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The success of anti-estrogen tamoxifen treatment of breast cancer and the discoveries that in a large number of breast tumors, proliferation of cells is stimulated by estrogen while retinoic acid exerts inhibitory effects have generated extensive interest among both clinicians and basic scientists. Presented studies have revealed a novel nuclear receptor coactivator of transcription referred to as p/CAF that is required for estrogen-, thyroid hormone- and retinoic acid-dependent gene expression. DNA bound nuclear receptor directly recruits its coactivator p/CAF, and the association of p/CAF with a nuclear receptor depends upon ligand-dependent nuclear receptor corepressor (NCoR) dismissal. The critical role of the intrinsic acetyltransferase enzymatic activity of p/CAF in hormone regulated transcription provides a link between the function of this coactivator and steroid-dependent regulation of cell growth in pathogenesis of breast cancer. A substitution mutation eliminating acetyltransferase activity has been introduced into the *p/CAF* gene locus in order to examine biological function of this gene in mouse. These studies have advanced our understanding of the molecular mechanism of hormone regulated gene expression and might open a new direction for improved treatment of breast cancer.

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INTRODUCTION

This project focuses on the role of nuclear receptor coactivators in the regulation of gene expression that has been linked to pathogenesis of breast cancer. The success of anti-estrogen tamoxifen treatment of breast cancer and the discoveries that in a large number of breast tumors, proliferation of cells is stimulated by estrogen while retinoic acid exerts inhibitory effects have generated extensive interest among both clinicians and basic scientists. The molecular mechanisms through which these hormones exert their effects and regulate cell growth remain obscure. The most promising aspect of endocrine therapies is a low toxicity rate when compared to the conventional cancer chemotherapy although this treatment is not a cure and patients suffer from a few serious side effects. Thus there is a sustained necessity to develop newer therapies that extend the effectiveness of those currently available. This will be possible only when our knowledge increases to the level that uncovers detailed molecular mechanisms controlling the hormone-dependent gene expression. Nuclear receptors exhibit ligand-dependent previously described interactions with a series of coactivators that include CBP/p300 (1, 2, 3) and the biochemically-identified p160 proteins (4) which have been identified as the structurally-related SRC-1/NCoA-1 (3, 5), TIF2/GRIP-1/NCoA-2 (6, 7) and p/CIP (8) as well as with p/CAF (9) for which critical function in nuclear receptor action was discovered during realization of this research project and reported in Annual Summary for 1998. This report presents data showing selectivity in the specific histone acetyltransferase activity (HAT) required for function of distinct classes of transcription factors. This enzymatic activity might directly destabilize promoter bound nucleosomes facilitating activation of transcription. The histone acetyltransferase activity of p/CAF, but not of CBP, appears to be indispensable for nuclear receptor activation, where CBP is likely to be recruited based on interaction with a complex containing p/CAF and SRC-1. Conversely, the HAT activity of CBP, directly recruited by phosphorylated CREB is required for its transcriptional function. In the case of nuclear receptors, p/CAF and SRC-1/p/CIP bind receptors in a ligand-independent and ligand-dependent fashion, respectively, with p/CAF recruitment apparently dependent upon dismissal of the NCoR, corepressor complex. Recently p/CAF has been found in the complex with more than 20 distinct subunits (10) homologous or identical to components of TFIID complex indicating that p/CAF complex might play an alternative role to TFIID complex in transcription. Together, these data imply that p/CAF plays a central role in nuclear receptor-dependent gene expression. The recent observation that overexpression of histone acetyltransferase targeted to chromatin may contribute to cancer development (11, 12) emphasizes importance of this field of study for the cancer research program. The later phase of this research project was mainly focusing on a genetic analysis and manipulation of the *p/CAF* gene. The examination of *p/CAF* gene function is essential for unraveling of the molecular mechanism of hormone action and provides a tissue-targeted means of manipulating hormone-regulated gene expression for a study of steroid-dependent breast cancer pathogenesis.

BODY

Methods

Generation of Anti-p/CAF IgG

A cDNA fragment corresponding to p/CAF amino acids 466-832 generated by restriction digestion from AC611 cDNA clone was subcloned into the pQE31 vector (Quiagen) containing an in-frame His tag and recombinant HIS-tagged proteins were generated and purified by nickel chelate chromatography to homogeneity. The purified recombinant proteins were injected into guinea pig, and antibodies were generated and affinity purified on Protein A-Sepharose FPLC using standard procedures (13).

Isolation of p/CAF cDNA

The screen for nuclear receptor coactivator with histone acetyltransferase activity was carried out employing Gene Trapper cDNA Positive Selection System (Life Technologies. Inc.). The advantage of this particular screening technology is a very fast data collection process but it requires using a plasmid cDNA library. In this system the biotinylated oligonucleotide complementary to a segment of target cDNA is hybridized to ssDNA, a product of conversion of ds phagemid DNA containing cDNA library using Gene II endonuclase and E. coli Exonuclease III. Hybrids are captured on streptavidine-coated paramagnetic beads and retrieved by magnet from solution. After release, the desired cDNA clone is recovered by specifically prime conversion to dsDNA following by transformation. A cDNA clone, AC611 (2 Kb) was isolated from a human fetal cDNA library (Gibco BRL) using oligonucleotide: TTG GAT ACT TTA AGA AAC AGG G corresponding to amino acids 218-225 of yGCN5 protein. The oligonucleotide sequence was based on the sequence found in randomly sequenced human complementary DNAs (Database of Gene Bank EST Division), clone # N39522 which revealed significant homology to yeast GCN5 protein. Obtained clone turned out to be a partial clone of the recently published p300 and CBP associated factor (p/CAF) corresponding to amino acids 301-832 of full length p/CAF (14). Subsequently, a full length p/CAF cDNA clone, pCX-Flag-p/CAF, was kindly provided by Y. Nakatani (14).

Yeast Two-hybrid Interaction Assays

The yeast strain EGY 48, the LexA-b galactosidase reporter construct (PSH 18-34) and the B42 parental vectors (PEG 202 and PJG 4-5) were all previously described (15). Various p/CAF fragments obtained by PCR or restriction digestion were subcloned into PEG 202 bait vector. DNA fragments of p/CIP , SRC-1, CBP or RAR proteins were generated either by using an appropriate restriction digest or by PCR and subcloned into PJG 4-5 prey vectors. EGY 48 cells were transformed with the *lac Z* reporter plasmid pSH 18-34 with the appropriate bait and prey vectors, and plated out on -Ura-His-Trp medium containing 2% galactose. Isolated yeast colonies were then allowed to grow in the same liquid medium, followed by assaying for b galactosidase, as previously described (16).

Transient Transfections and Reporter Assays

Transfection experiments were conducted in either HeLa cells using the standard calcium phosphate procedure (16). Cells were co-transfected with 1.0ug of a (UAS)3-luciferase reporter and the 5ug of GAL4 fusion proteins, and harvested 48 hrs later in order to perform luciferase activity assay.

Immunoprecipitations, Western Blot, GST-TNT Interaction Assays

Whole cell extracts were prepared by lysing the cells in NET-N buffer containing 50 mM Tris (pH 7.6), 5 mM EDTA, 0.3 M NaCl, 1mM DTT, 0.1% NP-40 and protease inhibitors (.2 mM PMSF, 10 ug/ml each of leupeptin, pepstatin and aprotinin), centrifuged at 30K for 1 hr at 4°C and the supernatant was stored at -80° C until use. For co-immunoprecipitation assays, 1 mg of cell extract was incubated in the presence of 2 ug of anti-p/CIP, or anti-Flag, or anti-retinoic acid receptor (RAR) IgG for 2 hrs at 4°C. The immune complexes were then precipitated with Protein-A Sepharose. Protein complexes were resolved by SDS-PAGE (17) and Western blotted and probed using 1 ug/ml of an anti-HA, or anti-Flag, or anti-p/CIP. TTNPB and LG629 were kindly provided by R. Heyman.

GST-RAR and GST-CBP (1458-1891) were generated as described (3). 25 ul of GST-sepharose beads containing 3-6 ug of the GST recombinant proteins were incubated with $5x10^5$ CPM of ^{35}S-labelled p/CAF proteins generated by *in vitro* transcription and translation for 2 hrs at 4° C. The complexes were washed 5 times with NET-N buffer, resolved by SDS-PAGE and fluorographed.

Mutagenesis

Mutation in p/CAF were introduced by site-directed mutagenesis using the Quick Change mutagenesis kit according to the manufacturers instructions (Stratagene). Double stranded oligonucleotides were designed in such a way that the wild type sequence corresponding to amino acids Y615/F616 (acetyl CoA binding site) in p/CAF cDNA were substituted with alanines in order to generate mutant of p/CAF lacking HAT activity, pCMV-p/CAF$_{HAT^-}$. A similar strategy was used to obtain mutant of CBP. CBP was expressed in Baculovirus and tested for histone acetyltransferase activity in solution using histones as a substrate (18).

Single Cell Microinjection Assay

Insulin-responsive Rat-1 fibroblasts were seeded on acid washed glass coverslips at subconfluent density and grown in MNE/F12 medium supplemented with 10% fetal bovine serum, gentamicin and methotrexate. Prior to the injection, the cells were rendered quiescent by incubation in serum-free medium for 24-36 hours. Plasmids were injected into the nuclei of cells at a final concentration of 100 mg/ml. Peptides were injected at a concentration of 200 mM. Either preimmune IgG of the appropriate species or antibodies directed against p/CAF, p/CIP, NCoA-1, or CBP were co-injected and allowed the unambiguous identification of the injected cells. Microinjections were carried out using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Approximately one hour after injection, the cells were stimulated, where indicated, with the appropriate ligand. In the case of rescue experiments, the cells were stimulated with ligand six hours after injection, to allow protein expression. After

overnight incubation, the cells were fixed and then stained to detect injected IgG and b-galactosidase expression (3, 19). Injected cells were identified using staining method with tetramethylrhodamine-conjugated donkey anti-rabbit IgG.

p/CAF expression pattern in mouse embryo.

In situ hybridization on mouse embryos (129/Sv inbred mouse strain) were performed as previously described (20) using the p/CAF cRNA as a probe on frozen cryostat sagittal sections from mouse embryos. At the time of sacrifice on gestational day (GD) 11, 12, 14, 15, 16, or 18, pregnant dams were anesthetized. Embryos were removed from the uterus, fixed with 4% paraformaldehyde, frozen in embedding medium (50% Tissue-Tech, Miles, Inc., 50% Aqua-Mount, Lerner Lab., Inc.), and cut frozen.

Generation of p/CAF$_{HAT^-}$ mutant mice.

The basic procedures for generating mutations in mice by homologous recombination are extensively utilized in M.G. Rosenfeld laboratory (21, 22, 23). Briefly, the procedure consists of the following steps: 1) A targeting vector is built that will undergo homologous recombination with the gene to be disrupted. 2) Mutations are produced in embryonic stem (ES) cells in culture by homologous recombination of the target gene with the introduced DNA. The genomic DNA from each clone is analyzed for homologous recombination by a PCR strategy and by examination of the restriction fragment length polymorphism (RFLP) at the locus. For PCR analysis, one primer recognizing specifically introduced mutation (or initiating in the Neo resistance cassette) and one primer initiating in the appropriate genomic locus will be used. For Southern blots of restriction enzyme-cleaved genomic DNA, the fragment chosen as a probe will be from a region of the locus that is either upstream or downstream from the area contained in the replacement vector. The probe is designed that the restriction fragment of the locus is altered in size as a result of a targeted disruption. 3) Mutant ES cells are introduced into host blastocysts that are in turn introduced into pseudopregnant mice and allowed to develop into chimeric mice. 4) The chimeric mice are bred to determine if the ES cells contribute to the germ line of the chimera. If so, mutations that they carry can be transmitted to future generations.

Results

The recent finding that some of the coactivators have an intrinsic histone acetyltransferase activity directed our attention towards nuclear proteins that possess homology to HAT domain found in yeast coactivators and are required in nuclear receptor dependent gene activation. HAT activity in coactivator complex is thought to play essential role during activation of transcription by regulating acetylation of nearby histones. Histone acetyltransferase, GCN5 was one of several proteins identified by genetic selection including ADA2 (24, 25) that were required for function of certain classes of activation domains (26, 27, 28, 29). This prompted a screen for coactivators involved in nuclear receptor gene activation containing a histone acetyltransferase domain homologous to yeast GCN5 (SOW: Task 1). This screen was performed using Gene Trapper cDNA Positive Selection System (Life Technologies. Inc.) (see Methods). Sequence analysis of

isolated clone revealed that it was a partial clone of the p300 and CBP associated factor (p/CAF) corresponding to amino acids 301-832 of full length p/CAF (14) (SOW: Task 2).

In order to define the potential function of p/CAF in nuclear receptor-dependent regulation of gene transcription a single cell microinjection of specific blocking IgG against p/CAF (aa 465-832) (Methods) developed in guinea pig (SOW: Task 4, 5) revealed that p/CAF was required for ligand-dependent activation, not only of estrogen-, but also of thyroid hormone- and retinoic acid-dependent transcription units (Fig. 1). Other transcription units including a promoter under the control of multiple SP1 sites were unaffected by anti-p/CAF IgG (Fig. 1) suggesting that p/CAF is not required for a function universal to all transcription units (SOW: Task 5). In cases where no specific IgGs were used, preimmune rabbit or guinea pig IgG was co-injected, allowing the unambiguous identification of the injected cells in addition to serving as a preimmune control for the experiment (3, 19). The observed specificity of p/CAF function is consistent with observations concerning selective functions of GCN5 in yeast (26, 27, 28, 29).

Co-immunoprecipitation assays performed from cell extracts revealed that p/CAF exhibits ligand-dependent recruitment to the retinoic acid receptor coactivator complex. p/CAF binding was abolished by presence of an RAR antagonist (Fig. 2A). However, there was only minimal ligand-dependence of this interaction in a yeast two-hybrid assay, with the interaction domain mapping to amino acids 1 to 351 of p/CAF (Fig. 2B). Thus, the N-terminal domain of p/CAF is directly involved in maintaining the interaction with nuclear receptor (SOW: Task 11). Using the avidin, biotin complex DNA assay to assess protein interactions on DNA bound receptors, there was no detectable ligand-dependence for association of p/CAF with RAR/RXR heterodimers (Fig. 3). However, the interaction between nuclear receptor and p/CAF was inhibited by the nuclear receptor corepressor (NCoR) (30), and restored on ligand-induced dismissal of NCoR from the receptor (Fig. 3A), indicating that ligand-dependent association of p/CAF in cells (Fig, 2A) depends upon release of the nuclear receptor corepressor complex (31). In contrast to the p160 coactivators, p/CAF interactions occurred independent of the AF2 domain (Fig. 3B). Together, these data reveal that the ligand-dependent recruitment of p/CAF to the activated nuclear receptor is distinct from the AF2-dependent mechanism of interaction between SRC-1/p/CIP and nuclear receptors (4, 32, 33, 34).

GST pull-down and yeast two hybrid assays revealed that, in addition to the previously described interaction between p/CAF and the C/H3-E1A interaction domain of CBP (14), the N-terminal region of p/CAF was also capable of direct interaction with the N-terminal region of CBP (Fig. 5, 6) (SOW: Task 6, 8). Consistent with the observation that both NCoA-1/SRC-1 (3, 5, 35) and p/CIP (8) could be co-immunoprecipitated with p/CAF present in cell extracts (Fig. 4A and (8, 35, 36), yeast two hybrid assays (Fig. 4B) demonstrated that the N-terminus of p/CAF mediated interactions with SRC-1 (Fig 4B), while the most effective p/CIP interaction domain with p/CAF was delineated to aa 649-725 (Fig. 4B), corresponding to the conserved regions of yeast and human GCN5 that interact with ADA2 (26, 27, 28, 29). These findings revealed the presence of multiple potential interaction interfaces between members of the coactivator complex (Fig. 6).

Based on a number of mutagenesis studies demonstrating that single amino acid substitutions, particularly in acetyl-CoA binding region of acetyltransferases, resulted in loss of enzymatic activity (37), a p/CAF mutant protein harboring a substitution of two conserved residues (Y616/F617 to A616/A617) was generated. This mutant had no intrinsic HAT activity (p/CAF_{HAT^-}) as was shown in liquid histone acetyltransferase assay (Fig. 7C). Two amino acid mutation (L1690/C169 to K1690/L1691) in CBP abolished its histone acetyltransferase activity (CBP_{HAT^-}) (Fig. 7C).

These mutants of p/CAF and CBP lacking a histone acetyltransferase activity allowed for an evaluation of the role of the histone acetyltransferase activity of p/CAF and CBP in transactivation function of specific classes of transcription factor. Blockade of both CBP and p/CAF activity by coinjection of both specific blocking IgGs, which almost abolished the transcriptional activity of retinoic acid receptor or CREB, was entirely reversed by coinjection of expression vectors expressing wild-type CBP and p/CAF (Fig. 1 and 7A, B). Conversely, expression of both factors, mutated to abolish acetyltransferase function mutation (HAT^-), failed to effectively rescue activation (Fig. 7A, B). A failure to rescue retinoic acid receptor activity was also observed with expression of wild-type CBP and p/CAF_{HAT^-} (Fig. 7A, B). However expression of wild-type p/CAF in the presence of CBP_{HAT^-} fully restored the ligand-dependent activation function of the retinoic acid receptor (Fig. 7A, B). In contrast, the HAT activity of CBP was not required for hormone regulated gene expression and the HAT activity of p/CAF was not required for CREB-dependent gene activation (Fig. 7A, B). These transcription units served as internal controls, because both HAT^- coactivators were functional, with differences reflecting the distinct requirements for specific HAT activity by different classes of transcription factors. Thus intrinsic acetyltransferase enzymatic activity of p/CAF was essential for hormone action and a point mutant of p/CAF with eliminated HAT activity was unable to facilitate hormone dependent gene activation (SOW: Task 9). It is noteworthy that specific requirement for acetyltransferase might be determined by signaling pathway activating given promoter as was observed on Pit-dependent promoter (38). The HAT activity of CBP or p/CAF were required for Pit function that was stimulated by cyclic AMP or growth factors, respectively (38).

In order to evaluate a function of different interaction domains of p/CAF, microinjection assays were performed, using retinoic acid receptor- or CREB- dependent promoter, to assess the ability of N- and C-terminally truncated p/CAF proteins to function in retinoic acid- and cAMP-dependent transcription (SOW: Task 9 -12). Deletion of the N-terminus of p/CAF (aa 1-518) did not significantly impair its function on either retinoic acid- or cAMP-stimulated transcription; however, the C-terminus was required for function of both transcription factors (Fig. 8). These data suggest that alternative interaction interfaces can be utilized in recruitment of specific, required factors into the coactivator complex. P/CAF activity is lost with deletion of the p/CIP interaction domain (Fig. 8), consistent with the functional effects of ADA2 domain in GCN5 (26, 27, 28, 29).

Although we have been unable to examine directly nuclear receptor coactivator mutations in tissues from biopsies, the performed extensive mutational analysis of p/CAF function gave a

new insight in the mechanism of nuclear receptor function and allowed us to characterize functional domains and essential regions of p/CAF for hormone dependent transactivation (Figures: 4, 5, 6, 7, and 8) (SOW: Task 7).

While nuclear receptors required p/CAF, p/CIP, SRC-1, and CBP, the protein kinase A-dependent activation of CREB (1) required CBP, p/CAF and p/CIP, but not SRC-1 (Fig. 9). Because the C'-terminal domain of p/CAF that selectively associates with p/CIP is distinct from the SRC-1 interaction domain (Fig. 4, 6), p/CAF could potentially provide a molecular platform for differential positioning of components of the p/CAF/p/CIP/CBP/p300/SRC-1 complex in a promoter-specific manner. While STAT-1 is associated with, and requires the action of, both CBP/p300 (39, 40) and p/CIP, STAT-1 proved not to require either p/CAF or SRC-1, as blocking IgGs against these factors (8) each failed to inhibit activity of the interferon-γ responsive element (Fig. 9). Thus different classes of transcription factors - nuclear receptors, CREB and STAT - appear to functionally require distinct components of the coactivator complex, including CBP/p300, SRC-1, p/CIP, and p/CAF. These data are most compatible with a model in which there are multiple possible configurations of the specific components of the coactivator complex recruited by different promoters (Fig. 10).

The discovery that several coactivators of transcription such as p/CAF, GCN5 as well as CBP/p300 harbor intrinsic acetyltransferase activities for histones and possible for other nuclear substrates has led to the model for the role of these factors in the regulation of transcription on chromatinized DNA templates. Indeed, in cell based-assays the acetyltransferase activity of p/CAF and CBP has been already demonstrated to be critical for transcriptional functions during the course of this study (41). This important enzymatic activity of p/CAF could be modulated, providing additional level of the regulation of gene expression. The adenoviral immediate early gene product, E1A is well known inhibitor of transcription and can interact with CBP protein (14, 42). We have demonstrated in biochemical assays that EIA protein directly inhibits acetyltransferase activity of both p/CAF and CBP (43). This data suggest a mechanism in which E1A exerts its inhibitory function on transcription by eliminating the acetyltransferase activity of factors such as p/CAF or CBP emphasizing the indispensable role of this enzymatic activities in a gene regulation (SOW: Task 16). Moreover histone acetyltransferases has been recently implicated in pathogenesis of cancer. In acute myeloid leukemia, the translocation t(8;16)(p11;p13) fuses the MOZ zinc finger motifs and putative acetyltransferase domain with a largely intact CBP causing possibly aberrant chromatin acetylation (11). The amplification of AIB1 gene, a human homologue of p/CIP has been reported in estrogen-positive breast and ovarian cell line as well as in specimens of primary breast cancer (12). High expression of AIB1 which can bind directly to estrogen receptor in ligand-dependent manner, enhances estrogen-dependent transcription and has histone acetyltransferase activity may contribute to development of steroid dependent cancers (12, 36).

The analysis of p/CAF expression in developing mouse embryo revealed ubiquitous expression through development although there are some differences in the level of expression in different tissues. Figures 11 and 12 are only representative from hundreds of sections prepared

from animals collected between gestational day 11 and at birth (Task 3). The pattern of *p/CAF* expression in mouse embryo suggests a general importance of this gene for a cellular function.

The critical role of the intrinsic acetyltransferase enzymatic activity of p/CAF in hormone regulated transcription provides a link between the function this coactivator and steroid dependent regulation of cell function including breast cancer pathogenesis. The two amino acids substitution mutation in human p/CAF acetyl-CoA binding domain, Y616/F617;A616/A617 resulted in total elimination of the intrinsic acetyltransferase activity as well as in the impairment of nuclear receptor-dependent gene expression tested in cell-based assays as was reported in the first year Annual Summary. This finding have provided the unique opportunity to analyze the biological function of *p/CAF* gene using genetic approach and helped us to design the strategy for generation of mouse harboring a p/CAF mutation (Tasks 13-15, 17-21). The gene knock out approach is very useful in deciphering the biological function of the examined gene but is not always efficient when the presence of homologous variants of the genes can compensate a genetic deletion. Expressed in mouse a p/CAF homologue, GCN5 can potentially compensate p/CAF function (44). Thus the method of choice here was a generation of the mutant mouse expressing p/CAF_{HAT^-} protein which should have exactly the same expression pattern and be recruited to the same promoters in its target complexes as a wild type but which would not posses intrinsic acetyltransferase activity allowing to monitor where this enzymatic activity is required for biological function.

p/CAF_{HAT^-} mutant mice are generated by homologous recombination in the ES cell line using the Cre/loxP system. The outline of strategy as well as ES mutant clones analysis is provided in Figure 13. BAC clones containing genomic locus of the murine *p/CAF* gene have been isolated from a mouse ES cell library. A targeting vector was constructed from 5 kb of homologous sequences harboring two amino acid substitution mutation (as described in Methods section) in acetyl-Co binding domain encoding exon, a *neo* selection cassette flanked by two loxP sites and a 3' genomic 4.5 kb fragment. Targeting vector (20 μg) was electroporated into 10^7 ES cells (Fig. 13). Stably transfected clones were isolated after selection with 200 μg/ml G418 in presence of 1 μM gancyclovir. Homologously recombined clones identified by Southern blot are transiently transfected with 20 μg of a Cre recombinase expression plasmid in order to remove *neo* cassette by recombination of two loxP sites from mutated allele, thus to guarantee faithful expression of *p/CAF* gene from mutated allele (Fig. 14). The presence of the mutation as well as absence of *neo* cassette was tested by specially designed PCR (Fig. 14d). The selected ES clones are distinct from the wild type only by introduced the two amino acids substitution mutation and one loxP site in the 3' intron flanking acetyl-CoA binding domain encoding exon. Positive mutant clones were used to produce chimeric animals by blastocyst injection. We have already generated chimeric mice but we have not gotten yet a germline transmission. At this point we are testing three independently selected ES clones harboring mutation for germline transmission. Generated heterozygous mice will intercross to originate homozygous mutant mouse. The analysis of generated mutant animals will give us the insight in the biological function of p/CAF gene as well as an impact on mouse model for the breast cancer.

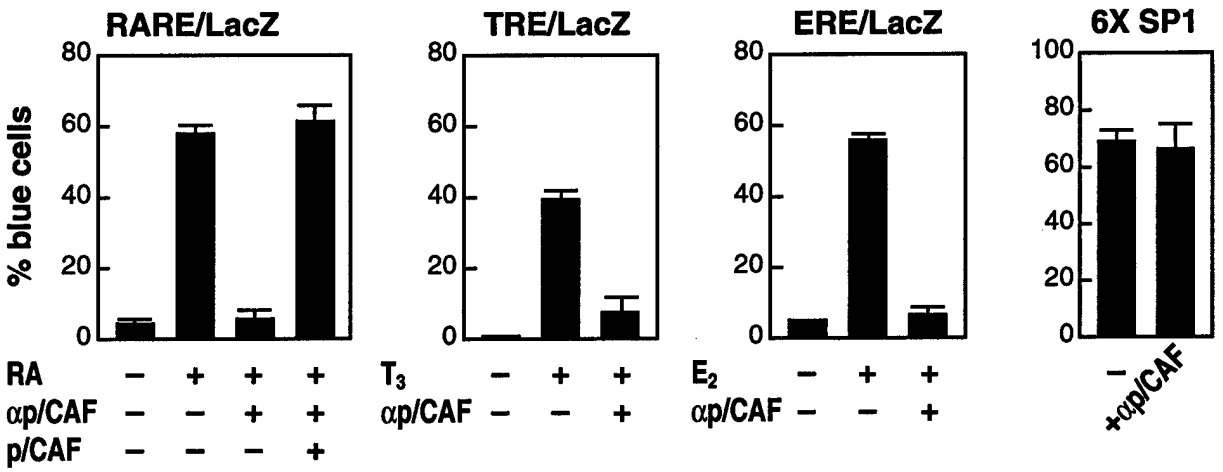


Figure 1. P/CAF is a nuclear receptor coactivator.

Microinjection of purified anti-p/CAF IgG blocked retinoic acid(RA)-, thyroid hormone (TR)- or estrogen receptor (ER)-dependent activation of promoters containing corresponding response elements in Rat-1 cells, but not SP1-driven reporter expression. Similar results were obtained in three independent experiments. All results are representative of experiments performed in triplicate, in which more than one thousand cells were injected for each experimental condition.

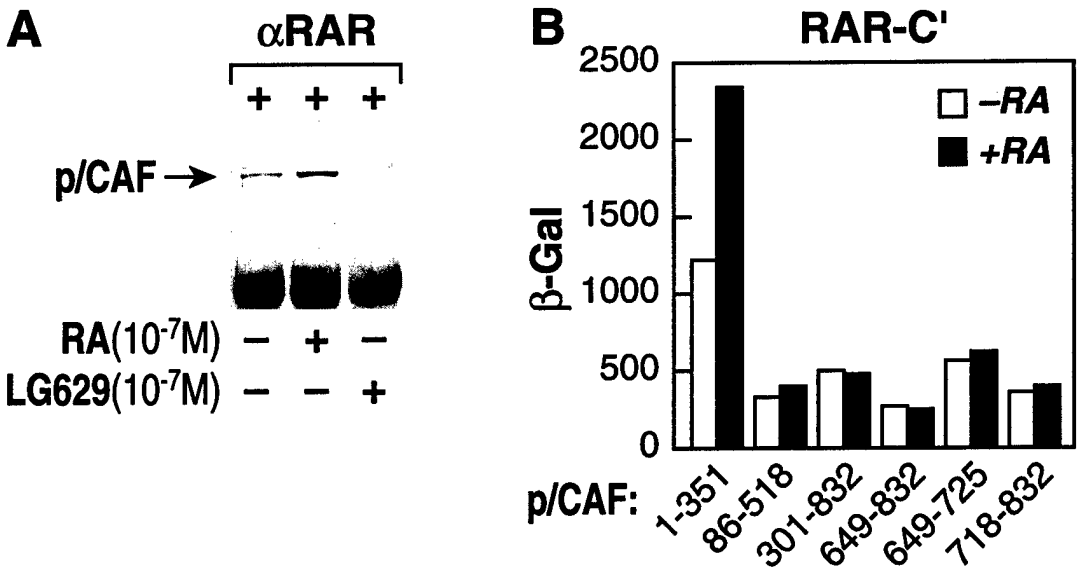


Figure 2. P/CAF interacts directly with nuclear receptor.

(A) Immunoprecipitation with anti-RAR antibodies of nuclear extracts from HeLa cells transfected with Flag-tagged p/CAF, and treated with all-trans-RA (10^{-7}M) or an antagonist, LG629 (10^{-7}M) reveals ligand-dependent co-immunoprecipitation of p/CAF and RAR, detected using monoclonal anti-Flag IgG. (B) A yeast two hybrid assay recorded specific interaction between indicated p/CAF fragments (baits) and the C-terminal domain of retinoic acid receptor (prey) in the presence or absence of retinoic acid. Duplicates differed by less than 10%.

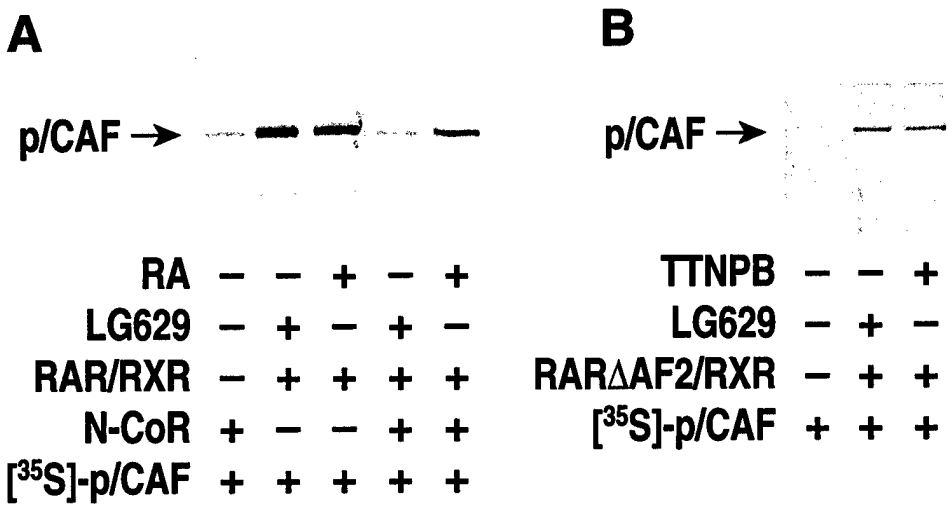


Figure 3. Ligand-dependent association of p/CAF with a nuclear receptor coactivator in cells depends upon release of the nuclear receptor corepressor complex.

Interaction between p/CAF and RAR was tested in previously described DNA-dependent assay (avidin, biotin complex DNA) for protein-protein binding. Bacterially-expressed RAR, or RARΔAF2, and RXR were bound to biotinylated oligonucletides corresponding to a DR5 (RARE), captured on streptavidin-agarose and incubated with [^{35}S]-labelled p/CAF in the presence of pan-agonist 9-cis-RA(10^{-6}M) or antagonist, LG629 (10^{-6}M), and analyzed by SDS-PAGE electrophoresis. Bacterially expressed NCoR (1mg) was incubated with receptor-DNA complexes before p/CAF addition.

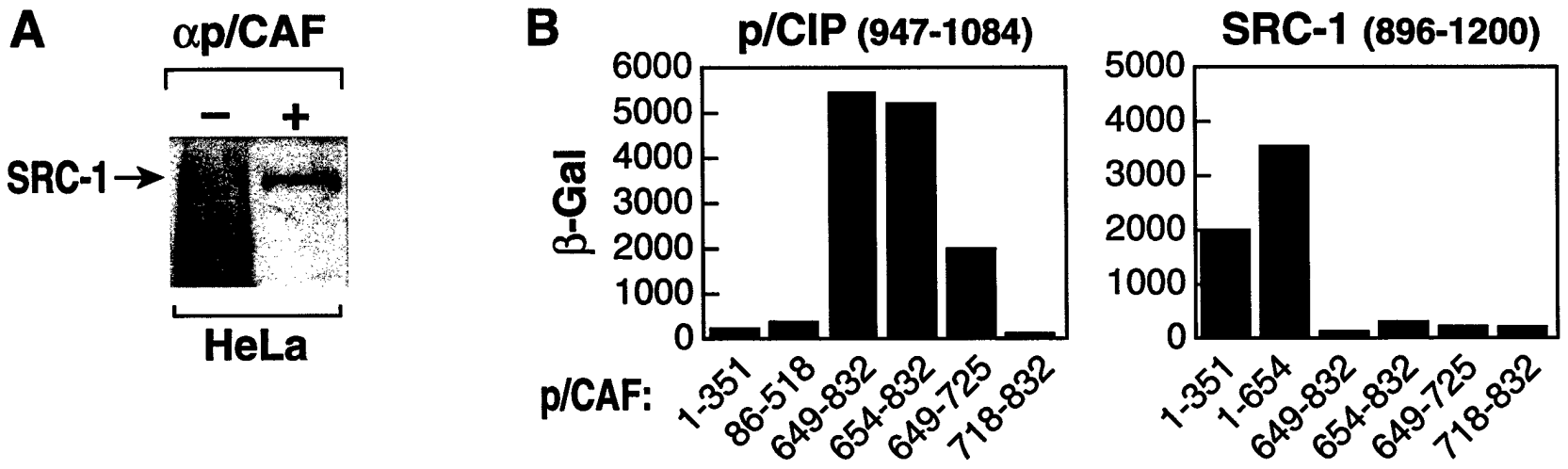


Figure 4. p/CAF interactions with CBP and NCoAs.

(A) Immunoprecipitations of HeLa extracts from cells cotransfected with Flag-tagged p/CAF and HA-tagged NCoA-1/SRC-1 expression vectors were subjected to Western blot analysis employing anti-HA for detection Yeast two hybrid interaction between (B) p/CIP (aa 947-1084) (prey) or SRC-1 (aa 896-1200) (prey) and fragments of p/CAF (baits) revealed a selective C-terminal p/CIP interaction domain and N-terminal SRC-1 interaction domains

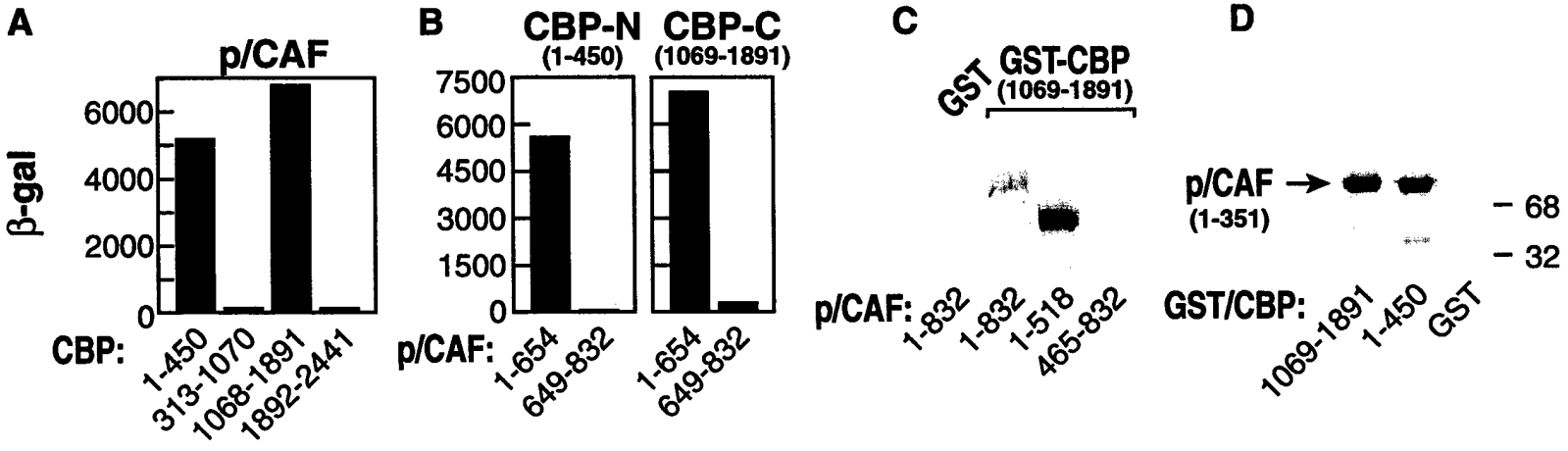


Figure 5. p/CAF interactions with CBP and NCoAs.

(A) A yeast two hybrid interaction assay using p/CAF(1-654) (bait) and CBP fragments (preys), revealing two independent interaction domains: (aa 1-450 and 1068-1891) of CBP. (B) Both CBP interaction domains interacts with N-terminal domain of p/CAF in yeast two hybrid assay but not with C-terminal domain of p/CAF. (C) GST pull-down assays with the CBP C-terminal interaction domain and fragments of [35S]-labelled p/CAF. 5 ug of GST-CBP(aa 1069-1891) protein was incubated with various [35S]-labelled p/CAF derived fragments, and specifically bound fragments detected as above; and (D) GST pull-down of [35S]-labelled p/CAF (1-351) with N- and C-terminal GST-CBP fragments.

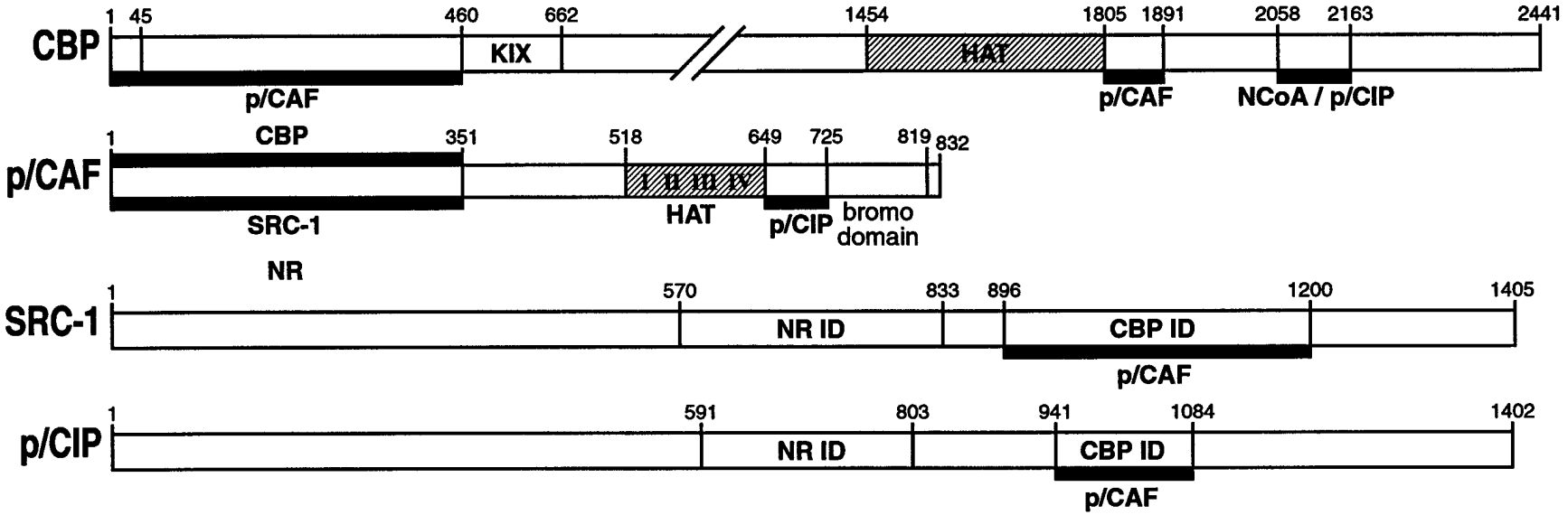


Figure 6. p/CAF interactions with CBP, coactivators aand nuclear receptor.

Schematic of CBP, p/CAF, SRC-1, and p/CIP, and their interaction domains. KIX, interaction with kinase-inducible domain of CREB; NR ID, nuclear receptor interaction domain; CBP ID, CBP interaction domain.

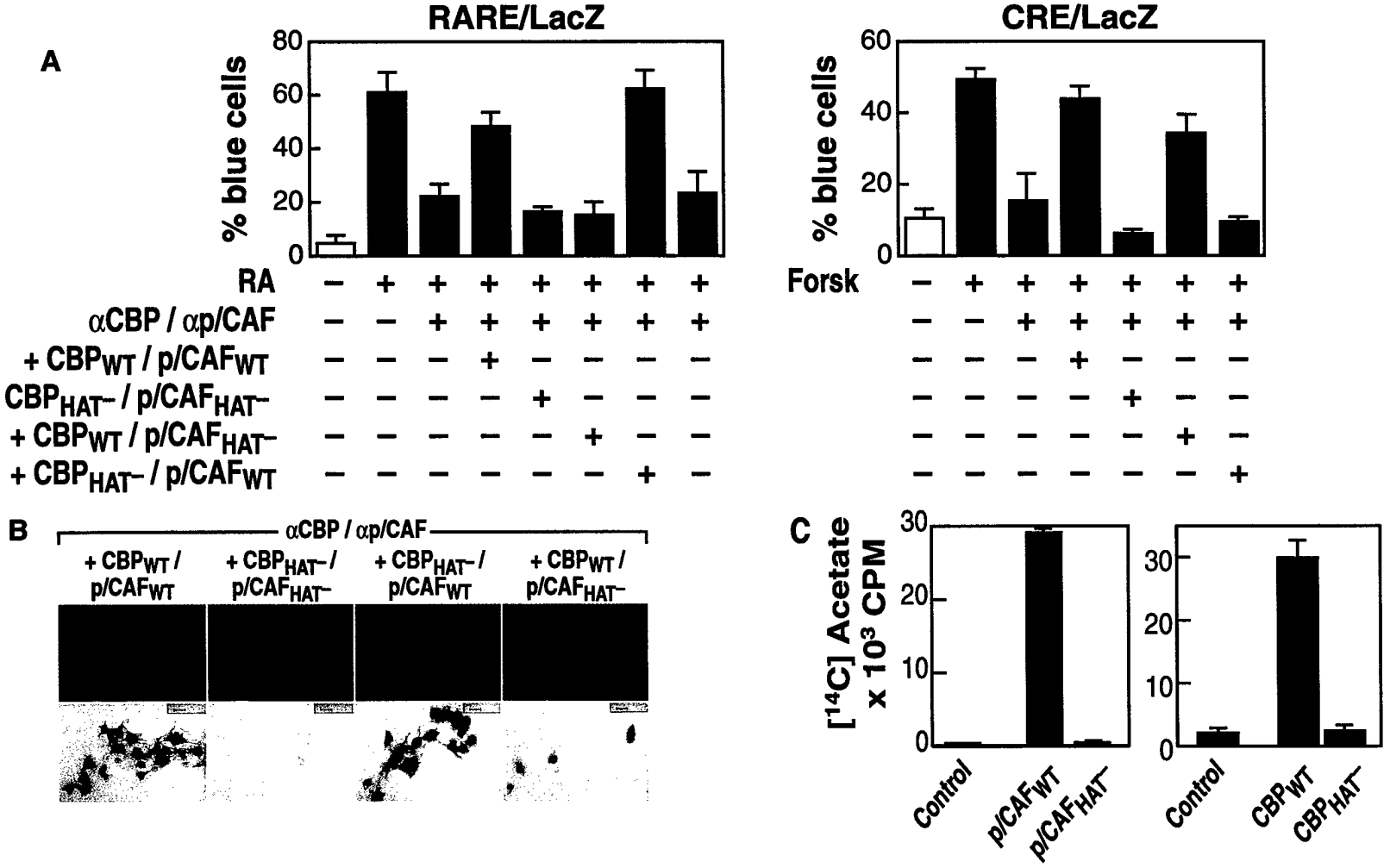


Figure 7. Histone acetyltransferase activity of p/CAF is required for RARE-dependent gene activation.

(A, B) Requirement for p/CAF and CBP acetyltransferase activity in retinoic acid receptor and CREB function tested in single cell microinjection assay. In these experiments, specific IgGs against CBP and p/CAF were coinjected with vectors directing expression of wild type wild-type or HAT- mutant of p/CAF and CBP. The ability of retinoic acid (10^{-7}M) or forskolin (10^{-6}M) to activate the appropriate reporter gene was then determined. Similar results were obtained in three independent experiments of similar design. (C) Generation of mutation in the p/CAF (Y616/F617;A616/A617) (p/CAF$_{HAT-}$)) that abolish detectable acetylation of histones using [^{14}C]acetyl-CoA as substrate. Similarly, the assay was performed with CBP mutant, CBP$_{HAT-}$. Activity was determined by liquid histone acetyltransferase assay using baculovirus-expressed p/CAF or CBP.

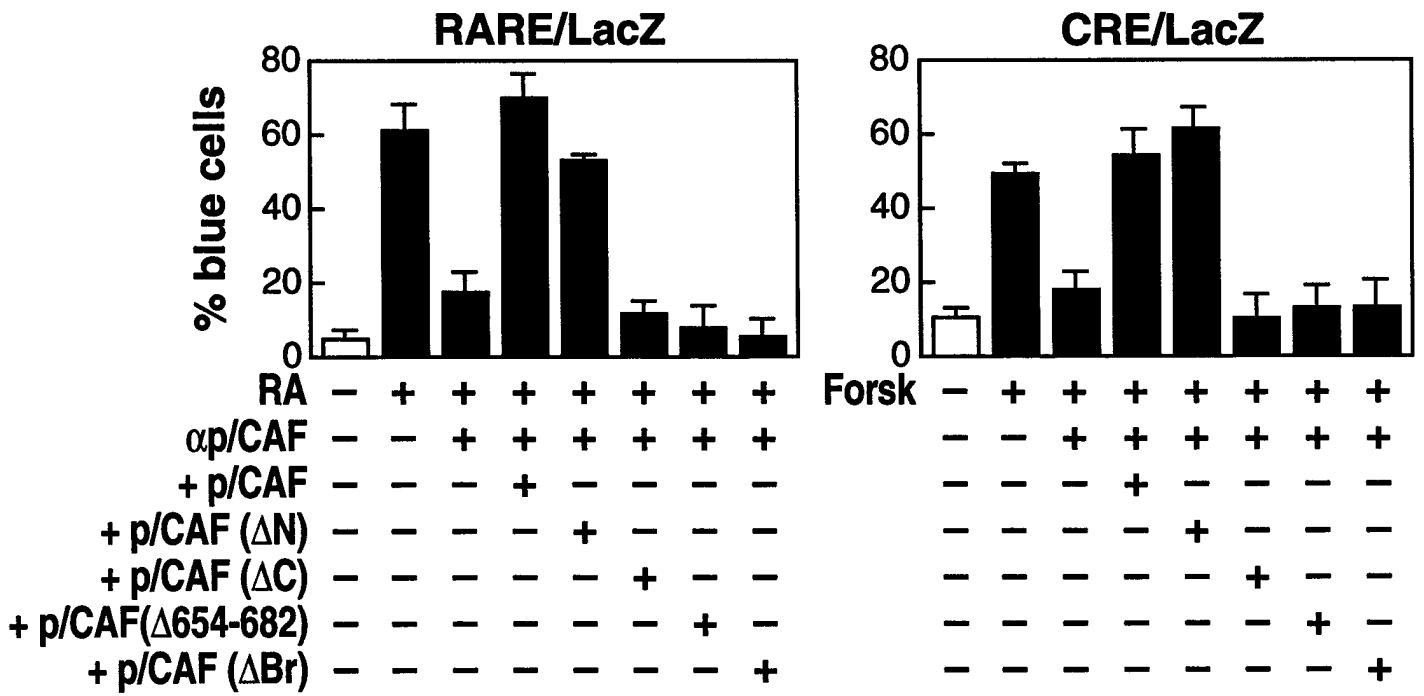


Figure 8. Role of HAT and other domains in functions of the coactivator complex on RARE- and CREB-dependent gene activation.

The ability of wild type p/CAF, p/CAF(ΔN) (aa518-832), p/CAF(ΔC) (aa1-654), p/CAF(Δ654-682) or p/CAF(ΔBr) (Δ745-832) to rescue retinoic acid-dependent activation of a RARE/LacZ reporter (left) or forskolin-dependent activation of the CREB-dependent reporter (right) in single cell microinjection assays; similar results were obtained in three separate experiments.

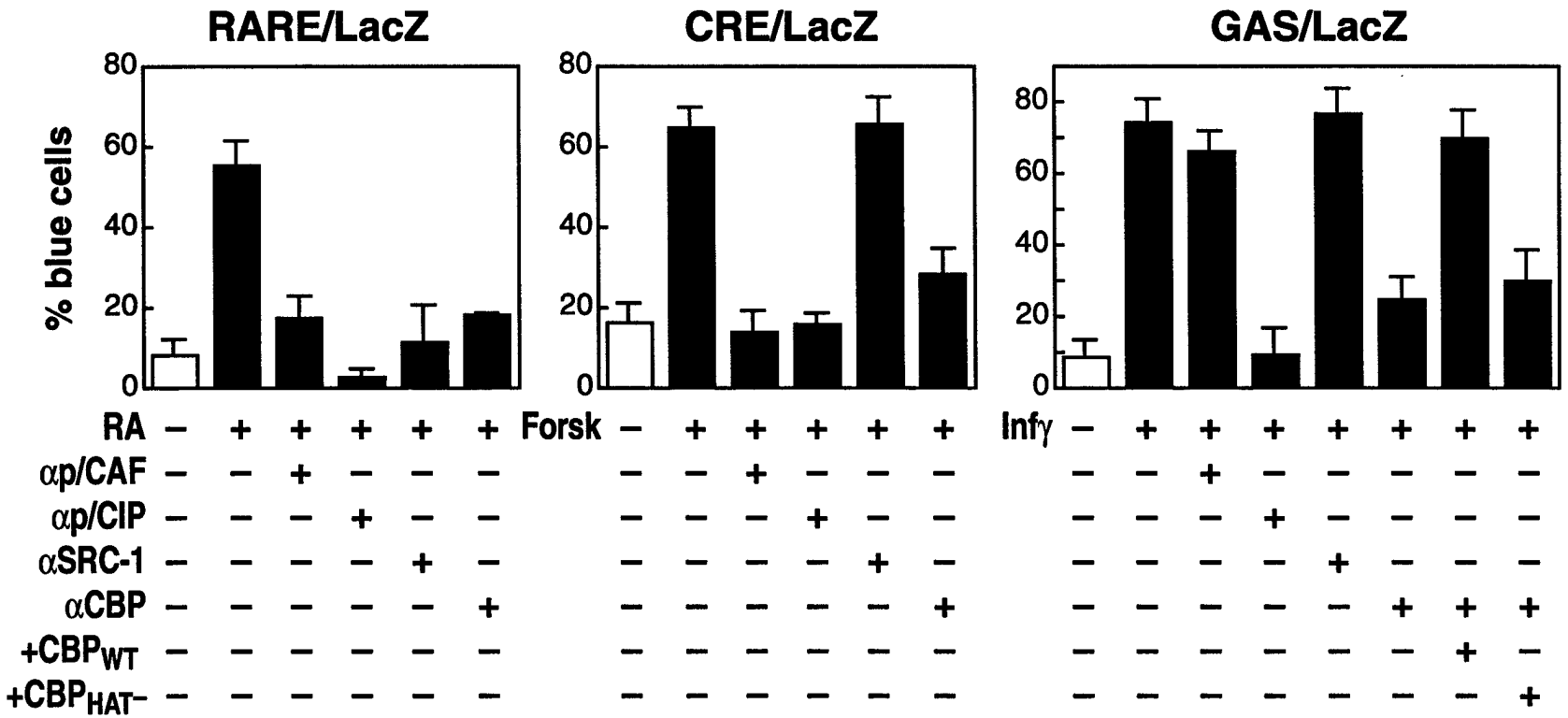


Figure 9. Promoter specificity in required coactivator complex components.

(Left) Inhibition of retinoic acid receptor activity by microinjection of anti-p/CAF, -p/CIP or -NCoA-1/SRC-1 or -CBP IgG; (midle) Inhibition of CREB activation by anti-CBP, anti-p/CIP or anti-p/CAF IgG, but not by anti-SRC-1 IgG; (right) Inhibition of Interferon γ-dependent activation of the GAS-dependent promoter by anti-CBP or anti-p/CIP IgG but not by anti-p/CAF and anti-SRC-1.

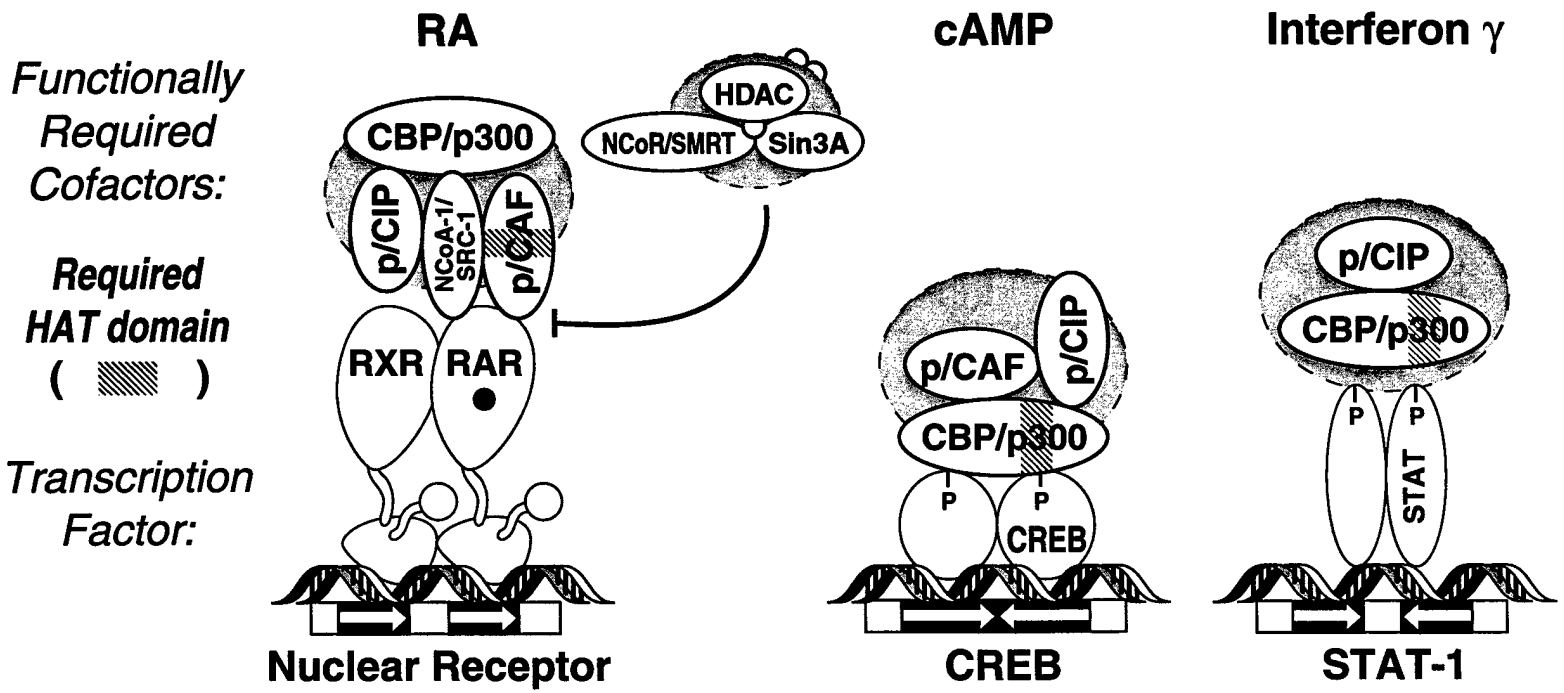
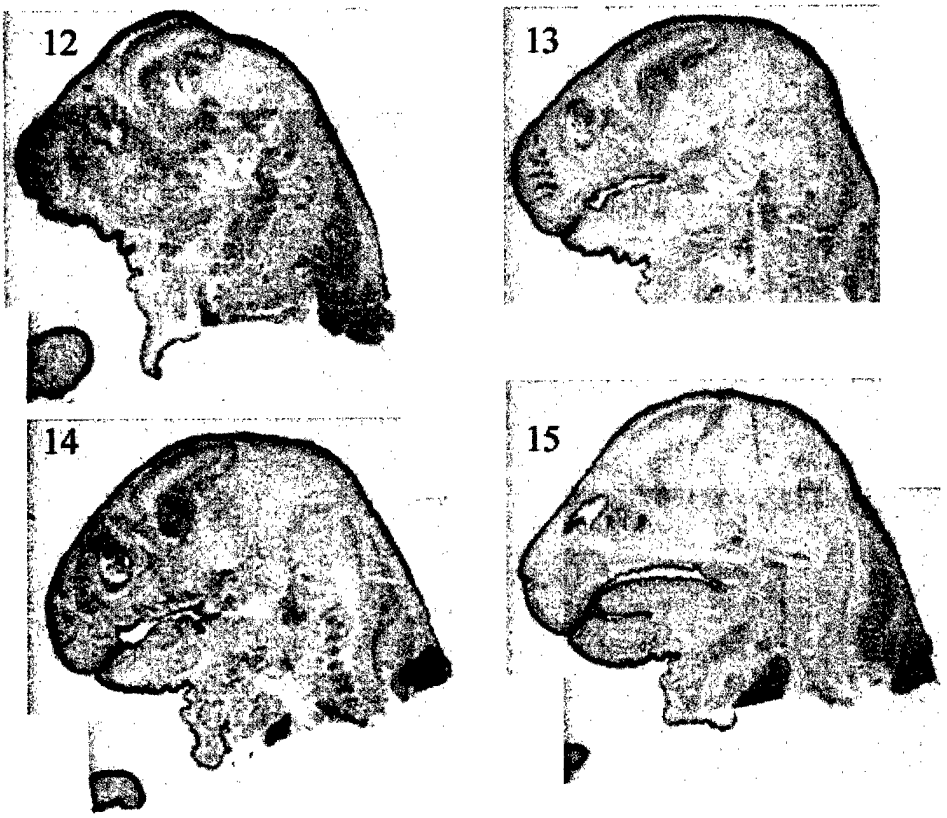


Figure 10. Model of promoter-specific requirements for the CBP, p/CIP, p/CAF and SRC-1 in the coactivator complex.

The functionally required components of the coactivator complex appear to be distinct for different promoters, and the required HAT activity is also promoter-specific. In the case of nuclear receptor-driven promoter, p/CAF and SRC-1 appear to interact with liganded receptor by direct interactions, with p/CAF binding upon dismissal NCoR from the receptor.



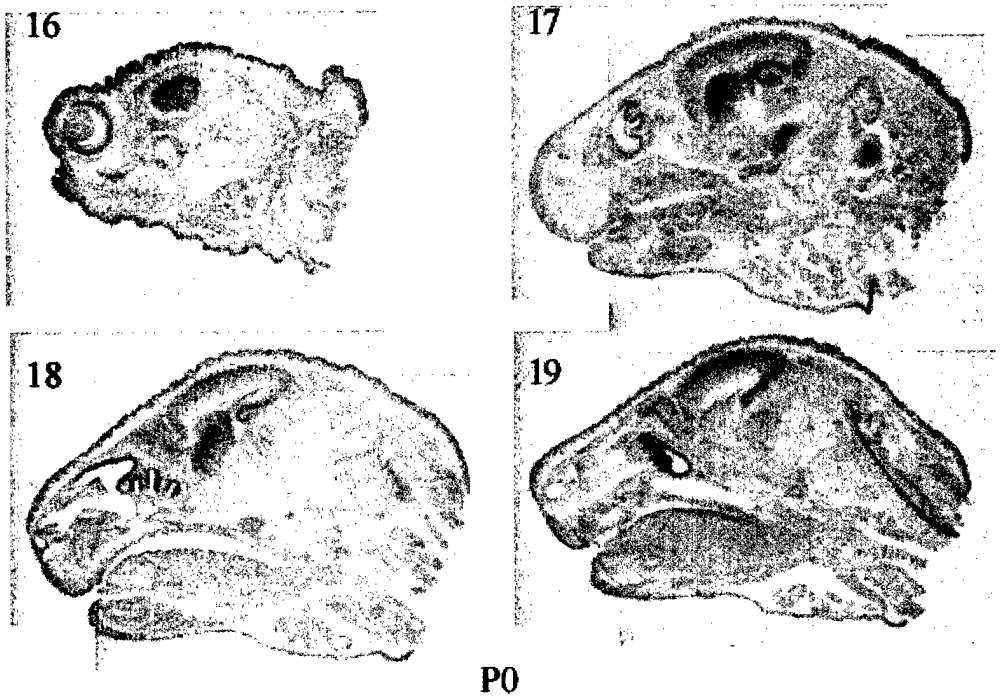


Figure 11. Expression pattern of *PCAF* gene in mouse at 18 day of gestation and at birth. *In situ* hbridization with PCAF cRNA probe was performed on sagital sections prepared from mouse embryos at gestational day 18 (GD 18; 12-15) and at birth (P0; 16-19)

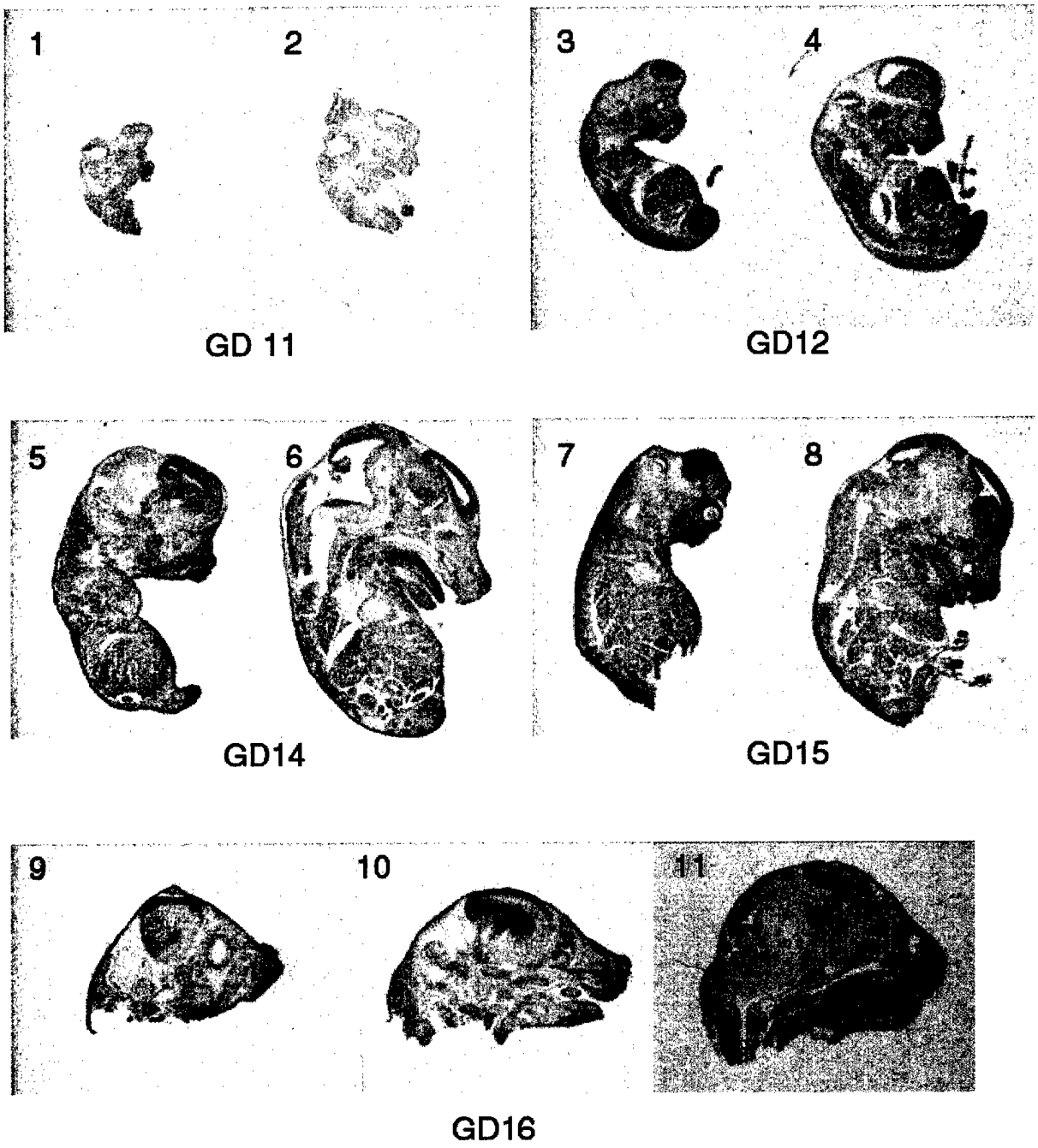


Figure 12. Expression pattern of *PCAF* gene during mouse embryo development. Sagital sections of mouse embryos prepared on gestational day (GD) 11 (1,2), GD 12 (3,4), GD 14 (5,6), GD 15 (7,8), GD 16 (9-11) were analyzed by in situ hybridization with PCAF cRNA probe.

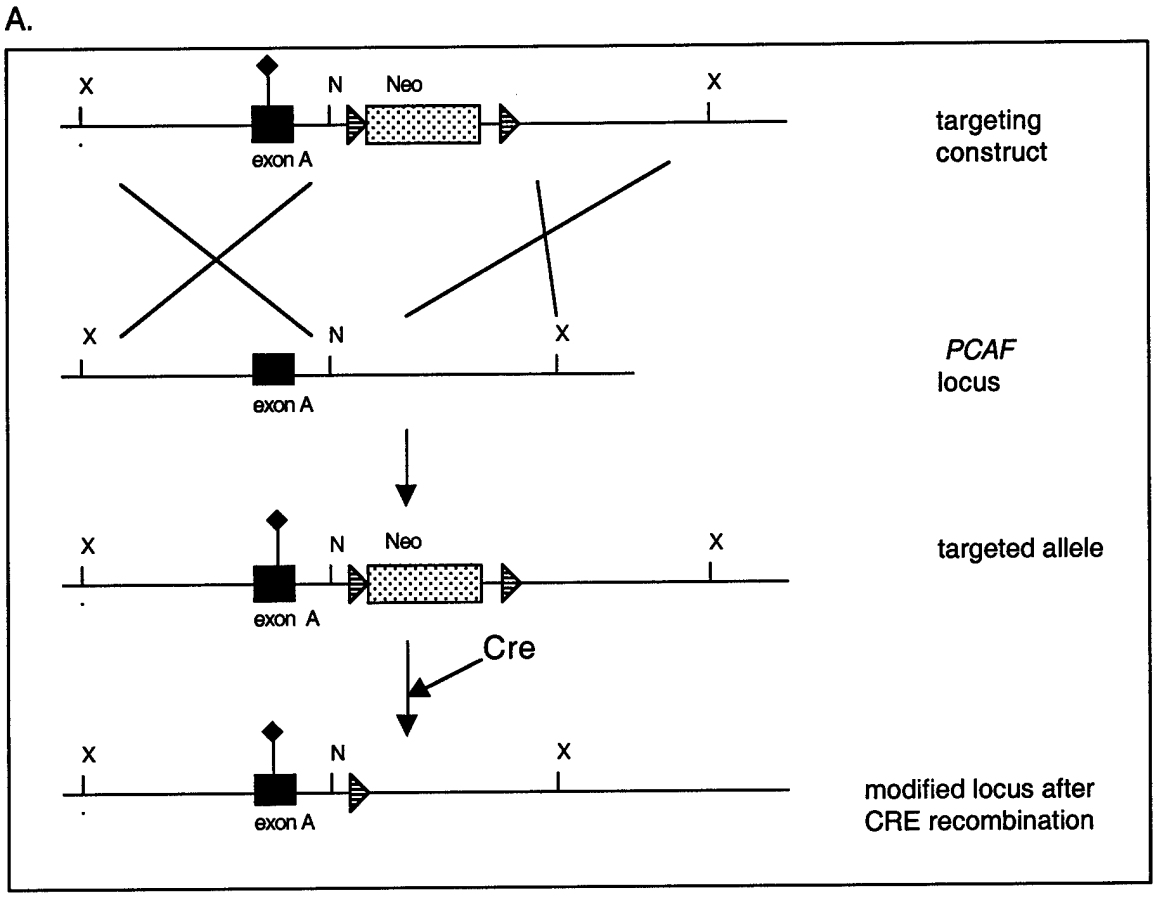


Figure 13. Strategy to prepare the p/CAF$_{HAT}$- mutants with a gene knock in procedure.

Targeting strategy for homologous recombination. The used targeting construct contains PGK-*neo* cassette encoding selection marker flanked by two LoxP sites and PGK-*tk* encoding thymidine kinase, negative selection marker in order to increase homologous recombination. The diamond marks the position of the mutation Y616/F617;A616/A617 in exon encoding acetyl-Co binding domain of PCAF protein. The targeted allele represents the structure of the *p/CAF* locus after homologous recombination, and the modified locus marks the structure after subsequent recombination of the two loxP sites by Cre recombinase.

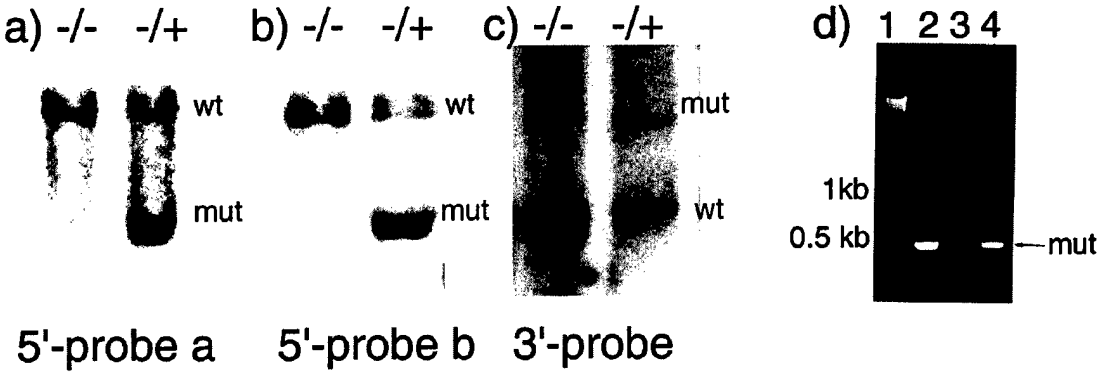


Figure 14. Analysis of generated p/CAF$_{HAT}$- mutant.

Southern blot analysis of heterozygous p/CAF$_{HAT}$- mutants using two DNA probes containing a sequences homologous to upstream from 5' arm of targeting construct: (a), 5'-probe a, (b), 5'probe b and one DNA probe homologous to downstream from 3' arm of targeting construct: (c), 3'-probe. Restriction enzyme digestions were performed on genomic DNA isolated from wild type (wt) used as a control and DNA isolated p/CAF$_{HAT}$- heterozygote ES clones with appropriate restriction enzymes. (d), PCR analysis with the appropriate primers designed against mutated sequences revealed the presence of introduced substitution mutation into the *p/CAF* locus.

KEY RESEARCH ACCOMPLISHMENTS:

- Characterization of a novel coactivator of nuclear receptor, p/CAF.
- Mapping of interactions between p/CAF and other nuclear proteins involved in hormone regulated gene expression.
- Mapping of functional domains in p/CAF gene.
- Generation of p/CAF mutant with eliminated acetyltransferase activity.
- Discovery of promoter specific requirements for coactivator acetyltransferase activity.
- Pattern of expression of *p/CAF* gene during mouse embryogenesis
- Isolation and mapping of mouse genomic DNA fragment encoding *p/CAF* gene.
- Generation of construct for targeted genomic mutagenesis of *p/CAF* locus in order to study the function of p/CAF acetyltransferase activity *in vivo*.
- Isolation of mutated ES clones for generation of p/CAF$_{HAT^-}$ mutant mouse.
- Generation of p/CAF mutant chimeric mouse.

Compliance with the goals outlined in the Statement of Work:

Task 1: Months 1-4: Completed
Task 2: Month 5: Completed
Task 3: Month 6: Completed
Task 4: Months 7-8: Completed
Task 5: Months 8-14: Completed
Task 6: Months 9-11: Completed
Task 7: Months 12-14: Completed
Task 8: Months 14-16: Completed
Task 9: Months 17-18: Completed
Task 10: Months 19-22: Completed
Task 11: Months 22-23: Completed
Task 12: Months 24-25: Completed
Task 13-15: Months 19-24: Completed
Task 16: Months 26-32: Completed
Tasks 17-21 Months 28-36: Completed or partially completed. We have obtained chimeric animals that is a key step in genetic analysis of p/CAF function and subsequently will allow us to advance our knowledge on the hormone-dependent gene regulation.

LIST OF REPORTABLE OUTCOME

- Publications:

Korzus, E., Torchia, J., Rose, D. W., Xu, L., Kurokawa, R., McInerney, E. M., Mullen, T. M., Glass, C. K., and Rosenfeld, M. G. (1998). Transcription factor-specific requirements for coactivators and their acetyltransferase functions. Science *279*, 703-7. (This paper was ranked on "The Red Hot Research Papers of 1998" by Institute of Scientific Information, *The Scientist* 11, June 21, 1999).

Xu, L., Lavinsky, R. M., Dasen, J. S., Flynn, S. E., McInerney, E. M., Mullen, T. M., Heinzel, T., Szeto, D., **Korzus, E.**, Kurokawa, R., Aggarwal, A. K., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1998). Signal-specific co-activator domain requirements for Pit-1 activation. Nature *395*, 301-6.

Perissi, V., Dasen, J. S., Kurokawa, R., Wang, Z., **Korzus,** E., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1999). Factor-specific modulation of CREB-binding protein acetyltransferase activity. Proc Natl Acad Sci U S A *96*, 3652-7.

- Poster Presentation:

Department of Defense Breast Cancer Research Program Meeting: Era of Hope. June 8-11, 2000; Atlanta, GA

- Platform Presentation:

Department of Defense Breast Cancer Research Program Meeting: Era of Hope. June 8-11, 2000; Atlanta, GA

CONCLUSIONS

These data indicate a selectivity in the specific histone acetyltransferase activity required for function of distinct promoters. The histone acetyltransferase activity of p/CAF, but not of CBP, appears to be indispensable for nuclear receptor activation, where CBP is likely to be recruited based on interaction with a complex containing p/CAF and SRC-1. The enzymatic activity of p/CAF protein is blocked by adenoviral gene product, E1A. E1A is the well-known inhibitor of transcription. In the case of nuclear receptors, p/CAF and SRC-1/p/CIP bind receptors in a ligand-independent and ligand-dependent fashion, respectively, with p/CAF recruitment apparently dependent upon dismissal of the N-CoR, corepressor complex (Fig. 3). Recently p/CAF has been found in the complex with more than 20 distinct subunits (10) homologous or identical to components of TFIID complex suggesting that p/CAF complex might play an alternative role to TFIID complex in transcription. Together, these data indicate that p/CAF plays a central role in nuclear receptor-dependent gene expression.

Analysis of *p/CAF* gene expression in mouse embryo has shown ubiquitous expression pattern suggesting general importance of the gene for a cellular function. To examine a biological function of *p/CAF*, a gene "knock in" technology has been employed in order to generate a mutant mice expressing p/CAF_{HAT^-} harboring two amino acids substitution mutation which eliminates the enzymatic activity. The results from this experiment should provide information about the biological function of *p/CAF* gene as well as an insight into molecular mechanism of action. The knowledge gained from this experiment will have impact on a design a mouse model for breast cancer study by providing a genetic tool (dominant negative *p/CAF*) for tissue targeted manipulation of hormone-dependent cellular functions.

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APPENDICES

Appendices include copies of reportable outcomes supported by this award:

1) Meeting abstract. Department of Defense Breast Cancer Research Program Meeting: Era of Hope. June 8-11, 2000; Atlanta, GA

2) Three published articles:

Korzus, E., Torchia, J., Rose, D. W., Xu, L., Kurokawa, R., McInerney, E. M., Mullen, T. M., Glass, C. K., and Rosenfeld, M. G. (1998). Transcription factor-specific requirements for coactivators and their acetyltransferase functions. Science *279*, 703-7.

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REQUIREMENT FOR THE ACETYLTRANSFERASE P300 AND CBP ASSOCIATED FACTOR IN HORMONE REGULATED GENE EXPRESSION

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The identification of hormonal factors including estrogen, progesterone, thyroid hormone, and retinoids as essential regulators of development of the normal breast, carcinogenesis of its epithelium, and progression of breast cancer directly links ligand-dependent nuclear receptor coactivator-mediated transactivation with gene regulation in these processes. Here, a novel nuclear receptor coactivator of transcription, p300 and CBP associated factor (PCAF) has been demonstrated to be required for estrogen-, thyroid hormone-, and retinoic acid-dependent gene expression. PCAF directly recruits a nuclear receptor from mammalian cell extracts in a ligand-dependent manner. The ligand-dependent association of PCAF with a nuclear receptor depends upon nuclear receptor corepressor (NCOR) dismissal. Although it is unclear how the transactivation occurs, it is postulated that the nuclear receptor/PCAF complex formation enables direct association with the RNA polymerase II complex. Using PCAF variant in which the enzymatic activity is abolished by mutation in nuclear microinjection assay it has been shown that intrinsic acetyltransferase activity of PCAF is selectively required for hormone-dependent transcription. The critical role of the intrinsic acetyltransferase enzymatic activity of PCAF in hormone regulated transcription provides a link between the function of this coactivator and steroid-dependent regulation of cell growth in pathogenesis of breast cancer. To examine a biological function of *PCAF*, a genetic approach has been employed in order to generate mutant mice with eliminated PCAF acetyltransferase activity. These studies have greatly advanced our understanding of the molecular mechanism of hormone regulated gene expression, and together with knowledge from analysis of the mutant mice, might lead to improved treatment strategies of breast cancer.

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SCIENCE

Transcription Factor–Specific Requirements for Coactivators and Their Acetyltransferase Functions

Edward Korzus, Joseph Torchia, David W. Rose, Lan Xu, Riki Kurokawa, Eileen M. McInerney, Tina-Marie Mullen, Christopher K. Glass, and Michael G. Rosenfeld*

Transcription Factor–Specific Requirements for Coactivators and Their Acetyltransferase Functions

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Different classes of mammalian transcription factors—nuclear receptors, cyclic adenosine 3′,5′-monophosphate–regulated enhancer binding protein (CREB), and signal transducer and activator of transcription-1 (STAT-1)—functionally require distinct components of the coactivator complex, including CREB-binding protein (CBP/p300), nuclear receptor coactivators (NCoAs), and p300/CBP-associated factor (p/CAF), based on their platform or assembly properties. Retinoic acid receptor, CREB, and STAT-1 also require different histone acetyltransferase (HAT) activities to activate transcription. Thus, transcription factor–specific differences in configuration and content of the coactivator complex dictate requirements for specific acetyltransferase activities, providing an explanation, at least in part, for the presence of multiple HAT components of the complex.

Nuclear receptors exhibit ligand-dependent interactions with coactivators such as CBP/p300 (*1–3*) and p160 proteins (*4*), steroid receptor coactivator-1 (SRC-1)/NCoA-1 (*2, 5*), TIF2/GRIP-1/NCoA-2 (*6*), and p300/CBP-interacting protein (p/CIP) (*7, 8*). CBP/p300 interacts with other coactivators and with the p300/CBP-associated factor (p/CAF) (*9*), which is homologous to the yeast transcriptional adaptor GCN5 (*10*). Both CBP/p300 and p/CAF exhibit strong histone acetyltransferase (HAT) activities (*9, 11*), whereas the p160 factors possess weak COOH-terminal HAT activity (*8, 12*).

To determine whether p/CAF is recruited into the nuclear receptor coactivator complex (*2–5, 13*), we performed coimmunoprecipitation assays with cell extracts. p/CAF exhibited ligand-dependent recruitment to the retinoic acid receptor (RAR) coactivator complex, and p/CAF binding was abolished by binding of an RAR antagonist (Fig. 1A). A minimal ligand-dependent interaction was observed in a yeast two-hybrid assay, and this interaction domain mapped to the NH_2-terminus (amino acids 1 to 351) of p/CAF (Fig. 1B). Using the avidin-biotin complex DNA assay (*14*) to assess protein interactions on DNA-bound receptors, we found that p/CAF bound to the RAR/RXR (retinoid-X receptor) heterodimer, but there was no detectable ligand-dependence for this association (Fig. 1C). The interaction between nuclear receptor and p/CAF was inhibited by the nuclear receptor corepressor (NCoR) (*15*)

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and was restored on ligand-induced elimination of NCoR from the receptor (Fig. 1C). This suggests that ligand-dependent association of p/CAF in cells depends on release of the NCoR complex (*16*). The interaction of p160 coactivators with RAR depends on a helical transactivation domain of RAR, referred to as AF2, through an allosteric mechanism induced by ligand binding (*4–7, 17*). However, p/CAF interaction with RAR was independent of the AF2 domain (Fig. 1C). Thus, the ligand-dependent recruitment of p/CAF to the activated nuclear receptor is distinct from the AF2-dependent mechanism of interaction between SRC-1–p/CIP and nuclear receptors.

Because *GCN5* was one of the genes identified by genetic selection, including also *ADA2* (*18*), that was required for function of certain classes of activation domains (*10*), we investigated the function of p/CAF in nuclear receptor–dependent regulation of gene transcription. Single-cell microinjection of highly specific blocking antibody against p/CAF (amino acids 465 through 832) [anti-p/CAF immunoglobulin G (IgG)] revealed that p/CAF was required for ligand-dependent activation, not only of RAR, but also of thyroid hormone– and estrogen receptor–dependent promoters (Fig. 1, D and E). A promoter that was under the control of multiple SP1 sites was unaffected by anti-p/CAF IgG (Fig. 1D), suggesting that p/CAF is not required for transcription of all promoters. When no specific antibodies were used, preimmune rabbit or guinea pig IgG was coinjected to identify the injected cells and to serve as a preimmune control (*2, 7*). The observed specificity of p/CAF function is consistent with observations concerning functions of GCN5 in yeast (*10*).

Glutathione S-transferase (GST) pull-down and yeast two-hybrid assays revealed that, in addition to the previously described interaction between p/CAF and the C/H3-E1A interaction domain of CBP (*9*), the NH_2-terminal region of p/CAF was capable of direct interaction with the NH_2-terminal region of CBP activity (Fig. 2, A and B). Consistent with the possible functional importance of this NH_2-terminal domain of CBP, as previously suggested for CREB (*19*), transcriptional activation by a GAL4-CBP(1-450) fusion protein was significantly inhibited by specific IgG against p/CAF, but not by specific IgG against either p/CIP or NCoA-1/SRC-1 (*20*). Both NCoA-1/SRC-1 (*2, 5, 12*) and p/CIP (*7*) can be coimmunoprecipitated with p/CAF that is present in cell extracts (Fig. 2C) (*8, 12, 20*). GST pull-down (*20*) and yeast two-hybrid assays (Fig. 2D) demonstrated that the NH_2-terminus of p/CAF mediated interactions with NCoA-1/SRC-1, whereas the most effective p/CIP interaction domain with p/CAF was localized to amino acids 649 to 725 (Fig. 2D), which corresponds to the conserved regions of yeast and human GCN5 that interact with ADA2 (*10*). Thus, there are multiple potential interaction interfaces between members of the coactivator complex (Fig. 2A).

To investigate the potential roles of specific enzymatic functions and interaction domains of these coactivators in transcription factor–specific gene activation events, we evaluated the roles of the acetyltransferase functions of p/CAF and CBP in RAR- and CREB-dependent transcription. On the basis of mutagenesis studies demonstrating that single amino-acid substitutions, particularly in the acetyl coenzyme A (acetyl-CoA)–binding region of acetyltransferases, resulted in loss of enzymatic activity (*21*), we generated a p/CAF mutant protein harboring a substitution of two conserved residues ($Tyr^{616}/Phe^{617} \rightarrow Ala^{616}/Ala^{617}$). This mutant has no intrinsic HAT activity (p/CAF_{HAT-}) (Fig. 3A). Similarly, a two–amino acid mutation ($Leu^{1690}/Cys^{1691} \rightarrow Lys^{1690}/Leu^{1691}$) in CBP abolished its HAT activity (CBP_{HAT-}) (Fig. 3A). The use of HAT^- proteins permitted an evaluation of the role of the HAT domains in transactivation function of specific classes of transcription factors. Blockade of both CBP and p/CAF activity by coinjection of both specific antibodies, which almost abolished the transcriptional activity of RAR or CREB, was completely reversed by coinjection of vectors expressing wild-type CBP and p/CAF (Fig. 3B). Conversely, expression

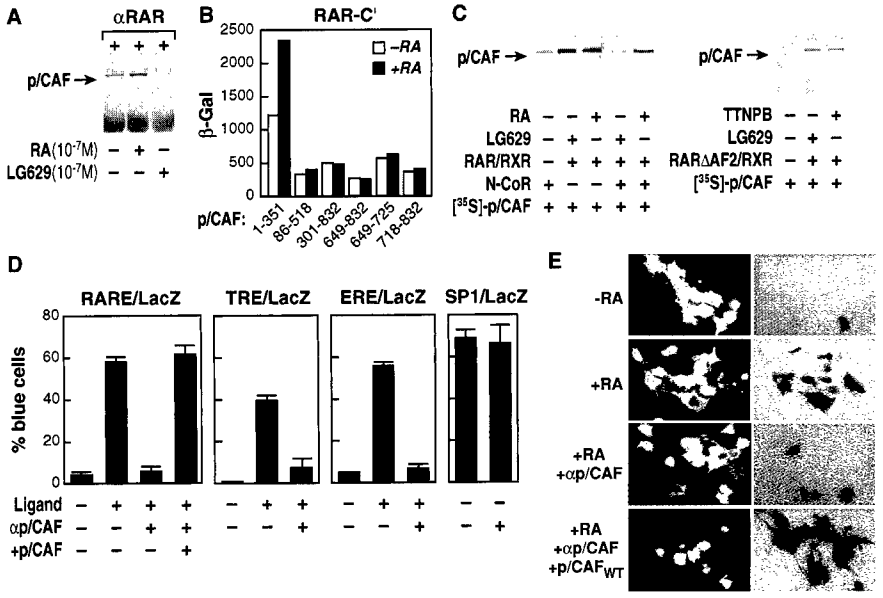

Fig. 1. p/CAF is present in a complex associated with nuclear receptors and is required for transcriptional activation induced by retinoic acid (RA). (**A**) Immunoprecipitation (*25*) with anti-RAR antibodies of nuclear extracts from HeLa cells transfected with Flag-tagged p/CAF and treated with all-*trans*-RA (10^{-7} M) or an antagonist, LG629 (10^{-7} M), reveals ligand-dependent coimmunoprecipitation of p/CAF and RAR, detected with monoclonal anti-Flag IgG. (**B**) A yeast two-hybrid assay (*26*) showed specific interaction between the indicated p/CAF fragments (denoted by ranges of residue positions) and the COOH-terminal domain of RAR (RAR-C') in the presence or absence of RA. Duplicates differed by less than 10%. (**C**) Interaction between p/CAF and RAR was tested in a DNA-dependent assay (avidin-biotin complex DNA) for protein-protein binding (*14*). Bacterially expressed RAR or RARΔAF2 (*17*) and retinoid-X receptor (RXR) (*14*) were bound to biotinylated oligonucleotides corresponding to a retinoic acid–responsive element (RARE), DR5, immobilized on streptavidin-agarose and incubated with ^{35}S-labeled p/CAF in the presence of pan-agonist 9-*cis*-RA (10^{-6} M), agonist (E)-4[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphtalenyl)-1-propenyl]benzoic acid (TTNPB) (10^{-6} M), or antagonist, LG629 (10^{-6} M), and analyzed by SDS-PAGE. Bacterially expressed NCoR (1 μg) (*15*) was incubated with receptor-DNA complexes before p/CAF addition. (**D**) Microinjection (*27*) of purified anti-p/CAF IgG (αp/CAF) blocked retinoic acid–, thyroid hormone (TR)–, or estrogen receptor (ER)–dependent activation of promoters containing the corresponding response elements (RARE, TRE, or ERE, respectively) in Rat-1 cells, but not SP1-driven reporter expression. Coinjection of p/CAF expression plasmid (p/CAF) reversed the blocking effect of anti-p/CAF IgG. All results are representative of experiments performed in triplicate, in which more than 1000 cells were injected for each experimental condition. (**E**) Photomicrographs of rhodamine-fluorescence (red) and Lac-Z (blue) staining of Rat-1 cells with the RARE reporter gene corresponding to the experiment in (D).

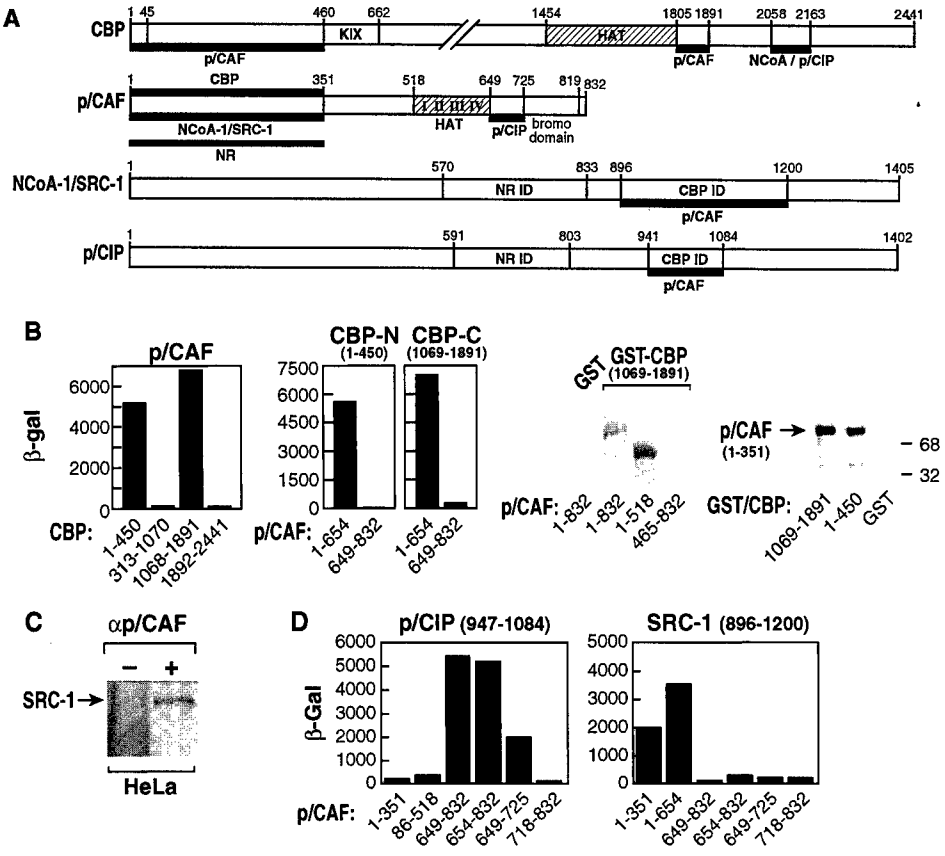

Fig. 2. Mapping of interactions between p/CAF, nuclear receptor, coactivators, and CBP. (**A**) Schematic of CBP, p/CAF, SRC-1, and p/CIP, and their interaction domains. KIX, interaction domain with kinase-inducible domain of CREB; NR ID, nuclear receptor interaction domain; CBP ID, CBP interaction domain. (**B**) (Left) A yeast two-hybrid interaction assay (*26*) using p/CAF(1-654) and CBP fragments, revealed two independent interaction domains (amino acids 1 to 450 and 1068 to 1891) of CBP (CBP-N, NH_2-terminal fragment; CBP-C, COOH-terminal fragment); (right) GST pull-down assays with the CBP COOH-terminal interaction domain and fragments of ^{35}S-labeled p/CAF (*28*). We incubated 3 μg of GST-CBP(1069-1891) protein with various ^{35}S-labeled p/CAF-derived fragments, and the specifically bound fragments detected above. GST pull-down assay of ^{35}S-labeled p/CAF(1-351) with NH_2- and COOH-terminal GST-CBP fragments. (**C**) Immunoprecipitations of HeLa extracts from cells cotransfected with Flag-tagged p/CAF and HA-tagged NCoA-1/SRC-1 expression vectors were subjected to protein immunoblot analysis employing anti-HA for detection. (**D**) Yeast two-hybrid interaction between p/CIP (947-1084) or NCoA-1/SRC-1 (896-1200) and fragments of p/CAF revealed a selective COOH-terminal p/CIP interaction domain and NH_2-terminal NCoA-1/SRC-1 interaction domains. Duplicate determinations differed by <10%.

of HAT$^-$ factors mutated to abolish acetyltransferase function mutation (HAT$^-$), failed to effectively rescue activation (Fig. 3B). A failure to rescue RAR activity was also observed with expression of wild-type CBP and p/CAF_{HAT-} (Fig. 3B). However, expression of wild-type p/CAF in the presence of CBP_{HAT-} fully restored the ligand-dependent activation function of the RAR (Fig. 3B). In contrast, the HAT activity of CBP was required for CREB function, whereas that of p/CAF was of minimal importance (Fig. 3B). These transcription units served as internal controls, because both HAT$^-$ coactivators were functional, with differences reflecting the distinct requirements for specific HAT activity by different classes of transcription factors.

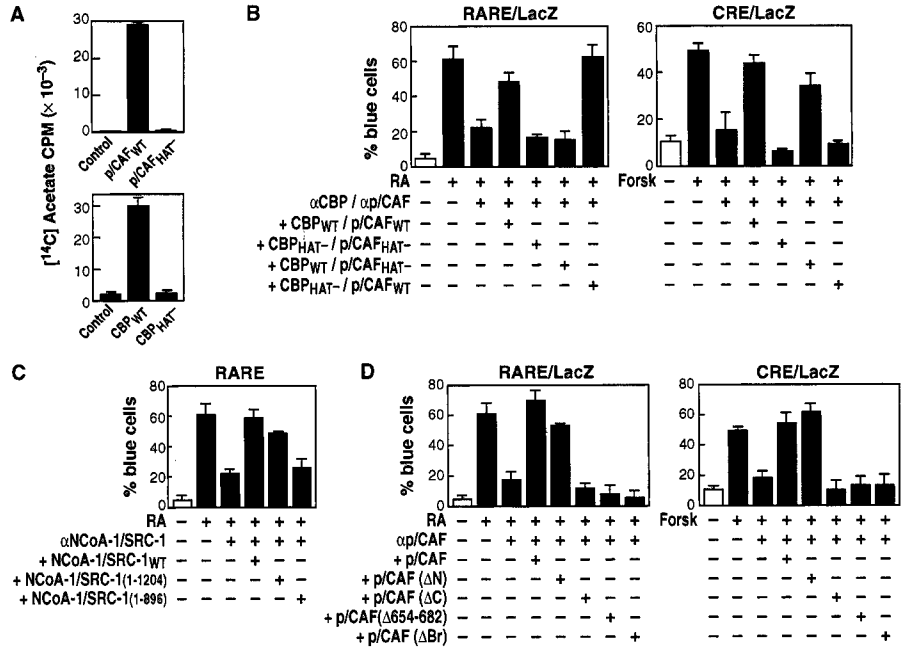

Fig. 3. Role of HAT and other domains in functions of the coactivator complex on RARE- and CREB-dependent gene activation. (**A**) Generation of mutations in the p/CAF (Tyr^{616}/Phe^{617} → Ala^{616}/Ala^{617}) (p/CAF_{HAT-}) or CBP (Leu^{1690}/Cys^{1691} → Lys^{1690}/Leu^{1691}) (CBP_{HAT-}) that abolish detectable acetylation of histones using [^{14}C]acetyl-CoA as substrate. Activity was determined by liquid HAT assay (*29*) using bacterially expressed p/CAF or baculovirus-expressed CBP. (**B**) Requirement for p/CAF and CBP acetyltransferase activity in RAR and CREB function tested in single-cell microinjection assays. In these experiments, specific IgGs against CBP and p/CAF were coinjected with vectors directing expression of wild-type (WT) or HAT$^-$ mutant of p/CAF and CBP. The ability of retinoic acid (10^{-7} M) or forskolin (10^{-6} M) to activate the appropriate reporter gene was then determined. Similar results were obtained in three independent experiments of similar design. (**C**) NCoA-1/SRC-1 deleted in the COOH-terminal domain containing acetyltransferase activity (1-1204) remained effective for RARE-dependent gene activation, whereas further deletion of the CBP interaction domain (1-896) abolished its function. (**D**) The ability of wildtype p/CAF, p/CAF(ΔN) (amino acids 518-832), p/CAF(ΔC) (amino acids 1-654), p/CAF(Δ654-682) (missing residues 654 to 682) or p/CAF(ΔBr) (missing residues 745 to 832) to rescue RA-dependent activation of a RARE/Lac-Z reporter (left) or forskolin-dependent activation of the CREB-dependent reporter CRE/Lac-Z (right) in single-cell microinjection assays; similar results were obtained in three separate experiments.

We also tested, in an RAR activation assay, the requirement of the NCoA-1/SRC-1 (*7*) COOH-terminus, which encompasses a domain with reported HAT function (*12*). Removal of the HAT domain in NCoA-1/SRC-1 did not significantly diminish its function in retinoic acid–dependent gene activation (Fig. 3C), whereas further COOH-terminal truncation to remove the CBP/pCAF interaction domain abolished NCoA-1/SRC-1 activity.

On the basis of presence of the multiple interaction interfaces in p/CAF, we performed microinjection assays using RAR- or CREB-dependent promoters to assess the ability of NH_2- and COOH-terminally truncated p/CAF proteins to function in retinoic acid– and adenosine 3′,5′-monophosphate (cAMP)–dependent transcription. Deletion of the NH_2-terminus of p/CAF (amino acids 1 to 518) did not significantly impair its function on either retinoic acid– or cAMP-stimulated transcription; however, the COOH-terminus was required for function of both transcription factors (Fig. 3D). The ability of p/CAF to be recruited even in the absence of a CBP/nuclear receptor NH_2-terminal interaction domain is in agreement with the findings that the p/CAF interaction domain in CBP is not required for RAR function (*22*). Thus, alternative interaction interfaces appear to be used in recruitment of specific, required factors into the coactivator complex. p/CAF activity was also lost with deletion of the p/CIP interaction domain (Fig. 3D), consistent with the functional effects of the *ADA2* interaction domain in *GCN5* (*10*).

Activation of a given transcription factor may exhibit differential requirements for the components of the potentially dynamic coactivator complex. Whereas nuclear receptors required p/CAF, p/CIP, NCoA-1/SRC-1, and CBP, the protein kinase A–dependent activation of CREB (*1*) required CBP, p/CAF, and p/CIP, but not NCoA-1/SRC-1 (Fig. 4A). Because the COOH-terminal domain of p/CAF that selectively associates with p/CIP is distinct from the NCoA-1/SRC-1 interaction domain (Fig. 2), p/CAF may provide a molecular platform for differential positioning of components of the p/CAF–p/CIP–CBP/p300–SRC-1 complex in a transcription factor–specific manner. Whereas STAT-1 is associated with and requires the action of both CBP/p300 (*23*) and p/CIP, STAT-1 did require either p/CAF or NCoA-1/SRC-1, because blocking antibodies against these factors (*7*) failed to inhibit activity of the interferon-γ–responsive element (Fig. 4A). Furthermore, rescue experiments revealed that STAT-1 function required an intact CBP HAT activity (Fig. 4A).

We suggest that there are multiple possible configurations of the specific components of the coactivator complex recruited by different transcription factors. In the case of nuclear receptors, p/CAF and SRC-1/NCoA-1 bind receptors in a ligand-independent and ligand-dependent fashion, respectively, with p/CAF recruitment apparently dependent on dismissal of the NCoR complex (Fig. 4B). Our data also suggest a selectivity in the specific HAT activity required for function of distinct classes of transcription factors. The HAT activity of p/CAF, but not of CBP, appears to be indispensable for nuclear receptor activation, where CBP is likely to be recruited on the basis of interaction with a complex containing p/CAF and NCoA-1 (Fig. 4B). Conversely, the HAT activity of CBP, directly recruited by phosphorylated CREB (*1*), is required for transcriptional function of CREB and STAT-1 (*24*).

In concert with the finding of factor-specific interfaces at which E1A acts to block transcriptional function (*22*), our studies suggest transcription factor specificity in requirements for various functional domains (such as HATs) in components of the coactivator complex, which we speculate reflects the use of alternative interaction interfaces in coactivator complex assembly.

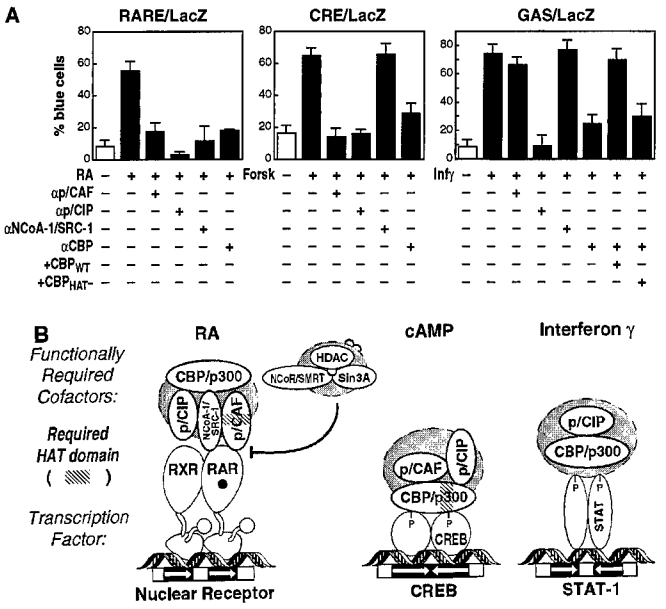

Fig. 4. Transcription factor specificity in required coactivator complex components. (**A**) (Left) Inhibition of RAR activity by microinjection of anti- p/CAF, anti-p/CIP, anti-NCoA-1/SRC-1, or anti-CBP IgG; (middle) inhibition of CREB activation by anti-CBP, anti-p/CIP or anti-p/CAF IgG, but not by anti-NCoA-1/SRC-1 IgG; (right) inhibition of interferon-γ (Infγ)–dependent activation of the interferon-γ activation sequence (GAS)–dependent promoter by anti-CBP or anti-p/CIP IgG but not by anti-p/CAF and anti-SRC-1. (**B**) Model of transcription factor–specific requirements for the CBP, p/CIP, p/CAF, and NCoA-1/SRC-1 in the coactivator complex. The functionally required components of the coactivator complex appear to be distinct for different classes of transcription factors, and the required HAT activity is also factor-specific. In the case of nuclear receptor, p/CAF and NCoA-1/SRC-1 appear to interact with liganded receptor by direct interactions, with p/CAF binding upon dismissal of NCoR from the receptor.

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25. Whole-cell extracts were prepared by lysing the cells in NET-N buffer containing 50 mM tris (pH 7.6), 5 mM EDTA, 0.3 M NaCl, 1 mM dithiothreitol, 0.1% NP-40, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of leupeptin, pepstatin, and aprotinin). The mixture was then centrifuged at 30,000*g* for 1 hour at 4°C, and the supernatant was stored at −80°C until use. For coimmunoprecipitation assays, 1 mg of cell extract was incubated in the presence of 2 μg of anti-p/CIP, anti-Flag, or anti-RAR IgG for 2 hours at 4°C. The immune complexes were then precipitated with protein A–Sepharose. Protein complexes were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) [U. K. Laemmli, *Nature* **227**, 680 (1970)], protein immunoblotted, and probed using 1 μg/ml of anti-Flag, anti-p/CIP, or an anti-hemagglutinin (anti-HA).
26. The yeast strain EGY 48, the LexA-b galactosidase reporter construct (PSH 18-34), and the B42 parental vectors (PEG 202 and PJG 4-5) were all previously described [J. Gyuris *et al.*, *Cell* **75**, 791 (1993)]. Various p/CAF fragments or fragments of other coactivators were obtained by polymerase chain reaction or restriction digestion and subcloned into PEG 202 bait vector or PJG 4-5 prey vectors, respectively. EGY 48 cells were transformed with the *lac Z* reporter plasmid pSH 18-34 with the appropriate bait and prey vectors, and were plated out on Ura$^-$His$^-$Trp$^-$ medium containing 2% galactose. Isolated yeast colonies were then allowed to grow in the same liquid medium and were subsequently assayed for β-galactosidase, as previously described [F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1995)]. All constructs were sequenced and functionally tested in yeast two-hybrid assays, showing interaction with other proteins as previously described (*2*, *7*). In the case of p/CAF constructs, each fragment interacted positively with at least one other coactivator except the fragment with amino acids 86 through 518.
27. Insulin-responsive Rat-1 fibroblasts were seeded on acid-washed glass cover slips at subconfluent density and grown in D-MEM/F-12 (Life Technologies) medium supplemented with 10% fetal bovine serum, gentamicin, and methotrexate. Before the injection, the cells were rendered quiescent by incubation in serum-free medium for 24 to 36 hours. Plasmids were injected into the nuclei of cells at a final concentration of 100 μg/ml. Immunoglobulin G specific for p/CAF was prepared from guinea pig serum raised against a bacterially expressed fragment (amino acids 466 to 832) of p/CAF; this IgG recognized the single band of p/CAF in protein immunoblot analysis. Either preimmune IgG or the appropriate specific antibodies directed against p/CAF, p/CIP, SRC-1, or CBP were coinjected and allowed the unambiguous identification of the injected cells (*7*). Preimmune controls were included in all experiments. Microinjections were carried out using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Approximately 1 hour after injection, the cells were stimulated, where indicated, with the appropriate ligand. In the case of rescue experiments, the cells were stimulated with ligand 6 hours after injection to allow protein expression. After overnight incubation, the cells were fixed and then stained to detect injected IgG and β-galactosidase expression [D. W. Rose *et al.*, *J. Cell Biol.* **119**, 1405 (1992); (*2*)]. Injected cells were identified by staining with tetramethylrhodamine-conjugated donkey anti-rabbit IgG. All experimental results are expressed as the mean ± SEM of at least three experiments in which at least 1000 cells were injected.
28. GST-RAR and GST-CBP fragments were generated as described (*2*). We incubated 25 μl of GST-Sepharose beads containing 3 to 6 μg of the GST recombinant proteins with 5×10^5 cpm of ^{35}S-labeled p/CAF proteins generated by in vitro transcription and translation for 2 hours at 4°C. The complexes were washed five times with NET-N buffer, resolved by SDS-PAGE, and fluorographed.
29. Mutations in p/CAF and CBP were introduced by site-directed mutagenesis using the Quick-Change mutagenesis system (Stratagene) according to the manufacturer's instructions. Double-stranded oligonucleotides were designed such that the wild-type sequence corresponding to amino acids Tyr^{616}/Phe^{617} (acetyl-CoA–binding site) in p/CAF cDNA were substituted with alanines in order to generate a mutant of p/CAF lacking HAT activity (pCMV–p/CAF$_{HAT-}$). A similar strategy was used to obtain mutants of CBP. Mutants of p/CAF and CBP were expressed in bacteria and baculovirus, respectively, and tested for HAT activity in solution using histones as substrates [J. E. Brownell and C. D. Allis, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6364 (1995)].
30. We thank R. Heyman for use of TTNPB and LG629, S. L. Berger for discussion and providing hGCN5 expression vector, and Y. Nakatani and X. J. Yang for providing a tagged p/CAF expression vector. We also thank C. Nelson for experimental assistance, L.-M. Phillips for excellent technical assistance, and P. Myer for expertise in figure preparation. E.K. is supported by a U.S. Army Medical Research Program Award, J.T. by the National Cancer Institute of Canada, E.M.M. by an NIH Postdoctoral Fellowship, D.W.R. by an American Diabetes Association Career Development Award, and L.X. by an American Heart Association Predoctoral Fellowship. Supported by grants from NIH to C.K.G. and M.G.R.

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Signal-specific co-activator domain requirements for Pit-1 activation

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POU-domain proteins, such as the pituitary-specific factor Pit-1, are members of the homeodomain family of proteins which are important in development and homeostasis, acting constitutively or in response to signal-transduction pathways to either repress or activate the expression of specific genes[1]. Here we show that whereas homeodomain-containing repressors such as Rpx[2] seem to recruit only a co-repressor complex, the activity of Pit-1 (ref. 3) is determined by a regulated balance between a co-repressor complex that contains N-CoR/SMRT[4,5], mSin3A/B[6–8] and histone deacetylases[6–8], and a co-activator complex that includes the CREB-binding protein (CBP)[9] and p/CAF[10]. Activation of Pit-1 by cyclic AMP or growth factors depends on distinct amino- and carboxy-terminal domains of CBP, respectively. Furthermore, the histone acetyltransferase functions of CBP[11,12] or p/CAF[10] are required for Pit-1 function that is stimulated by cyclic AMP or growth factors, respectively. These data show that there is a switch in specific requirements for histone acetyltransferases and CBP domains in mediating the effects of different signal-transduction pathways on specific DNA-bound transcription factors.

A potential connection between the nuclear receptor co-repressor (N-CoR) and homeodomain-containing transcription factors was indicated by screening of λgt11 complementary DNA libraries using the radio-labelled, bacterially expressed repression domain III (amino acids 970–1,502) of N-CoR. Unexpectedly, one-third of the sequenced positive isolates encompassed the homeodomain or POU domain of several transcriptional activators. Therefore, we tested whether Pit-1, a tissue-specific POU homeodomain factor that is important in the differentiation of three pituitary cell types[3], could associate with N-CoR, and whether such an interaction might modulate Pit-1 function. When acting alone, Pit-1 weakly activated the minimal promoters under the control of a multimerized cognate Pit-1 response element, which is derived from the prolactin Pit-1 1P site (Fig. 1a). Blocking N-CoR, mSin3A/B or histone deacetylase

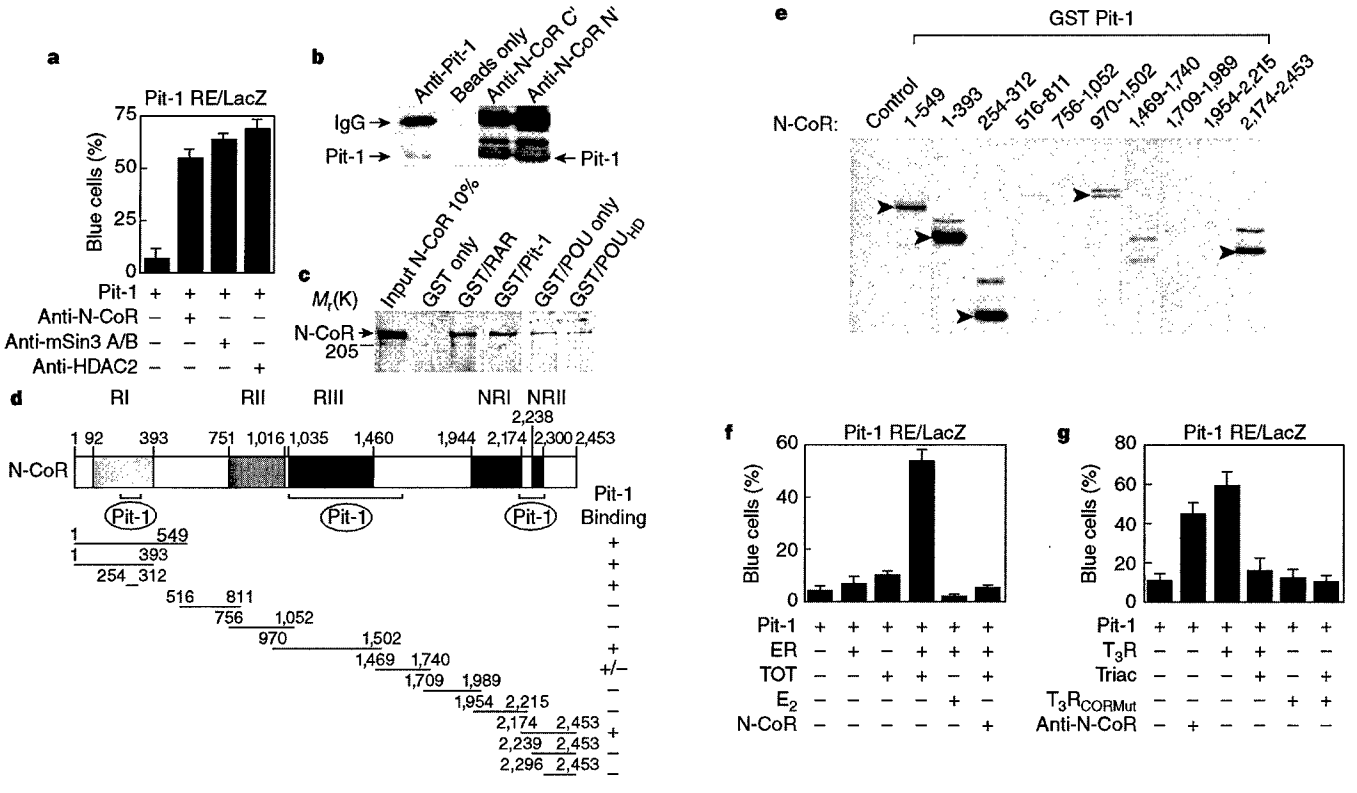

Figure 1 Role of N-CoR repression in Pit-1 function. **a**, Single-cell microinjection assay showing effects of anti-N-CoR, anti-mSin3 A/B, or anti-HDAC[13] IgG on Pit-1-mediated activation of a Pit-1-dependent reporter (Pit-1 RE/LacZ). **b**, Immunoprecipitation with whole-cell extract prepared from 293 cells transfected with Pit-1 expression vector, using specific antibodies against Pit-1 and the N or C terminus of N-CoR (N-terminal amino acids 1–132, C-terminal amino acids 2,239–2,456). The western blot was detected with anti-Pit-1 antibody. **c**, GST pull-down from extracts of 293 cells transfected with cytomegalovirus (CMV)-driven Flag-tagged N-CoR. GST fusions containing the retinoic acid receptor, Pit-1, the POU domain of Pit-1 or POU$_{HD}$ of Pit-1 were used. The western blot was detected with anti-Flag antibody (Kodak). **d**, Map of N-CoR showing locations of three independent Pit-1 interaction domains. R I, R II, R III: repression domains I, II and III (refs 4, 14); NR I, NR II: nuclear-receptor-binding domains I and II (refs 4, 14). **e**, Interaction assay using GST–Pit-1 and ^{35}S-labelled N-CoR fragments (TNT, Promega). **f**, The ability of TOT-occupied oestrogen receptor (ER) to 'transactivate' Pit-1 was studied using the reporter as in **a**. ER exhibited TOT-dependent (10^{-7} M), but not 17-β-oestradiol (E_2)-dependent (10^{-7} M), activation of the Pit-1-responsive reporter. **g**, The thyroid-hormone receptor CoR box mutant (T_3R CoRmut) was used to determine whether N-CoR binding by the thyroid-hormone receptor (T_3R) was responsible for the ability of unliganded T_3R to activate Pit-1 in 'trans'.

HDAC2 (ref. 13) functions by antibodies in the microinjection assay resulted in markedly increased Pit-1 activity (Fig. 1a). The same antibodies had no effects on control reporters (Fig. 2b, and data not shown). Immunoprecipitation with N-CoR antibodies resulted in co-precipitation of Pit-1 (Fig. 1b); and bacterially produced full-length Pit-1 or the POU domain alone were able to specifically pull down Flag-epitope-tagged N-CoR expressed in 293 cells (Fig. 1c). The Pit-1 POU domain interacted specifically with N-CoR repression regions I and III (refs 4, 14), and a C-terminal region of N-CoR that is N-terminal to the primary interaction domain of the nuclear receptor[4,14] (NR-I; Fig. 1d, e). These results indicate that the N-CoR complex exerts significant inhibitory effects on Pit-1 activity within the cell.

We next investigated whether the N-CoR complex could be 'limiting' in a fashion analogous to that suggested for co-activators in mediating transrepression between nuclear receptor and AP-1 (ref. 15). The observation that 4-*trans*-hydroxytamoxifen (TOT), an antagonist of the oestrogen receptor, but not the agonist 17-β-oestradiol, induces N-CoR interaction with the oestrogen receptor[16] allowed us to examine whether antagonist-bound oestrogen receptor would be able to confer 'transactivation' to Pit-1 by titrating the available N-CoR. Indeed, oestrogen receptor bound to TOT, but not to 17-β-oestradiol, resulted in transactivation of Pit-1, and this effect was abolished by overexpression of N-CoR (Fig. 1f).

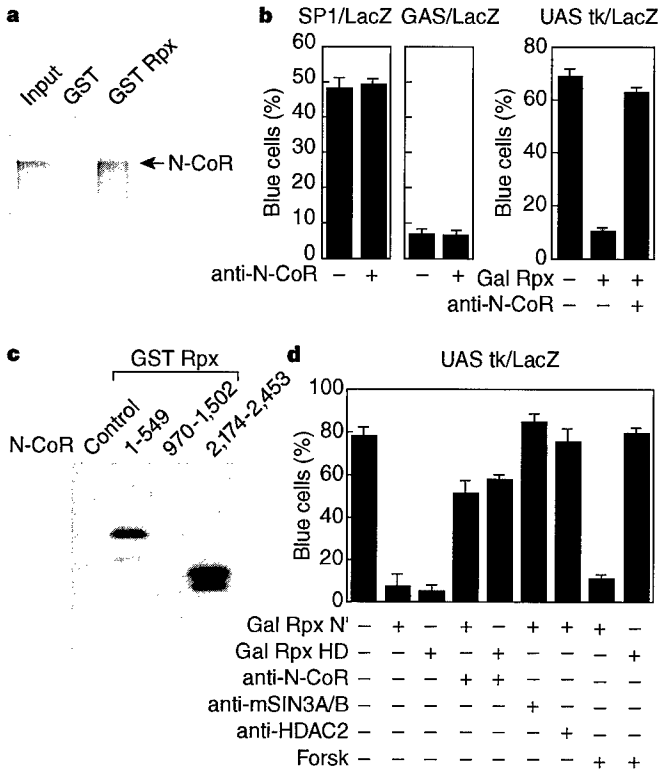

Figure 2 Requirement for N-CoR co-repressor complex in the repression function of Rpx. **a**, GST-Rpx interacted with full-length N-CoR expressed by *in vitro* transcription and translation (TNT, Promega). **b**, Rpx fused to the Gal4 DNA-binding domain was able to repress a high baseline reporter upstream activation sequence (UAS) tk/LacZ[6]. This repression was largely abolished by injection of anti-N-CoR IgG. Control reporters driven by Sp-1 sites or interferon-γ-responsive (GAS) elements were not affected in the same assay. **c**, Mapping of the Rpx-interaction domain on N-CoR. **d**, Injection of purified IgGs against N-CoR, mSin3 A/B and HDAC2 (ref. 13) blocked the repression function of both the N terminus and homeodomain of Rpx. Forskolin treatment reduced the repression function that was mediated by the homeodomain of Rpx. The reporter system is as described in (**b**).

Similarly, unliganded, wild-type thyroid-hormone receptor, which binds to N-CoR, caused strong transactivation of Pit-1, and this was reversed by dismissal of N-CoR from the thyroid-hormone receptor after the addition of ligand (Fig. 1g). This transactivation effect was not observed with an unliganded thyroid-hormone receptor CoR box mutant that is unable to bind N-CoR (Fig. 1g)[14]. These data support a model in which N-CoR, under certain conditions, can be partitioned between multiple transcription factors, providing an additional level of regulation (see below).

Consistent with its role as a co-repressor, N-CoR could interact with homeodomain repressors such as Rpx[2] (Fig. 2a, c, and data not shown). Repression mediated by either the N terminus or homeodomain of Rpx required N-CoR, mSin3A/B and HDAC2, as shown in the microinjection assay (Fig. 2b, d). Thus, the repression function of Rpx seemed to result from the cooperative recruitment of N-CoR to both the N terminus and the homeodomain. Repression that was mediated by the Rpx homeodomain, but not its N terminus, could be relieved by forskolin treatment, which increased the level of intracellular cAMP (Fig. 2d).

Because CBP is involved in activities of many families of transcription factors, we investigated whether the activation function of Pit-1 might be linked to recruitment of the CBP co-activator complex. CBP can interact with Pit-1, both by immunoprecipitation with Pit-1 antibody using cells transfected with Pit-1, and by glutathione *S*-transferase (GST) pull-down assay (Fig. 3a). The most robust interaction mapped primarily to the N terminus of CBP (amino acids 271–450), and represents a new interaction interface on CBP (Fig. 3a). Weaker interaction was also detectable between Pit-1 and a region of CBP that include the C/H3-E1A-binding domain[17]. Mapping indicated that this interaction probably involved both the POU_S and the POU_{HD} regions of Pit-1 (Fig. 3b). The role of CBP in Pit-1 function was tested initially by transient co-transfection assays, which showed that overexpression of CBP enhanced Pit-1 action markedly (Fig. 3c).

Analogous to the ligand-induced displacement of the N-CoR co-repressor complex by the CBP co-activator complex in nuclear-receptor activation, we found that N-CoR and CBP can compete directly for binding to Pit-1. By using a DNA-dependent protein–protein interaction assay[18] to test for possible direct competition between N-CoR and CBP for association with Pit-1, we found that the Pit-1-interaction domain of CBP (amino acids 1–450) did indeed compete with N-CoR for association with Pit-1 bound to its cognate DNA site (Fig. 3d). GST alone had no effect (Fig. 3e). Furthermore, anti-CBP IgG reversed the activation of Pit-1 caused by anti-N-CoR IgG (Fig. 3f), which shows directly the functional antagonism between the co-activator and co-repressor complexes. Extra evidence supporting the model that Pit-1 activity is determined by the relative strength of the interactions between N-CoR and CBP was obtained by systematically mutating surface residues in the Pit-1 homeodomain, on the basis of the crystal structure of Pit-1 on a cognate DNA site[19]. We found a single point mutation (E254A, in the linker preceding helix 3) that inhibited Pit-1 activity (Fig. 3g, h) without disrupting its ability to bind to DNA (data not shown) or to CBP (Fig. 3i), while exhibiting increased affinity for N-CoR (Fig. 3i).

Increasing intracellular cAMP levels, or activating mitogen-activated protein (MAP) kinases by growth factors such as epidermal growth factor (EGF) or insulin, stimulated the prolactin and Pit-1 promoters[20–24]. This effect was actually mapped to Pit-1-binding sites themselves, suggesting a connection between the activation of signal-transduction pathways and Pit-1 function[20,21,24]. In addition, a Pit-1/Ets compound site was defined on the prolactin 3P site and may mediate stimulation of prolactin expression by growth factors[22]. Here we used the reporter driven by the prolactin 1P element[2] (Pit-1 RE/Lac Z), which allowed us to examine the impact of signalling pathways on Pit-1 itself. Treating the cells with forskolin or 8-Br cAMP to elevate intracellular cAMP levels, or

activating MAP-kinase pathways by growth factors such as EGF or insulin, resulted in increased Pit-1 activity (Fig. 4a, b). A Pit-1 protein (Pit_{mut}) with mutations in the protein kinase A (PKA) site within the homeodomain[24] (T219A/T220A) was fully competent to respond to elevated cAMP level or to insulin/EGF treatment (Fig. 4a), indicating that regulation was not conferred by phosphorylation of Pit-1 itself, which is consistent with previous reports[23]. To further confirm that the actions of cAMP and growth factors were not due to cryptic factors binding to the Pit-1 site, we studied a Ga14–Pit-1 fusion protein. The activity of Ga14–Pit-1 could still be stimulated in response to both forskolin or growth factors, and this activation depends on CBP and p/CAF (Fig. 4b). The stimulation of

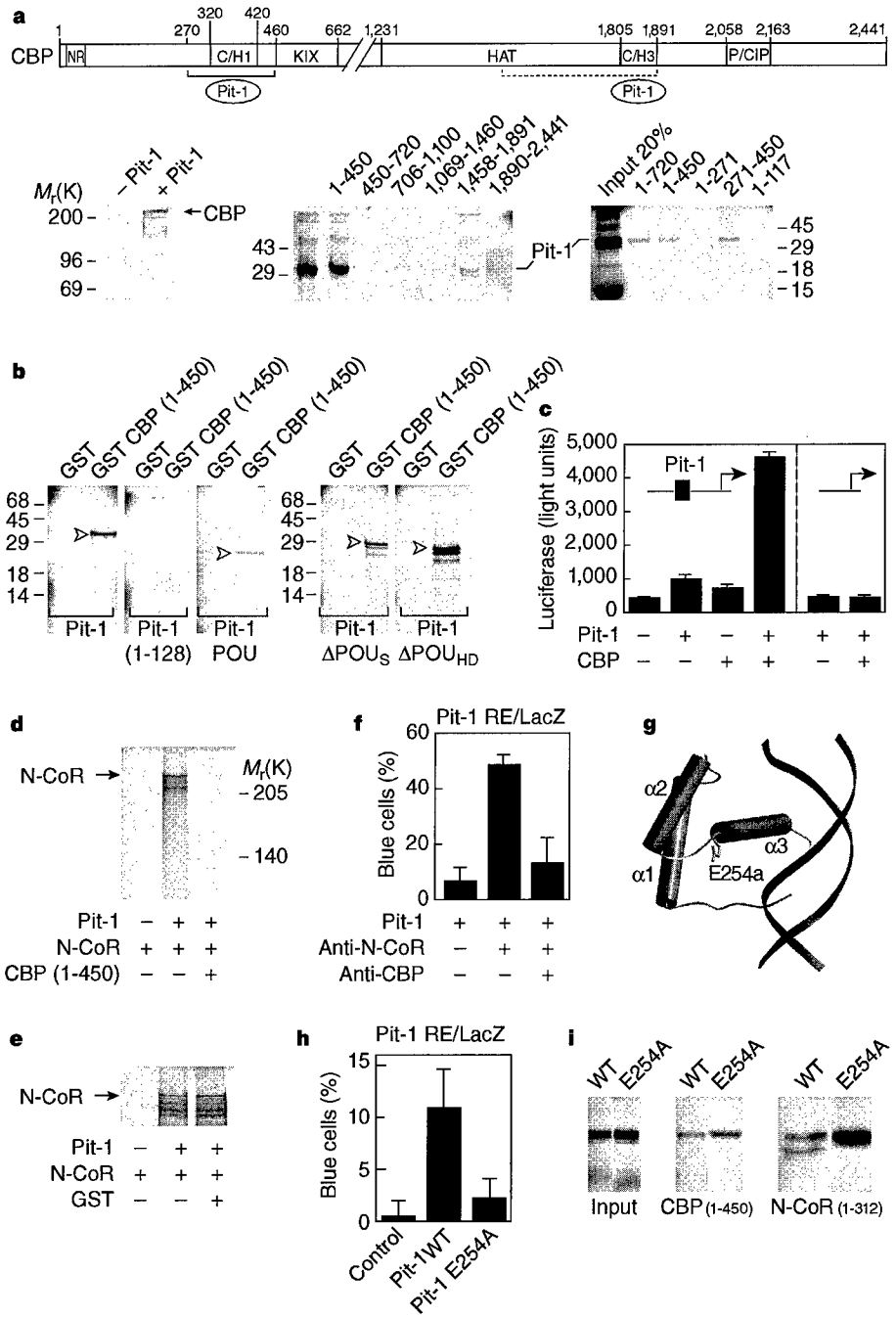

Figure 3 CBP is a required co-activator of Pit-1. **a**, The C/H1 domain of CBP mediates interaction with Pit-1. Whole-cell extracts prepared from 293 cells with or without transfected Pit-1 plasmid were mixed with extracts containing Flag-tagged CBP. Immunoprecipitation was done using anti-Pit-1 antisera and the western blot was detected with anti-Flag antibody (Kodak). GST-fusion proteins of CBP were tested for interaction with ^{35}S-labelled full-length Pit-1. A strong interaction domain was mapped to amino acids 271–450 of CBP, whereas a second weaker interaction domain was mapped to amino acids 1,458–1,891. **b**, Although the combination of POU_S and POU_{HD} (POU) is sufficient for interaction with CBP1–450, deletion of either POU_S or POU_{HD} alone did not eliminate the interaction. **c**, Co-transfection of Pit-1 and CBP led to synergy on a reporter driven by a Pit-1-responsive element. **d**, An avidin/biotin/DNA-dependent protein–protein interaction assay[18] was done using a biotinylated Pit-1 site (Prl-1P) and bacterially expressed His_6–Pit-1 holoprotein (1 μg), GST–CBP (1–450) (5 μg) and ^{35}S-labelled full-length N-CoR produced by TNT (Promega). GST–CBP1–450 effectively prevented N-Cor from binding to Pit-1. **e**, In the control experiment, GST alone did not block N-CoR binding to Pit-1. **f**, Antagonizing roles of N-CoR and CBP in regulating Pit-1 activity. Blockade of N-CoR repression function led to activation of the Pit-1 response element (RE)/LacZ reporter, and this activation was eliminated with anti-CBP IgG. **g**, Pit-1–DNA crystal structure[19] and position of the E254A mutation in Pit-1. **h**, The E254A mutant had much lower activity on the Pit-1-RE reporter. **i**, The E254A mutant had similar affinity for CBP (1–450) compared to the wild type, but had significantly higher affinity for N-CoR (1–312). WT, wild-type.

Pit-1 by forskolin, or by 8-Br cAMP, did not require SRC-1/NCoA-1 (ref. 25) or p/CIP[26], which are crucial for the function of nuclear receptors[26] (Fig. 4c).

As phosphorylation of Pit-1 does not appear to account for regulation of Pit-1 activity for forskolin, we considered the possibility that the required components of the CBP co-activator complex were altered by modulation by PKA. Thus, we investigated which functional domain of CBP is critical for the effect of cAMP on Pit-1 activity. Co-injection of expression plasmids that encode CBP holoprotein or CBP mutants with deletion of the CREB binding domain (ΔKIX)[9], the bromodomain (ΔBr) or the N-terminus (ΔN), all reversed the block of Pit-1 function by anti-CBP IgG, whereas CBP mutants lacking the histone acetyltransferase (HAT) function (ΔHAT)[27], the C/H3 domain (ΔC/H3) or the C terminus (CBP1–1,891) failed to do so (Fig. 4d). In contrast, the p/CIP-binding domain of CBP (Δp/CIP)[15,26] was not vital for cAMP-stimulated Pit-1 activity (Fig. 4d). All CBP constructs were expressed at roughly equivalent levels; more importantly, their functional integrity was confirmed by their activities in other signalling pathways (Fig. 5c, and data not shown). Mutation of the only perfect PKA consensus site (S1772) (ref. 9) in CBP resulted in almost complete loss of the ability of CBP to mediate cAMP induction of Pit-1 function (Fig. 4c), which could reflect either a requirement for phosphorylation of this residue by PKA or the importance of amino acid 1,772 in configuration of this region of CBP.

As both CBP and p/CAF are required for Pit-1 function, it is interesting to determine whether the HAT functions from these two proteins are both required, or if only one of them is involved. Thus, we analysed two point mutations—L1690K/C1691L for CBP, and Y616A/F617A for P/CAF—that abolished their ability to acetylate histones without affecting expression[27]. The ability of wild-type and HAT mutants to reverse the block of Pit-1 functions by anti-CBP and anti-p/CAF indicated that the HAT activity of CBP was required, whereas the HAT function of p/CAF appeared to exert only a minimal role in the activation of Pit-1 by cAMP (Fig. 4e). Our results show that the HAT function, C/H3 domain, and the C-terminal CBP domains are required for stimulation of Pit-1 activity by PKA.

To determine whether these CBP domains could function in a signal-specific fashion, we evaluated their roles in the stimulation of Pit-1 activity by growth factors. Surprisingly, although both CBP and p/CAF were still required, the crucial regions of CBP required to modulate the effects were different from those required for cAMP stimulation. The N-terminal 468 amino acids were vital, whereas the HAT domain and the C/H3 and the C-terminal regions of CBP

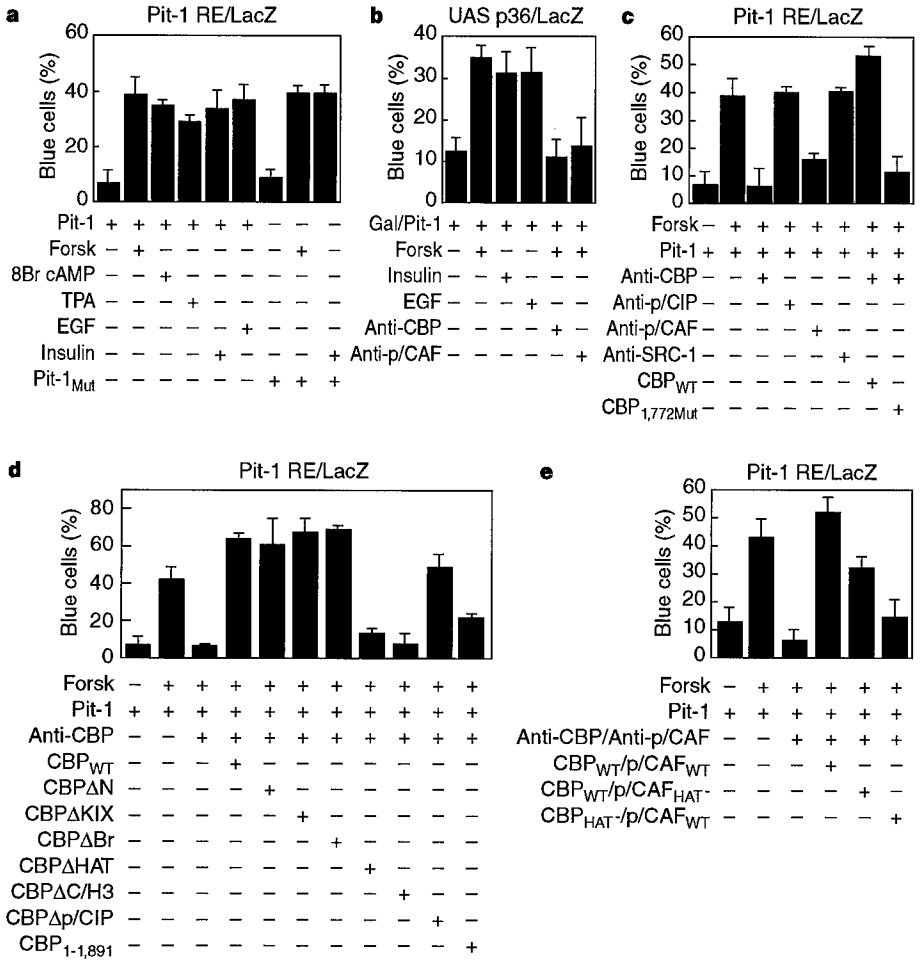

Figure 4 Activation of Pit-1 function by signal-transduction pathways is mediated through the CBP complex. **a**, Pit-1 function is activated by agents stimulating several signal-transduction pathways. Mutations of the PKA sites on Pit-1 (ref. 24) (Pit-1mut: T219A/T220A) did not abolish the ability of both forskolin and insulin to activate Pit-1 function. **b**, The activity of Gal4-Pit-1 can also be stimulated by various signal-transduction pathways. Forskolin-induced activation of Gal4-Pit-1 is dependent on CBP and p/CAF. The reporter contains three copies of UAS sites upstream of the p36 minimal promoter. **c**, Roles of other components of the CBP complex in Pit-1 function stimulated by forskolin. The consensus PKA site in CBP (S1772) was crucial for induction of Pit-1 activity by forskolin. **d**, Regions of CBP required for forskolin-stimulated Pit-1 function. Indicated CBP mutants were used to rescue the blockade of Pit-1 function by co-injected anti-CBP IgG. CBPΔN: Δ2–468; CBPΔKIX: Δ451–720; CBPΔBr: Δ1,100–1,171; CBPΔHAT: Δ1,257–1,413; CBPΔC/H3: Δ1,805–1,891; CBPΔp/CIP: Δ2,058–2,163. **e**, Requirement of the HAT functions of CBP and p/CAF for Pit-1 function. CBP_{HAT^-}: L1690K/C1691L; p/CAF_{HAT^-}: Y616A/F617A.

were no longer required (Fig. 5a). There was no apparent effect of either signal-transduction pathway on levels of expression of any of these mutant CBP proteins, assessed by western blot or by immunohistochemical analysis of injected cells (Fig. 5c, and data not shown). Thus, it appeared that different functional domains of CBP were used by cAMP and growth-factor-induced signalling pathways. Using a mammalian two-hybrid assay with Ga14–CBP1–450 and VP16–Pit-1, we found an enhanced interaction (from 3 to 15% blue cells) between CBP1–450 with Pit-1 in response to insulin, but not cAMP, indicating that a component of the insulin effect might be to increase interactions between CBP and Pit-1. These results also indicated that P/CAF may provide the HAT function required for growth-factor-stimulated Pit-1 activity. Indeed, the HAT$^-$ p/CAF was unable to mediate growth-factor-induced Pit-1 activation (Fig. 5b), in contrast to its role in cAMP stimulation of Pit-1 activity (Fig. 4e). These results indicate use of different HAT activities and distinct domains of CBP for cAMP- or growth-factor-induced Pit-1 activity.

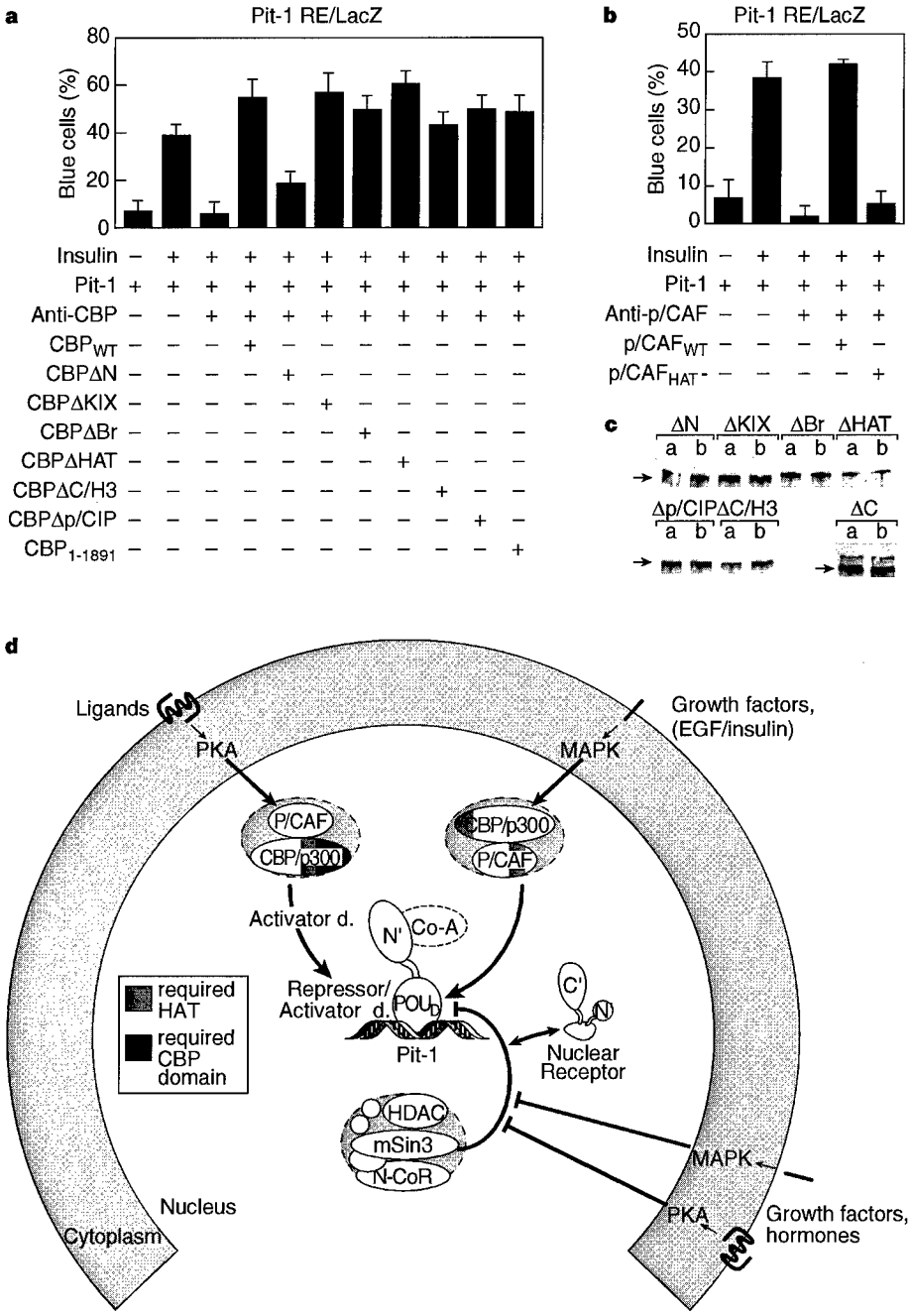

Figure 5 Requirements of HAT functions and domains of CBP for growth-factor-stimulated Pit-1 function. **a**, Regions of CBP required for growth-factor-stimulated Pit-1 function. The CBP mutants are as described in Fig. 4d; note that CBPΔHAT retains full function in this case. **b**, The HAT function of p/CAF is required for growth-factor-stimulated Pit-1 activity in microinjection assays. **c**, CBP mutants were expressed at comparable levels in transfected cells treated with forskolin (a) or growth factor (b), under conditions similar to those in Fig. 4. In the case of ΔC, antibody against the CBP N terminus was used, and the upper band represents endogenous CBP. The others were detected with anti-Flag antibody. **d**, Model of modulation of Pit-1 activation by specific signal-transduction pathways. For the PKA-induced Pit-1 activation, the HAT function and C-terminal domain of CBP are required; for growth-factor-induced Pit-1 activation, the HAT function of p/CAF and the N terminus of CBP are required. A putative Pit-1 N-terminal-associated co-activator (Co-A) is proposed to cooperatively recruit the CBP co-activator complex to the POU domain and displace the N-CoR complex. The co-repressor complex, while exerting repression on both Pit-1 and the unliganded nuclear receptor, can be partitioned (two-headed arrow) between the two classes of transcription factors, resulting in 'transactivation'.

Together, these data show that the large family of activating homeodomain transcription factors, such as Pit-1, exert their functions through a balance between the N-CoR co-repressor complexes and the CBP co-activator complexes. Pit-1 activity is regulated by distinct signal-transduction pathways, through mechanisms that do not appear to involve modification of Pit-1 itself, but at least in part through regulation of the recruited co-activator complex. A surprising consequence of these events is that a single transcription factor, Pit-1, actually uses the HAT functions of different proteins, and requires the function of different domains of CBP when activated in response to cAMP or growth-factors (Fig. 5d). These results have implications for the mechanism of integration of signalling events that control complex patterns of gene expression by many classes of transcription factors. □

Methods

Protein–protein interaction assays. All the GST–fusion proteins were expressed and purified as described previously[4,15]. *In vitro* protein–protein interaction assay, immunoprecipitation, GST pull-down and DNA-dependent protein–protein interaction (ABCD) assays were done as described[4,6,18].

Nuclear microinjection, staining and fluorescence microscopy. Microinjection analysis was done as described previously using affinity-purified IgGs[6,15,26,28].

Transient transfection. HeLa and 293 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Calcium precipitation mediated transient transfection was done according to standard protocol.

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Biochemistry

Factor-specific modulation of CREB-binding protein acetyltransferase activity

(p300/CBP interacting protein/p300/CBP associated factor/E1A)

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ABSTRACT **CREB-binding proteins (CBP) and p300 are essential transcriptional coactivators for a large number of regulated DNA-binding transcription factors, including CREB, nuclear receptors, and STATs. CBP and p300 function in part by mediating the assembly of multiprotein complexes that contain additional cofactors such as p300/CBP interacting protein (p/CIP), a member of the p160/SRC family of coactivators, and the p300/CBP associated factor p/CAF. In addition to serving as molecular scaffolds, CBP and p300 each possess intrinsic acetyltransferase activities that are required for their function as coactivators. Here we report that the adenovirus E1A protein inhibits the acetyltransferase activity of CBP on binding to the C/H3 domain, whereas binding of CREB, or a CREB/E1A fusion protein to the KIX domain, fails to inhibit CBP acetyltransferase activity. Surprisingly, p/CIP can either inhibit or stimulate CBP acetyltransferase activity depending on the specific substrate evaluated and the functional domains present in the p/CIP protein. While the CBP interaction domain of p/CIP inhibits acetylation of histones H3, H4, or high mobility group by CBP, it enhances acetylation of other substrates, such as Pit-1. These observations suggest that the acetyltransferase activities of CBP/p300 and p/CAF can be differentially modulated by factors binding to distinct regions of CBP/p300. Because these interactions are likely to result in differential effects on the coactivator functions of CBP/p300 for different classes of transcription factors, regulation of CBP/p300 acetyltransferase activity may represent a mechanism for integration of diverse signaling pathways.**

The regulation of gene transcription by DNA-binding transcription factors has been linked to the recruitment of CBP/p300, the p300/CBP associated factor (p/CAF) complex, as well as other cofactors, such as the p160/SRC/TIF2/p/CIP (1–5) family of factors (6). The CREB-binding protein (CBP) (7, 8) and the p300 adenoviral protein E1A interacting protein (9) have been implicated in the actions of a large number of regulated transcription factors (10), based on experiments using neutralizing antibodies against CBP/p300, *in vivo* gene deletion, and specific ribozymes (3, 6, 11, 12). The discovery that GCN5 (13), CBP/p300 (14, 15), and p/CAF (16) harbor intrinsic acetyltransferase activities for histones and for other proteins (17, 18), has led to a model of the role of these factors in the regulation of chromatinized DNA templates (19, 20). Indeed, in both biochemical and cell-based assays the acetyltransferase functions of CBP and/or p/CAF have proved critical for transcriptional function (7, 21). CBP/p300 and p/CAF are large proteins that contain conserved domains, each of which appear to interact with large numbers of distinct DNA-binding transcription factors, which include nuclear receptors (6), CREB (8), and STAT proteins, (22–24). In addition, CBP/p300 interact with other classes of modulating proteins such as RNA helicase A, the p160/SRC-1/p/CIP family of factors, and S6 kinase (25, 26). This property may underlie the putative ability of CBP/p300 to serve as nuclear integrators of transcriptional responses (6, 27).

In addition to potential roles of CBP/p300 in the modification of chromatin structure, a number of additional substrates have been identified that include DNA-binding transcription factors, such as p53 (28), GATA-1 (29), T cell factor-1 (17), and high mobility group (HMG) I/Y (18). Acetylation of these proteins can increase DNA binding (28, 29), decrease binding (18), or inhibit protein–protein interaction (17).

The adenoviral immediate early gene product, E1A, is well characterized as an inhibitor of many classes of CBP-dependent transcription factors (19, 20). Although E1A was initially found to bind to the C/H3 domain in CBP/p300 (16) there appear to be additional interaction domains that are of differential functional importance for different classes of transcription factors. Because E1A binds to critical control regions of CBP, models predicting competition between E1A and functional CBP interacting proteins, including p/CAF, RNA helicases, and p/CIP have been suggested (25, 26). Thus, a series of DNA-binding transcription factors that directly interact with the C/H3 domain of CBP/p300 might directly compete for access to this cofactor.

The cAMP-dependent transcription factor CREB interacts strongly with CBP, in response to CREB phosphorylation at Ser-133. CBP is required for CREB function (27), dependent on its acetyltransferase activity (3, 27). Similarly, the IFN-γ-dependent transcription factor STAT1 also binds to CBP and requires the CBP-HAT (histone acetyltransferase) activity for function (21). In contrast, nuclear receptors require additional factors, including p160/SRC/p/CIP (6), to recruit CBP complexes. Intriguingly, the retinoic acid receptor appears to require the acetyltransferase function of p/CAF, rather than that of CBP (21), raising questions concerning the potential regulation of CBP/p300 HAT activities by these and other coregulatory molecules.

In this paper, we report that the interaction of E1A with the C/H3 region is capable of strongly inhibiting CBP HAT function on a variety of substrates, including histones H4 and H3, HMG I/Y, HMG 14/17 and, to a limited extent, on CBP

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Abbreviations: CBP, CREB-binding protein; E1A, adenoviral oncoprotein E1A; p/CAF, p300/CBP associated factor; HAT, histone acetyltransferase; p/CIP, p300/CBP interacting protein; HMG, high mobility group.

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itself. In contrast, binding of CREB, or even of a CREB/E1A fusion protein, to the KIX domain does not have this effect. Surprisingly, the CBP interaction domain of p/CIP (3) also strongly inhibits CBP acetyltransferase function, whereas on a number of substrates the presence of additional p/CIP domains overcomes the inhibition of CBP HAT function. These data suggest that p/CIP is an allosteric regulator of the acetyltransferase activity of CBP, and that this effect may be modified further by interacting factors and/or covalent modifications. Together we speculate that a component of the CBP integration function reflects substrate-specific regulation of its acetyltransferase functions. Similar events may modulate p/CAF acetyltransferase function.

MATERIALS AND METHODS

Single Cell Microinjection Assay. Quiescent insulin-responsive Rat-1 fibroblasts were seeded on acid-washed glass coverslips at subconfluent density and grown in MNE/F12 medium, supplemented with 10% FBS, gentacin, and methotrexate. Expression plasmids were injected into the nuclei of cells at 100 μg ml^{-1} by using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. About 1 hr after injection cells were stimulated, where indicated, with the appropriate ligand. In rescue experiments, cells were stimulated with ligand 6 hr after injection to allow protein expression. After overnight incubation, cells were fixed and stained to detect injected IgG and β-galactosidase expression (6).

Protein Preparation and Acetylation Assays. For bacterial expression all vectors were constructed as glutathione *S*-transferase or HisG fusion, and purified from cultures grown to OD = 0.6–0.8, and induced 90–120 min at 37°C with 1 mM isopropyl β-D-thiogalactoside. Bacteria were lysed and purified in the presence of a phenylmethylsulfonyl fluoride/Boehringer–Mannheim protease inhibitor cocktail by using glutathione agarose or nickel columns (1–2 hr, 4°C). Flag-tagged CBP was expressed in a baculoviral expression vector and purified by using an anti-Flag IgG column. For CREB and CREB (1–198)/E1A (29–242) fusion protein interactions with CBP, recombinant proteins were incubated with the protein kinase A catalytic domain (Sigma) in the presence of 0.2 mM ATP (30 min, 4°C), stopped, and the phosphorylated proteins purified on affinity beads. All proteins were checked for purification to apparent homogeneity by SDS/PAGE. Acetylation reactions were performed in 20 μl of 10 mM sodium butyrate, 50 mM Tris·HCl (pH 7.6), 0.5 mM DTT, ~2 μg of each protein, and 50 μM [^{14}C]acetyl-CoA (50 mCi/mmol), and were incubated at 30°C for 30 min (CBP) or 1 hr (p/CAF) before electrophoresis on SDS/polyacrylamide gels.

Mutations of CBP and E1A were generated with the PCR or the Quick-Change Mutagenesis kit (Stratagene), confirmed by sequence analysis and substitution of a region containing the mutation into the wild-type vector backbone.

RESULTS

E1A Actions on CBP HAT Function. The effects of E1A on the HAT activity of CBP were initially carried out by using Flag-tagged CBP expressed in baculovirus-infected SF-9 cells. The addition of bacterially expressed E1A caused a marked inhibition of both histone H4 and histone H3 acetylation (Fig. 1). Deletion of exon 2 information at amino acid 139 [E1A(139)] reduced but did not abolish the ability of E1A to inhibit histone acetylation activity (Fig. 1 *A* and *B*). However, truncation to amino acid 82, excluding CR2 and CR3, showed a marked loss in inhibitory activity and truncation to amino acid 36 abolished activity (data not shown).

To examine the substrate specificity of these effects, the acetyltransferase activity of CBP was assayed by using HMG I/Y, HMG 14, and HMG 17 as substrates. E1A(1–139) was an effective inhibitor of acetylation of all of these substrates (Figs. 1 and 2). Even CBP autoacetylation was somewhat impaired by E1A(1–139) (Fig. 1*C*). Based on the extensive study of E1A,

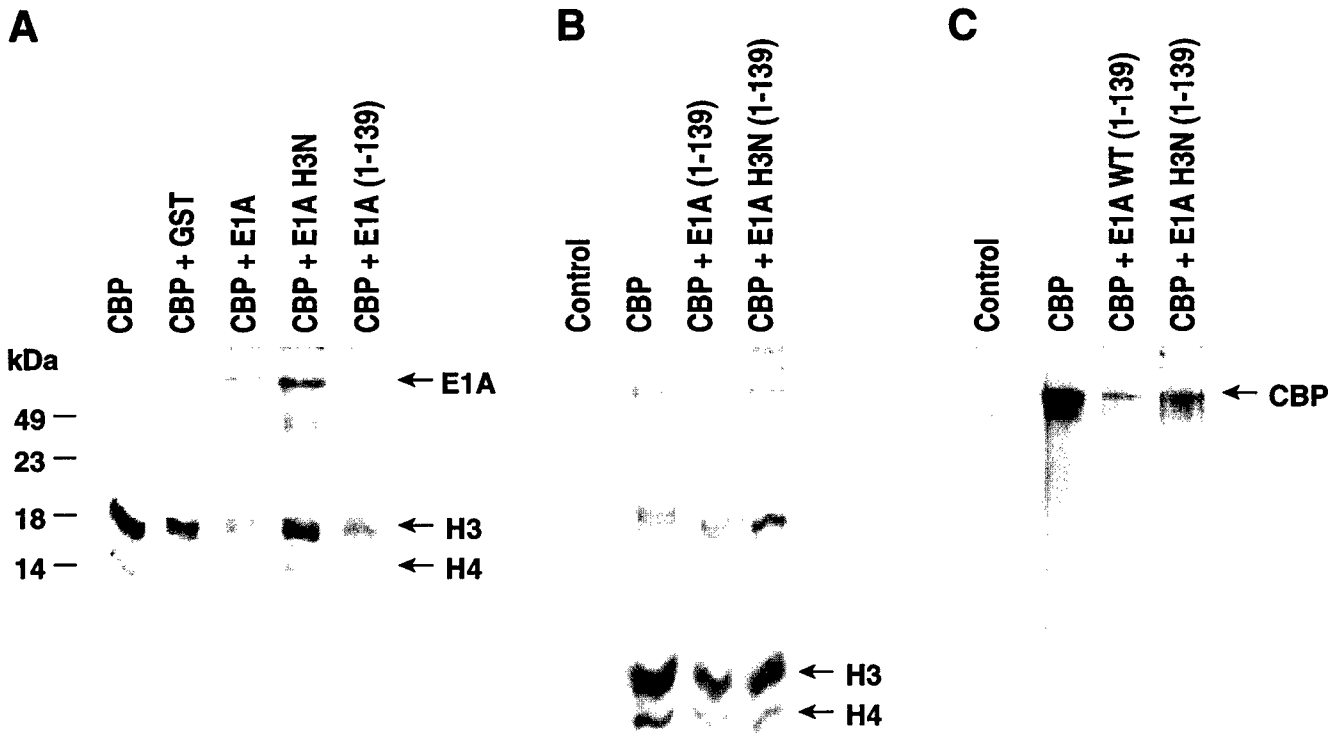

FIG. 1. Effects of E1A on CBP acetyltransferase function. (*A*) Effects of CBP-dependent histone H3 and histone H4 acetylation are shown using 12S E1A holoprotein (1–242) or mutant (H3N) E1A(1–242) or E1A(1–139). The H3N mutation causes loss of binding to the C/H3 domain of CBP. (*B*) Actions of E1A(1–139) or a H3N mutant of E1A(1–139). (*C*) Comparison of CBP autoacetylation in the presence of CBP(1–139) or the H3N mutant E1A(1–139). We note that the C terminus of E1A (amino acids 139–242) represents a substrate for CBP-dependent acetylation.

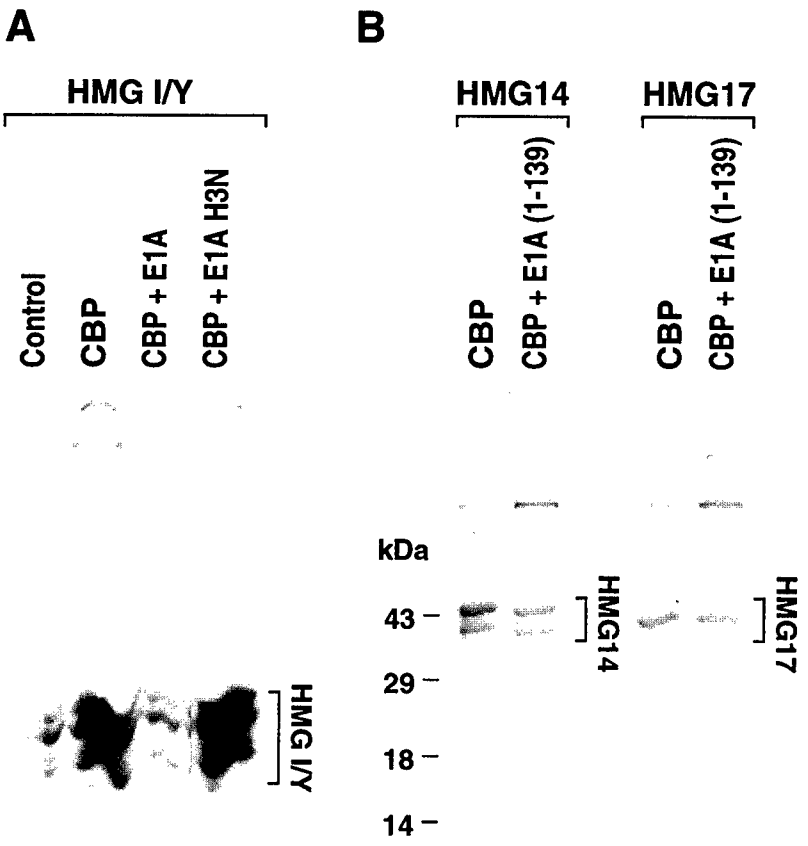

FIG. 2. Effects of E1A on CBP-dependent acetyltransferase of HMG I/Y, HMG 14, or HMG 17. (*A*) Effects of the addition of E1A or H3N E1A on CBP-dependent acetylation of HMG (I/Y). (*B*) Effects of E1A on HMG 14 and HMG 17 acetylation with less severe inhibition than with HMG I/Y.

mutants that impair specific functions have been identified. An H3N mutation at the N terminus inhibits binding of E1A to the C/H3 domain, but only modestly impairs binding of E1A to the N terminus and p/CIP interaction domains of CBP (12). This mutant E1A protein is nearly as effective as wild-type E1A in inhibiting retinoic acid receptor function, but is much less active as an inhibitor of STAT function (12). The H3N E1A holoprotein or H3N E1A(1–139) was a much weaker inhibitor of CBP acetyltransferase function (Fig. 1 *A* and *B*). In contrast to E1A wild-type protein, this mutant E1A does not effectively inhibit CBP HAT function on either histone H3, H4, or HMG I/Y (Figs. 1 and 2*A*). Inhibitory effects on HMG14 or HMG17 were less marked (Fig. 2*B*). Therefore, effects of E1A may correlate, to some extent, with the region of CBP with which it interacts, and the interaction with the C/H3 domain appears to be particularly important for E1A inhibitory effects on CBP acetyltransferase function. Interestingly, E1A also inhibited p/CAF HAT activity, and H3N E1A was almost equivalent to the wild-type protein with respect to this inhibition (Fig. 3*A*).

CREB Binding Does Not Inhibit CBP HAT Activity. Based on the actions of E1A, it became of interest to determine whether other factors, such as CREB, affect HAT function on interaction with CBP. CREB exhibits high-affinity binding to the KIX domain after activation by protein kinase A phosphorylation of Ser-133 (8, 30). Microinjection experiments have previously indicated that CREB requires CBP HAT function to activate CRE-dependent transcription. Therefore, bacterially expressed, purified CREB was phosphorylated by using protein kinase A and assessed for effects on CBP HAT function. As shown in Fig. 3*B*, addition of phosphorylated CREB did not inhibit CBP acetyltransferase activity by using either histone H3 and H4 or HMG I/Y as substrates (18), and there was actually a slight increase of CBP autoacetylation (Fig. 3*B*). Because C-terminal E1A information was required for effective inhibition of CBP acetyltransferase activity, we inquired as to whether fusing N-terminally truncated E1A to the CREB C terminus would alter its effects on CBP acetyltransferase function on substrates tested. Here, protein kinase A-phosphorylated CREB or a CREB/E1A fusion protein was used. Interestingly, in contrast to wild-type E1A, the CREB/E1A fusion protein exhibited no detectable ability to inhibit CBP HAT function. Consistent with a requirement for CBP HAT function for actions of CREB, interactions dependent on the KIX domain did not negatively control CBP acetyltransferase activity. One possible interpretation is that the specific interaction domain on CBP is a critical component in determining the ability of specific protein sequences to inhibit acetyltransferase activity.

p/CIP/CBP Functional Interactions. The SRC/NCoA family of p160 factors, including p/CIP, have been shown to exhibit ligand-dependent interactions with a number of nuclear receptors and to be capable of enhancing transcription on specific promoters. Indeed, nuclear receptor interactions with these factors, dependent at least in part on LXXLL-containing helical motifs (31), are more robust than with CBP, and it is postulated that CBP is recruited to liganded nuclear receptors by means of the p160 factors (6, 32). This recruitment involves a 100-aa domain that is highly conserved among the three known members of this family, SRC/NCoA (1–3), GRIP/TIF2/$NCoA_2$ (1, 2), or p/CIP/ACTR/AIB (3–5). This interaction appears to require a domain in CBP C terminal to the C/H3 domain (3, 31). Therefore, we assessed the effects of the CBP interaction domain of p/CIP (947–1084) on CBP acetyltransferase function. Unexpectedly, this region of p/CIP (p/CIP_{CBP}) was capable of marked inhibition of CBP acetyltransferase activity when histone H3, histone H4, HMG 14, or HMG 17 were used as substrates (Fig. 4 *A* and *B*). This result was particularly surprising because p/CIP often synergizes

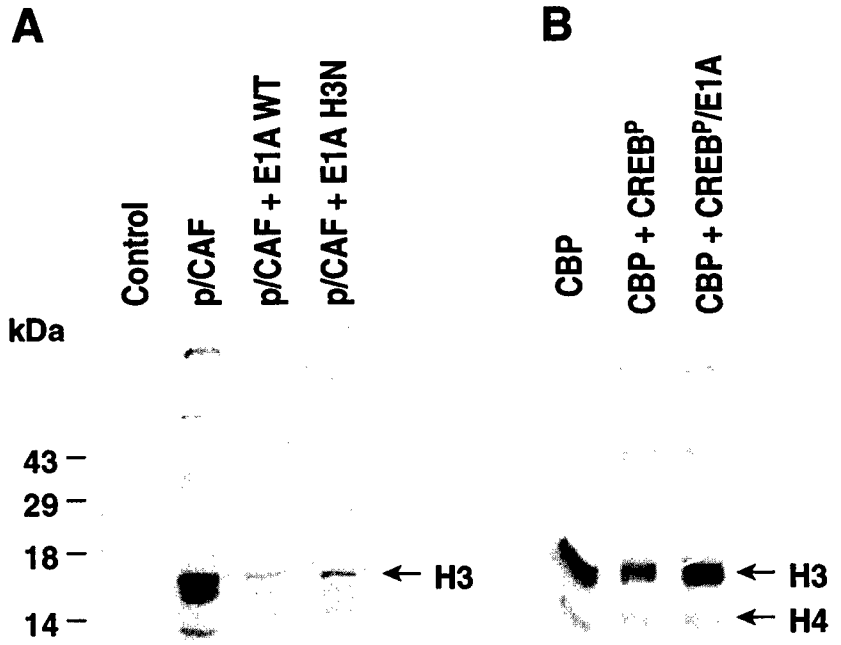

FIG. 3. HAT specificity and factor specificity of enzyme inhibitory actions. (*A*) Effects of E1A on p/CAF-dependent acetylation of histone H3. (*B*) Effect of CREB or CREB/E1A fusion protein or HAT activity of CBP on histone H3/H4. Neither CREB nor the CREB fusion protein inhibited CBP-dependent acetylation. Similar results were obtained with CBP/H3N E1A(1–242) fusion protein.

with CBP in receptor activation (3). Therefore, to further examine this issue, we expressed and purified a p/CIP protein that encompassed all known regulatory domains, including the nuclear receptor and CBP interaction domains, and a C-terminal domain that has been reported to have weak transactivation properties (3) and weak HAT activity (4, 33). However, when we evaluated the purified p/CIP protein, expressing this putative HAT domain, which migrated as a

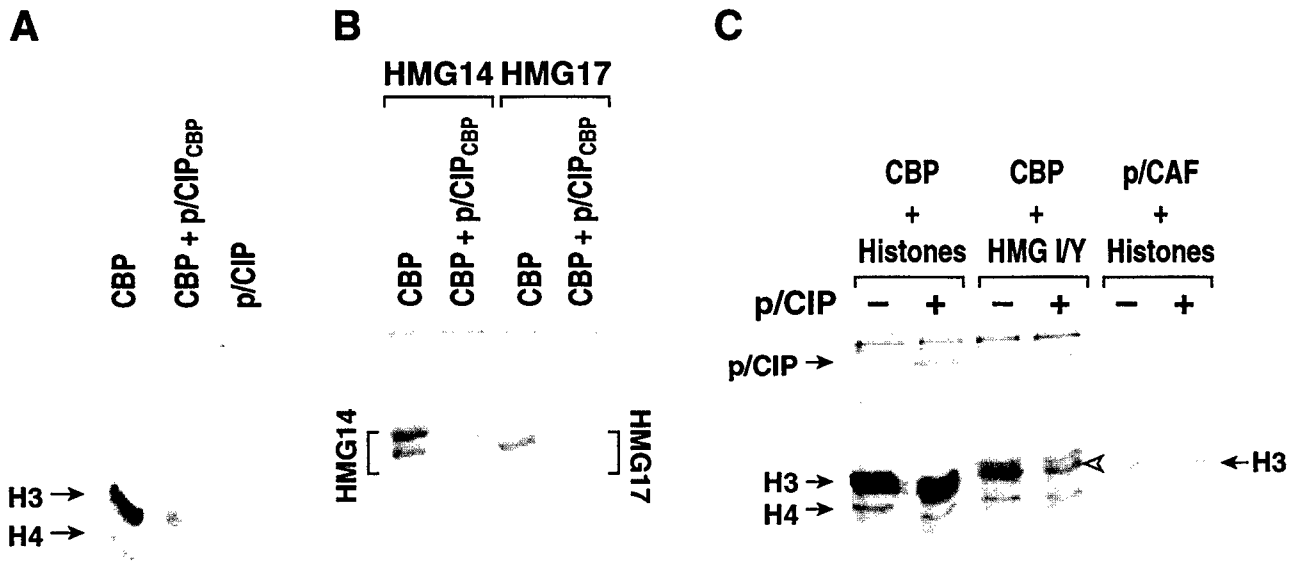

FIG. 4. Role of p/CIP on CBP protein acetyltransferase activity. (*A*) Inhibiting effects of the p/CIP CBP interaction domain (aa 947–1089), on histone acetylation. (*B*) Inhibiting effects of the p/CIP CBP interaction domain on HMG 14 and HMG 17 acetylation. (*C*) Inhibitory effects of p/CIP encompassing known functional domains (aa 636–1304) on CBP-dependent histone, HMG I/Y acetylation, or p/CAF-dependent histone H3 or histone H4 acetylation. While P/CIP$_{CBP}$ is not acetylated, the longer form is a CBP substrate.

single band of the expected molecular weight, for its ability to alone acetylate histones, no detectable activity was observed (Fig. 4*C*). When this longer version of p/CIP was bound to CBP and CBP acetyltransferase activity was assessed, there was no detectable inhibition of CBP acetyltransferase activity on any substrate tested. Therefore, the inhibitory activity apparently present in the CBP interaction domain is overcome by the presence of the adjacent domains of p/CIP.

Consistent with these differential actions of the p/CIP CBP interaction domain alone versus the extended molecule on CBP acetyltransferase activation we found that the presence of the p/CIP CBP interaction domain, in contrast to the p/CIP holoprotein, inhibited both CREB-dependent and IFN-γ-dependent transactivation events (Fig. 5 *A* and *B*) by using the single-cell microinjection assay. When the two p/CIP LXXLL-related helical motifs in the CBP interaction domain were mutated, which abolished interaction with CBP (31), it now failed to inhibit CREB or IFN-γ-dependent transcription (data not shown). Although there are many potential explanations for these data, they are consistent with the observation that HAT activity of CBP, required for CREB-dependent gene activation in response of CBP activation (19), is inhibited in a manner that depends on the specific domains of p/CIP that are expressed.

Finally, we wanted to determine whether there might be substrate specificity associated with the observed inhibitory effects of p/CIP. Therefore, we examined Pit-1 as a potential substrate, because the transcriptional actions of Pit-1 have been linked to CBP (34). Pit-1 holoprotein proved a poor substrate for activation by CBP HAT, but this acetylation was inhibited by E1A. Unexpectedly, the addition of the p/CIP CBP interaction domain, in contrast to actions of histone H3 or H4, or HMG I/Y acetylation, actually enhanced Pit-1 acetylation (Fig. 5*C*).

DISCUSSION

The discovery that CBP/p300 and p/CAF possess intrinsic HAT activities that are critical to their ability to activate transcription has intriguing implications for multifactorial regulation of gene expression. In this paper we report initial biochemical and cell culture experiments indicating that CBP and p/CAF HAT activities can be regulated, in some cases, in a substrate-specific or selective fashion.

The ability of E1A to inhibit multiple transcription factors can be correlated, for many cases, with the ability of E1A to bind to CBP/p300 (12). Because we find that the H3N E1A mutant protein is less effective than wild-type E1A at inhibiting CBP HAT activity and has selectively lost the ability to interact with the C/H3, but not with other domains of CBP, we suggest that the domain of CBP to which E1A binds is a critical component of its ability to exert inhibitory actions on HAT activity. Conversely, the requirement for C-terminal sequences of E1A for effective inhibition suggests that both specific

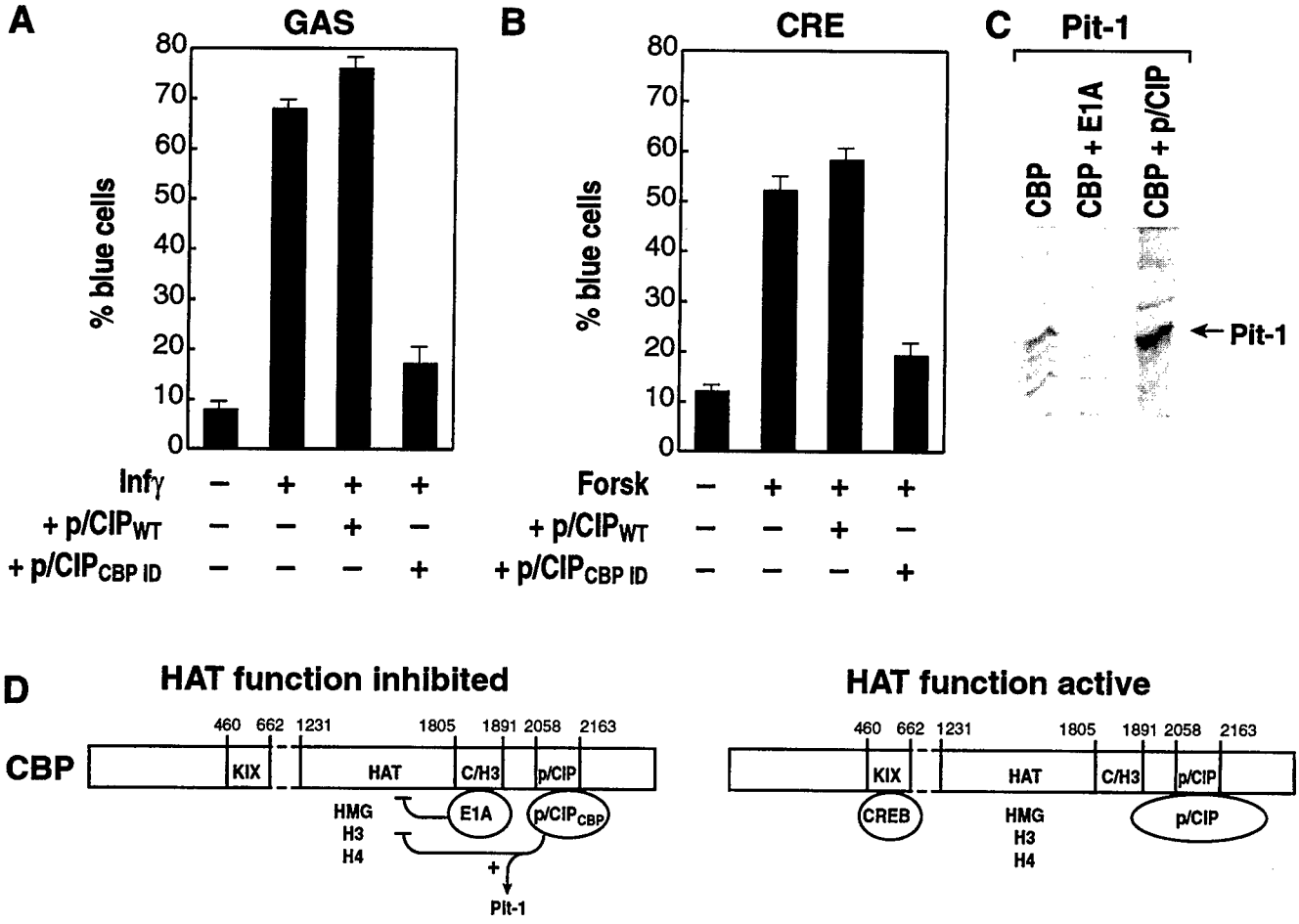

FIG. 5. Correlation of p/CIP effects on function and CBP HAT activity. (*A*) Effects of p/CIP_{CBP} or p/CIP holoprotein on interferon-dependent gene activation by using the nuclear microinjection assay and a gal4 activation sequence (GAS)-dependent reporter as described (3). (*B*) Effects of p/CIP holoprotein and p/CIP ligand binding interaction domain on forskolin-induced CREB-dependent activation as described (3). (*C*) Effects of E1A and the p/CIP CBP interaction domain on CBP HAT function, with Pit-1 as a substrate. (*D*) Factor interactions with CBP can result in inhibition of acetyltransferase functions or not interfere with the CBP acetyltransferase function. The differential effect of the p/CIP CBP interaction domain suggests combinatorial, allosteric regulation of CBP acetyltransferase function.

interaction regions within CBP and distinct domains within E1A appear to be necessary for effective inhibition.

In contrast, consistent with the requirement of CBP HAT function for its activity, CREB fails to inhibit CBP HAT function, and this is true also of a CREB/E1A fusion protein that harbors the region of E1A required for its inhibitory function. Therefore, when bound to the KIX domain, these sequences fail to inhibit HAT function, supporting further the idea that both the domain of interaction and the information in the interacting protein are determinants of the ability to perturbate CBP HAT activity.

Investigation of the interactions between the coactivators p/CIP and CBP has led to a second, potentially important aspect of regulation of CBP HAT activity. Unexpectedly, the minimal CBP interaction domain of p/CIP harbors information that, on binding to a specific C-terminal domain of CBP, has proven to inhibit CBP HAT function by using histones and HMG proteins as substrates, but to stimulate acetyltransferase activity when Pit-1 was used as a substrate. Furthermore, with the inclusion of additional functional domains, p/CIP failed to inhibit histone acetylation. In contrast to previous reports, we have been unable to document significant acetyltransferase activity of p/CIP itself, and such an activity does not account for the effects of p/CIP on Pit-1 acetylation. In concert, these observations raise the possibility that p/CIP may regulate CBP function *in vivo* by either stimulating or inhibiting its acetyltransferase. Such effects may in turn be regulated by interactions of p/CIP with other cellular factors or by posttranslational modifications. Our data are most consistent with an allosteric model for the effects of CBP interacting factors, because of the observation that the p/CIP interaction domain, which inhibited acetylation of histones H3 and H4, enhanced acetylation of Pit-1. Distinct regions of CBP may therefore act in a potentially combinatorial fashion to modulate its acetyltransferase function, providing an additional mechanism for integration of nuclear signaling events (see Fig. 5*D*).

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