct, in which the CAT gene transcription is driven by the Long Terminal Repeat of Rous Sarcoma Virus.

The results are shown in Panel A of Fig. 1: 4 hours treatment of HeLa cells with OA increases about 4-fold the transcription efficiency of the ferritin promoter as well as the transcription driven by the CRE element. Panel B of Fig. 1 shows a time-course experiment: 4 hours of continuous stimulation with OA determines the maximal response driven by the ferritin promoter.

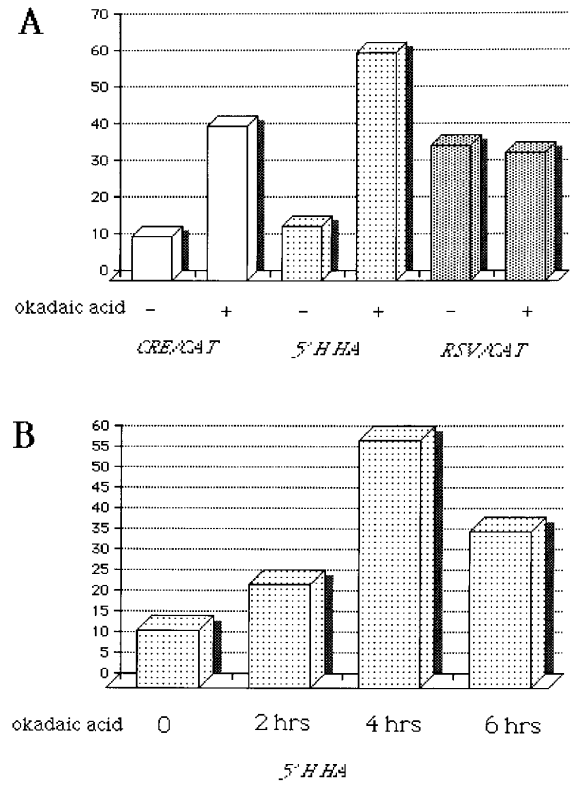

FIG. 1. Ferritin promoter activity in untreated and OA-treated HeLa cells. Panel A: CAT activity driven by the CRE element from somatostatin promoter (CRE-CAT), from the −100/+1 region of the H ferritin promoter (5′ H HA) and from the LTR of Rous Sarcoma Virus (RSV-CAT), transiently transfected in HeLa cells untreated and treated for 4 hours with OA. Panel B: CAT activity driven by the −100/+1 region of the H ferritin promoter (5′ H HA) in HeLa cells treated for the indicated times with OA. The values are the average of three independent transfection experiments, and are expressed as per cent of the acetylated form of the ^{14}C chloroamphenicol.

These experiments indicate that the increased H ferritin expression induced by the OA-treatment is due to an enhanced transcription of the gene. The region of the promoter from −100 to +1 contains the sequence element responsible for this induction.

Bbf Binding to DNA in Treated and Untreated HeLa Cells.

As already mentioned, the −100/+1 region of the H promoter binds a transcription factor, called Bbf (9, 11, 18, 19). Bbf binding to DNA is incremented in G418-treated cells (11) and during the spontaneous differentiation of Caco-2 cell line (9); in both cases, the increased binding of Bbf to the DNA transactivates the H promoter. Bbf also mediates the cAMP-stimulation of the H ferritin transcription (18).

As next step, we compared Bbf binding to its DNA element in unstimulated and OA-stimulated HeLa cells. Nuclear extracts prepared from the wild-type cells and from 4 hours-treated cells were challenged with the −100 to +1 region of the H promoter, radioactively labelled, and the protein/DNA complexes were analyzed by EMSA. The results, shown in Fig. 2, demonstrate that the intensity of the specifically retarded complex was essentially the same in all the samples (compare lane 2 with lanes 5 and 6), thus indicating that the binding of Bbf to the B element was not stimulated by OA.

Okadaic Acid Stimulates the Formation of Bbf/p300 Complex.

We have recently found that Bbf forms a functional complex with the nuclear protein p300, and that cAMP signalling, by stimulating the assembly of this complex, induces the H ferritin transcription (18). p300 essentially functions as a co-activator molecule, mediating the contact of several transacting factors with TFIIB (26). In most cases, such as CREB and Jun, phosporylation plays a critical role in inducing the assembly of this complex (26, 27).

Okadaic acid does not stimulate the formation of the Bbf/DNA complex, thus suggesting that the effect of the drug is exerted at the level of Bbf molecules already located on the DNA. We sought to analyze if OA effect was to stimulate the association of Bbf with p300. To this, HeLa total cell extracts were immunoprecipitated with an anti-p300 antibody; the immunoprecipitates were eluted with deoxycholate according to (24), and challenged with a radioactive B-site probe. The p300/Bbf complexes were then analyzed by EMSA. The results, depicted in Fig. 3, show that the intensity of the p300/Bbf immunocomplex rescued by the B-site probe in extracts from OA-treated cells is significantly more intense than that from untreated cells (compare lanes 2 and 6).

In *vivo* treatment of HeLa cells with okadaic acid thus results in an increase in the ability of Bbf to bind p300 and/or in a stabilization of the complex.

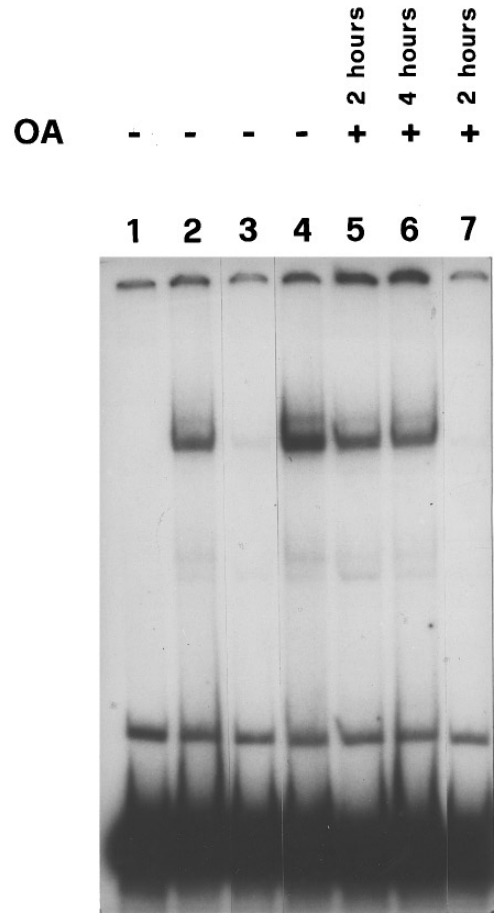

FIG. 2. EMSA of the −100/+1 region in untreated and OA-treated HeLa cells. The −100/+1 region from the H promoter, terminally labelled, was incubated with 4 μg of nuclear extracts from untreated (− OA) and from OA-treated HeLa cells (+ OA), as described under Materials and Methods. Lane 1: no extract. Lane 2: 4 μg of extract from untreated cells. Lane 3: as lane 2 plus 100-fold molar excess of unlabelled DNA fragment. Lane 4: as lane 2 plus a 100-fold molar excess of unrelated competitor oligo. Lane 5: no extract. Lane 6: 4 μg of extract from treated cells. Lane 7: as lane 6 plus a 100-fold molar excess of unlabelled DNA fragment. Lane 8: as lane 2 plus a 100-fold molar excess of unrelated competitor oligo.

Okadaic acid is a tumor promoting agent that specifically inhibits a variety of phosphatases, including PP1 and PP2A and the related PPX and PP5 (3). The phosphatase inhibitors have been extensively used to study the role of phosphorylation/dephosphorylation of transacting factors in mediantingtranscritpional control. The analysis on Bbf activity in OA-treated cells indicate that this H ferritin transcription factor does not belong to the class of transacting factors dependent on phosphorylation for DNA binding. Conversely, OA-treatment of HeLa cells results in an increased amount of the Bbf/p300 complex, which, in turns, transactivate the H promoter. It is likely that the increased associa-

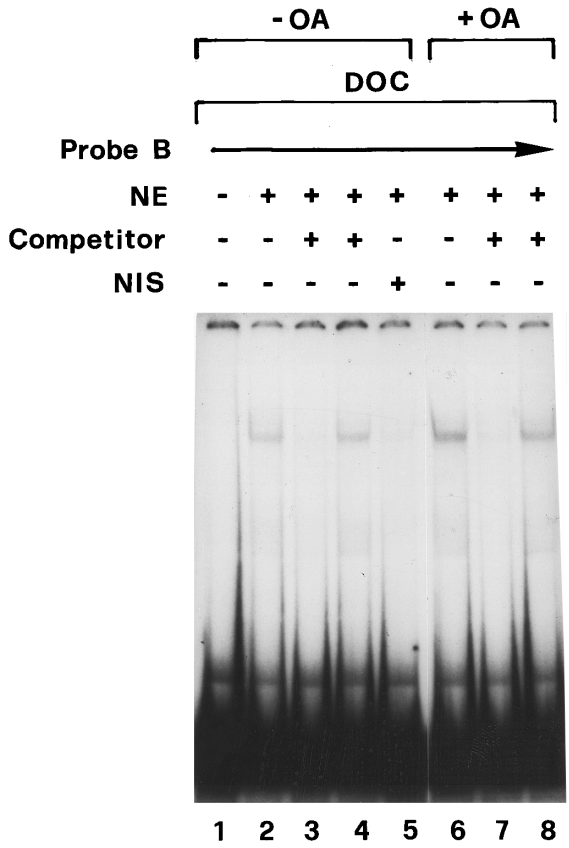

FIG. 3. Bbf/p300 complex in untreated and OA-treated HeLa cells. Whole-cell extracts from untreated (− OA) and from OA-treated HeLa cells (+ OA) were immunoprecipitated with anti-p300 antibody (lanes 2, 3, 4,. 6, 7, 8) or with not-immune antisera (NIS, lane 5). Deoxycholate elutions of the immunoprecipitates (DOC) were then analyzed by EMSA with the −100/+1 region terminally labelled (B-probe). Lane 1: no nuclear extract. Competitions were performed with a 100-fold molar excess of unlabelled DNA fragment (lanes 3 and 7) or with a 100-fold molar excess of unrelated competitor oligo (lanes 4. and 8).

tion of p300 with Bbf is due to the dephosphorylation block induced by the drug. This finding is in agreement with the stimulation of H gene transcription due to cAMP signalling (18).

The identification of the Bbf/p300 complex as the target of phosphorylation/dephosphorylation processes open the way to study the interplay between ferritin gene transcription and different signal transduction pathways.

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