

# Regulation of *Id2* expression by CCAAT/enhancer binding protein $\beta$

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## ABSTRACT

Mice deficient for *Id2*, a negative regulator of basic helix–loop–helix (bHLH) transcription factors, exhibit a defect in lactation due to impaired lobuloalveolar development during pregnancy, similar to the mice lacking the CCAAT enhancer binding protein (C/EBP)  $\beta$ . Here, we show that *Id2* is a direct target of C/EBP $\beta$ . Translocation of C/EBP $\beta$  into the nucleus, which was achieved by using a system utilizing the fusion protein between C/EBP $\beta$  and the ligand-binding domain of the human estrogen receptor (C/EBP $\beta$ -ERT), demonstrated the rapid induction of endogenous *Id2* expression. In reporter assays, transactivation of the *Id2* promoter by C/EBP $\beta$  was observed and, among three potential C/EBP $\beta$  binding sites found in the 2.3 kb *Id2* promoter region, the most proximal element was responsible for the transactivation. Electrophoretic mobility shift assay (EMSA) identified this element as a core sequence to which C/EBP $\beta$  binds. Chromatin immunoprecipitation (ChIP) furthermore confirmed the presence of C/EBP $\beta$  in the *Id2* promoter region. Northern blotting showed that *Id2* expression in C/EBP $\beta$ -deficient mammary glands was reduced at 10 days post coitus (d.p.c.), compared with that in wild-type mammary glands. Thus, our data demonstrate that *Id2* is a direct target of C/EBP $\beta$  and provide insight into molecular mechanisms underlying mammary gland development during pregnancy.

## INTRODUCTION

Transcription factors play a pivotal role in gene networks that control various events related to differentiation, proliferation

and responses to extra- and intracellular stimuli. To understand the molecular bases underlying complex biological processes, it is essential to elucidate the functional relationships of transcription factors and their regulators that are involved in a process of interest.

The mammary gland provides a good experimental model system to investigate mechanisms underlying cell proliferation, differentiation, apoptosis and tissue remodeling (1). In pregnancy, under the influence of various hormones and growth factors, mammary epithelial cells undergo rapid proliferation and subsequent differentiation into an expanded glandular structure via the process of lobuloalveolar development (1). Loss-of-function experiments in mice have revealed several genes that are involved in the distinct steps of mammary gland development (2). These genes include not only nuclear hormone receptors, i.e. the estrogen receptor and progesterone receptor, but also other transcription factors and cell cycle regulators (2).

Id proteins are negative regulators of basic helix–loop–helix (bHLH) transcription factors that are exemplified by MyoD and Neurogenin (3). They have a helix–loop–helix (HLH) motif but lack a DNA-binding domain. Due to these structural features, Id proteins dimerize with bHLH factors (mainly with ubiquitously expressed bHLH factors known as E proteins) in a dominant-negative manner, and prevent bHLH factors from forming hetero- and homodimers (3). Four members of the Id protein family, Id1 through Id4, have been identified in mammals (3). Since bHLH factors are involved in the regulation of multiple processes of cell differentiation, proliferation and cellular functions, Id proteins also participate in these biological situations. We previously reported that mice deficient for *Id2* show a lactation defect due to impaired lobuloalveolar development of the mammary gland during pregnancy (4). The observed defect is due mostly to the impaired proliferation and enhanced apoptosis of mammary epithelial cells in the early and late phases of pregnancy, respectively (4).

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The CCAAT enhancer binding proteins (C/EBPs) are transcription factors bearing a basic leucine zipper motif. Six members of the family, C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\gamma$ , C/EBP $\delta$ , C/EBP $\epsilon$  and C/EBP $\zeta$ , have been identified, and homo- and heterodimers formed by the members and their isoforms generated by differential usage of the start codon (5–7). C/EBPs act as important regulators of cellular differentiation, proliferation and function in a variety of tissues (5–7). Mice deficient for C/EBP $\beta$ , also known as NF-IL6 (8), display impaired lobuloalveolar development, which is associated with perturbed proliferation of mammary epithelial cells in the early phase of pregnancy (9,10), as in the case of *Id2*-null mice (4). Elevated expression of the progesterone receptor in mammary epithelial cells is found in both these types of null mutant mice (4,11).

To understand the molecular mechanisms underlying pregnancy-induced mammary gland development, we investigated the relationship between *Id2* and C/EBP $\beta$ , and found that *Id2* is under the control of C/EBP $\beta$ . Our present work revealed a gene network that regulates normal mammary gland development in response to gestation.

## MATERIALS AND METHODS

### Mice and animal experiments

Both *Cebpb* mutant and *Rag2* mutant mice were maintained under specific pathogen-free conditions. Transplantation of mammary glands to *Rag2*-null mice and whole mount staining of mammary glands with carmine were done as described previously (4). All experimental procedures followed the guidelines of University of Fukui for animal experiments.

### Plasmid construction

pC/EBP $\beta$ -ERT and pC/EBP $\beta$ -6Myc were constructed with fragments corresponding to nucleotides 897–994 and 1–994 of the mouse *Cebpb* cDNA, respectively, that were amplified by PCR with pMSV-C/EBP $\beta$  (12) as a template. For pC/EBP $\beta$ -ERT, the PCR fragment was used to substitute the corresponding region of pMSV-C/EBP $\beta$  and the XhoI–EcoRI fragment (the region encoding the human estrogen receptor ligand-binding domain, ERT) of pBS-ERT2 (gift from T. Nakano) was inserted into the XhoI and EcoRI sites of the resultant plasmid. For pC/EBP $\beta$ -6Myc, the endogenous NheI site present in the end of the coding region of the *Cebpb* cDNA was changed to BamHI or XhoI sites by using appropriate reverse primers. pC/EBP $\beta$ -6Myc was generated by inserting the amplified fragment into the SmaI and BamHI sites of pCMV-6Myc (13).

Construction of a series of reporter plasmids bearing various lengths of the *Id2* promoter was done using pGL2-basic (Promega) and the plasmid pBS-Eco10K, which harbors a mouse genomic fragment (~10 kb) containing the whole *Id2* gene locus (14). Endogenous restriction sites for BamHI, HindIII, SmaI and SacI in the *Id2* promoter region were utilized to generate pGL2-*Id2*/A, pGL2-*Id2*/C, pGL2-*Id2*/D and pGL2-*Id2*/E, respectively. Plasmids pGL2-*Id2*/F and pGL2-*Id2*/G were generated by subcloning the corresponding PCR fragments prepared with appropriate primer sets into pGL2-basic. Reporter plasmids bearing proximal deletion mutants,

pGL2-*Id2*/E  $\Delta$ 1 through  $\Delta$ 3, were generated by substituting the EcoRI–ApaLI fragment of pGL2-*Id2*/E with mutated fragments prepared by annealing synthesized sense and antisense oligonucleotides. All the reporter constructs contained the sequence 5' to the translation start codon of the *Id2* gene, including the endogenous TATA box. Authenticity of the plasmids was verified by sequencing.

### Cell cultures and DNA transfection

NIH3T3 cells were maintained in DMEM supplemented with 10% fetal calf serum plus 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. For transient transfection, NIH3T3 cells were plated at densities of  $4 \times 10^4$  cells/1.6-cm well or  $1 \times 10^6$  cells/10-cm dish 24 h before transfection. Transfections were performed by the lipofection method using TransIT-LT1<sup>TM</sup> (Pan Vera Corp.) according to the manufacturer's instructions.

### Establishment of a cell line constitutively expressing a C/EBP $\beta$ -ERT chimeric protein

NIH3T3 cells were co-transfected with pPGKneo (15) and pC/EBP $\beta$ -ERT using the calcium-phosphate DNA coprecipitation method in a molar ratio of 1:10. One day after transfection, the cells were subjected to neomycin selection for 10 days. Neomycin-resistant clones were transferred to 96-well plates and C/EBP $\beta$  protein expression were evaluated by immunostaining with polyclonal anti-C/EBP $\beta$  Ab (C-19) (Santa Cruz Biotechnology). Nuclear translocation of the C/EBP $\beta$ -ERT chimeric protein was induced by treating the cells with 1  $\mu$ M 4-hydroxytamoxifen (4HT) (Sigma).

### Northern blot analysis

Total RNA was extracted from cultured cells and mouse organs using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. Ten to twenty micrograms of total RNA were separated by electrophoresis on a 1.0% agarose-formaldehyde gel, transferred onto nylon filters, and cross-linked in a UV chamber. Radioactive DNA probes for *Id2*,  $\beta$ -actin (*Actb*) and cytokeratin 18 (*Cyk18*) were prepared by random-primed labeling of the mouse *Id2*, *Actb* and *Cyk18* cDNAs with  $\alpha$ -[<sup>32</sup>P]dCTP, respectively. Hybridization and washing were performed under high-stringency condition. The filters were exposed to X-ray films with an intensifying screen or an imaging plate for BAS2000 (Fuji Film) to calculate the radioactivity.

### Luciferase assay

Using the Dual-Luciferase<sup>TM</sup> reporter assay system (Promega), total cell lysates were prepared 48 h after transfection and luciferase assays were performed as described (13). As an internal control to normalize firefly luciferase activity, the *PGK* promoter-driven sea-pansy luciferase plasmid, pRL-*PGK*, which was constructed by inserting the *PGK* promoter into pRL-null (Promega), was used. Total light production was measured with a Luminescencer-JHR (ATTO) or Luminescencer-PSN (ATTO).

### Nuclear extracts preparation, EMSA and western blot analysis

Nuclear extracts of NIH3T3 cells were prepared, as described previously (16). The protein content was determined using the

BCA Protein Assay Reagent (Pierce). For EMSA, oligonucleotides labeled with  $\alpha$ - $^{32}\text{P}$ dCTP using the Klenow fragment were reacted with 5  $\mu\text{g}$  of nuclear extract and separated on a 6.0% polyacrylamide gel, as described previously (17). The sequence of the HpxA oligonucleotide was 5'-GATCC-TATTTGCAGTGATGTAATCAGCG-3' (18). For the inhibition assay, a 200-fold excess of unlabeled competitor oligonucleotide was added in the reaction. Competitor oligonucleotides were synthesized according to the sequences reported previously: Oct, YY and NF-Y (19), Myb (20), Sox (21) and NF- $\kappa\text{B}$  (22). For the supershift assay, 2  $\mu\text{g}$  of rabbit polyclonal IgG Ab (Ab) against c-Myc (A-14) (Santa Cruz Biotechnology) was preincubated with the nuclear extracts for 30 min at room temperature and the mixture was subjected to EMSA. Western blot analysis was done essentially as described previously (13). Antibodies used were anti-c-Myc (A-14) (Santa Cruz Biotechnology) and anti-C/EBP $\beta$  (C-19) (Santa Cruz Biotechnology).

### ChIP assay

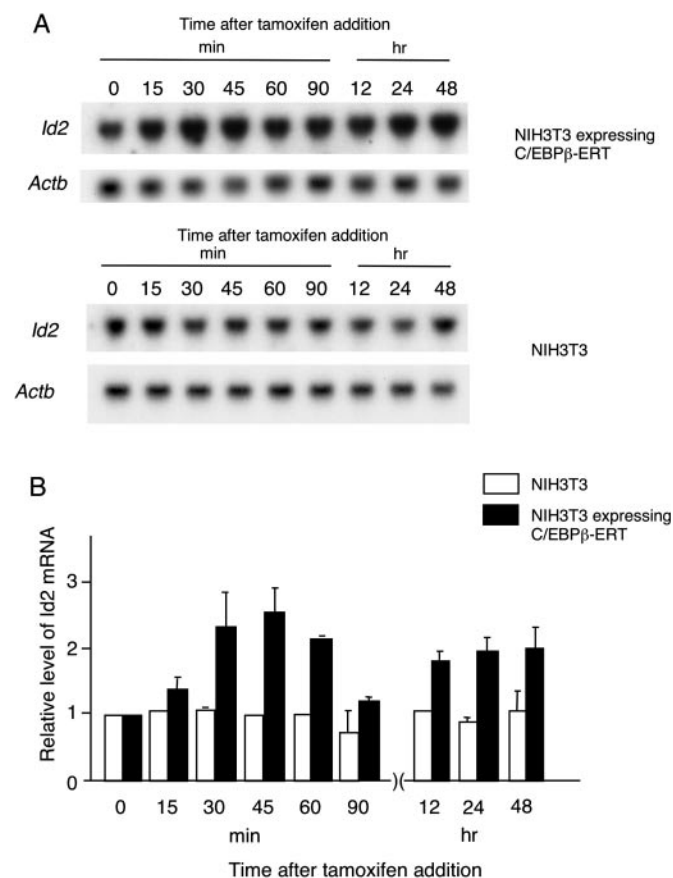
The ChIP assay was done as described previously (23), with the following modification. NIH3T3 cells ( $\sim 2 \times 10^6$  cells) were transfected with 12  $\mu\text{g}$  of pC/EBP $\beta$ -6Myc using the FuGENE 6 reagent (Roche Molecular Biochemicals), according to the manufacturer's instructions, and incubated for 48 h. After cross-linkage with 1% formaldehyde in the medium for 2 min at 37°C, cells were rinsed with ice-cold PBS twice, resuspended in 1 ml of SDS lysis buffer (23), and incubated on ice for 10 min. Cell lysates were then sonicated with a Bioruptor (Cosmo Bio) for 30 s at the maximum setting eight times with 1-min intervals, yielding chromatin fragments between 100 and 500 bp in size. The sonicated lysates were centrifuged for 10 min and the resulting supernatants were diluted in 1.8 ml of ChIP dilution buffer (23) and precleared by adding 30  $\mu\text{l}$  of protein A-Sepharose supplemented with 100  $\mu\text{g}/\text{ml}$  salmon sperm DNA at 4°C for 1 h. Immunoprecipitation was performed with 5  $\mu\text{g}$  of polyclonal anti-Myc Ab (A-14) or anti-C/EBP $\beta$  Ab (C-19) (Santa Cruz Biotechnology) at 4°C for 2 h. As controls, we omitted Ab or included normal rabbit IgG (Sigma). Immune complexes were then mixed with 30  $\mu\text{l}$  of protein A-Sepharose at 4°C for 2 h and washed sequentially with low salt buffer, high salt buffer and LiCl wash buffer (23). After precipitates were washed with TE and extracted with elution buffer, reversal of cross-linking and purification of DNA were done as described (23). Precipitated DNA was resuspended in 100  $\mu\text{l}$  of TE for input DNA and 20  $\mu\text{l}$  of TE for ChIP DNA. Obtained DNA fragments were analyzed by PCR using the following primer pairs: *Id2*, forward, 5'-GAATTCGCCTGGTATGATGGACGG-3' and reverse, 5'-ATGTTGTAGAGCAGACTCATCGGG-3'; IGF-1, forward, 5'-GTCTGCTAACCCTGTCAGAGACAC-3' and reverse, 5'-GGCTCTATCTGCTCTGAATTTAGC-3'.

## RESULTS

### Induction of endogenous *Id2* gene expression by C/EBP $\beta$

To determine if exogenous C/EBP $\beta$  expression can transactivate the endogenous *Id2* gene expression, we established

a stable line of NIH3T3 cells expressing the fusion protein of C/EBP $\beta$  with the ligand-binding domain of human estrogen receptor (C/EBP $\beta$ -ERT). The C/EBP $\beta$ -ERT protein is sequestered in the cytoplasm by heat shock proteins and the application of 4-hydroxytamoxifen (4HT) to the cells induces rapid translocation of C/EBP $\beta$ -ERT from the cytoplasm to the nucleus (24), which enables us to follow the effect of C/EBP $\beta$  on gene expression. As shown in Figure 1, the level of *Id2* mRNA was increased by >2-fold in a biphasic manner, showing a sharp peak at 30 min and a broad rise in the later phase, after the addition of 4HT. In contrast, *Id2* mRNA expression in the parental NIH3T3 cells was not changed significantly by the treatment (Figure 1). This indicates that the endogenous *Id2* gene expression is induced by C/EBP $\beta$ . The observed rapid and biphasic changes in the *Id2* mRNA level after the 4HT treatment suggested the short half-life of *Id2* mRNA and a complex regulation of the endogenous *Id2* gene expression by C/EBP $\beta$ .



**Figure 1.** Induction of endogenous *Id2* expression by the C/EBP $\beta$ -ERT fusion protein. A permanent cell line expressing C/EBP $\beta$ -ERT was established with NIH3T3 cells and treated with 1  $\mu\text{M}$  4HT to induce the translocation of the C/EBP $\beta$ -ERT fusion protein from the cytosol. RNA was extracted from the cells at various times and subjected to northern blot analysis. Representative results are shown in (A). The probes used are indicated on the left of each panel. Relative *Id2* expression is shown in (B). *Actb* expression was used as an internal control. Each *Id2* mRNA level is expressed as fold increase, compared to that in cells incubated without 4HT. White and black bars indicate relative *Id2* mRNA levels in the parental NIH3T3 cells and NIH3T3 cells stably expressing C/EBP $\beta$ -ERT, respectively. Results are shown as the mean and standard error values of the mean ( $n = 3$ ).

**C/EBPβ activates *Id2* gene expression through the proximal promoter region**

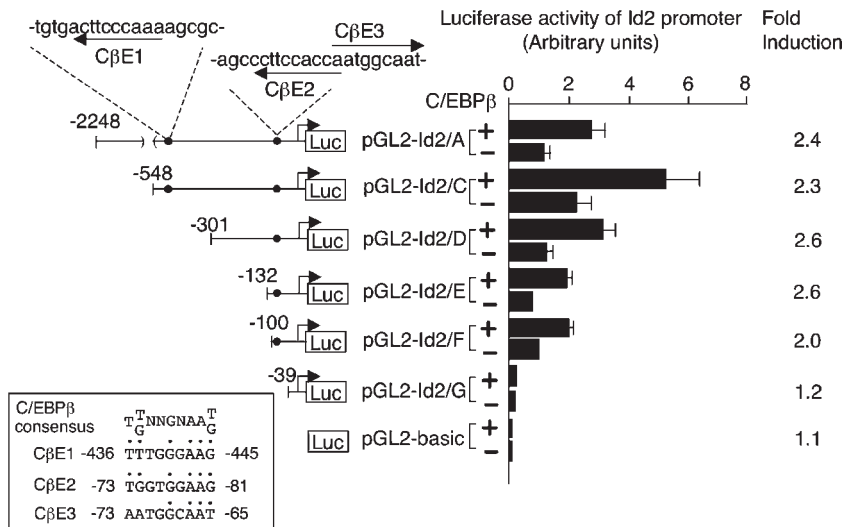
We next examined whether the promoter region of the *Id2* gene contains the C/EBPβ consensus binding site, TT/GNNGNAAT/G (8), and found three potential sites at positions -445 to -436, -81 to -73 and -73 to -65 (designated C/EBPβ related element CβE1, CβE2 and CβE3, respectively), as shown in Figure 2. In addition, a luciferase reporter construct containing the upstream region of the *Id2* gene, spanning from -2248 to +84 relative to the transcription initiation site, showed a 2.5-fold increase in activity in NIH3T3 cells co-transfected with a C/EBPβ expression vector, indicating that the *Id2* promoter is responsive to the transcription factor (Figure 2). We then constructed a series of deletion mutants of the *Id2* promoter and analyzed their activities in response to C/EBPβ using the same system. Deletion of the promoter region from -2248 to -101 bp resulted in retention of the responsiveness of the promoter to C/EBPβ, while further deletion from positions -100 to -40 abolished the response to C/EBPβ. These results demonstrated that the C/EBPβ-responsive element resides in the region containing CβE2 and CβE3 that spans -100 to -40 bp in the *Id2* promoter.

**C/EBPβ binds directly to the *Id2* promoter sequence containing its consensus binding sites**

To examine whether the transactivation of the *Id2* promoter by C/EBPβ is direct or indirect, we next performed an EMSA using oligonucleotide probes derived from the *Id2* promoter region. As the reporter assay demonstrated that the region spanning positions -100 to -40 from the transcription initiation site is responsible for the transactivation of the *Id2* promoter by C/EBPβ, we designed four oligonucleotides to cover this region in an overlapping manner (Figure 3A). Nuclear extracts were

prepared from NIH3T3 cells transfected with an expression vector of C/EBPβ tagged with 6xMyc or its parental vector. As shown in Figure 3A (lanes 1-3), three retarded bands were detected with an oligonucleotide derived from the promoter of the *HpxA* gene, which is one of the direct targets of C/EBPβ (18). It is known that a single *Cebpb* mRNA can generate three isoforms of the C/EBPβ protein, liver-enriched activating proteins (LAP1 and LAP2) and liver-enriched inhibitory protein (LIP), through leaky ribosomal scanning (7,18,25). Western blot analysis confirmed that transient transfection with the vectors of untagged C/EBPβ or 6xMyc-tagged C/EBPβ leads to the expression of the three isoforms in NIH3T3 cells, while endogenous C/EBPβ proteins were barely detectable in the parental NIH3T3 cells (Figure 3B). Therefore, according to the previous report (25), the detected bands seemed to correspond to dimers of LAP/LAP, LAP/LIP and LIP/LIP, as indicated in Figure 3A. Similarly, probe II containing the region covering positions -95 and -56 demonstrated the binding of C/EBPβ (lanes 7-9 in Figure 3A). In both cases, inclusion of Ab against the Myc-tag or C/EBPβ (data not shown) caused supershift of the retarded bands, demonstrating the specificity of the reaction. Furthermore, the formation of protein-DNA complexes with probe II was blocked by competition with a 200-fold molar excess of the cold HpxA oligonucleotide (lanes 16 and 17 in Figure 3A), although, as detected in lanes 7-9, nonspecific binding at the position of LAP/LAP was observed with nuclear extracts of both non-transfectants and transfectants of pC/EBPβ-6Myc (lanes 16 and 17). In contrast to the binding to probe II, no specific binding was detected in EMSA with probes I, III and IV (lanes 4-6 and 10-15 in Figure 3A). These results suggested that C/EBPβ binds directly to the *Id2* promoter via CβE2 and CβE3.

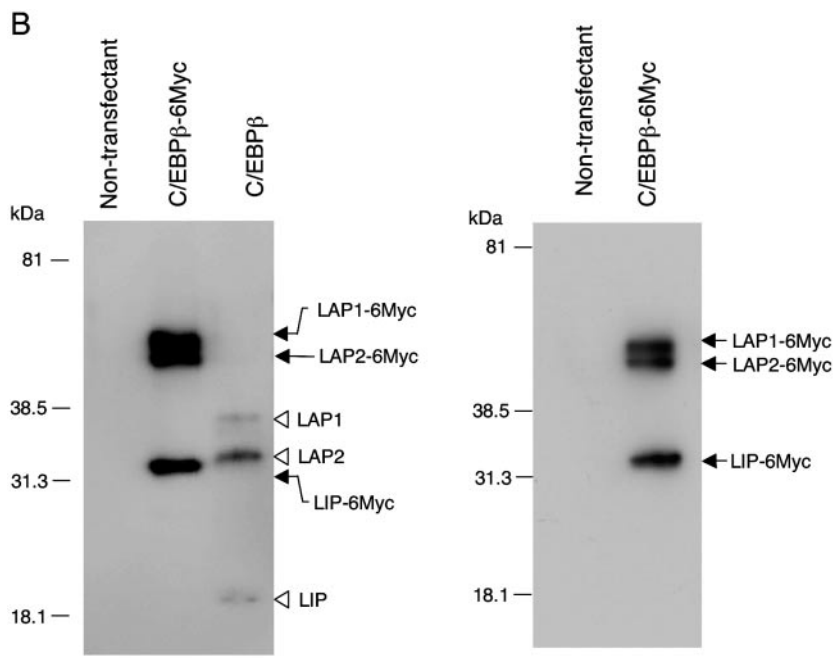
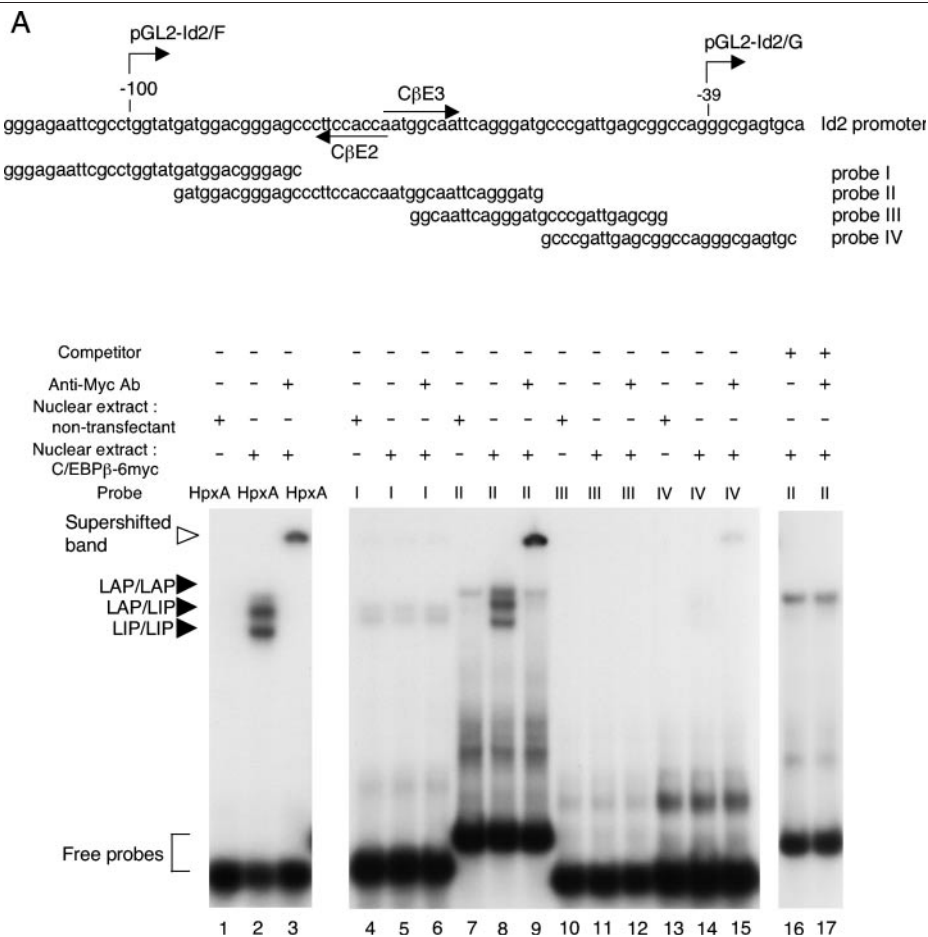
To delineate the binding site of C/EBPβ more precisely, we synthesized five oligonucleotides bearing deletions or

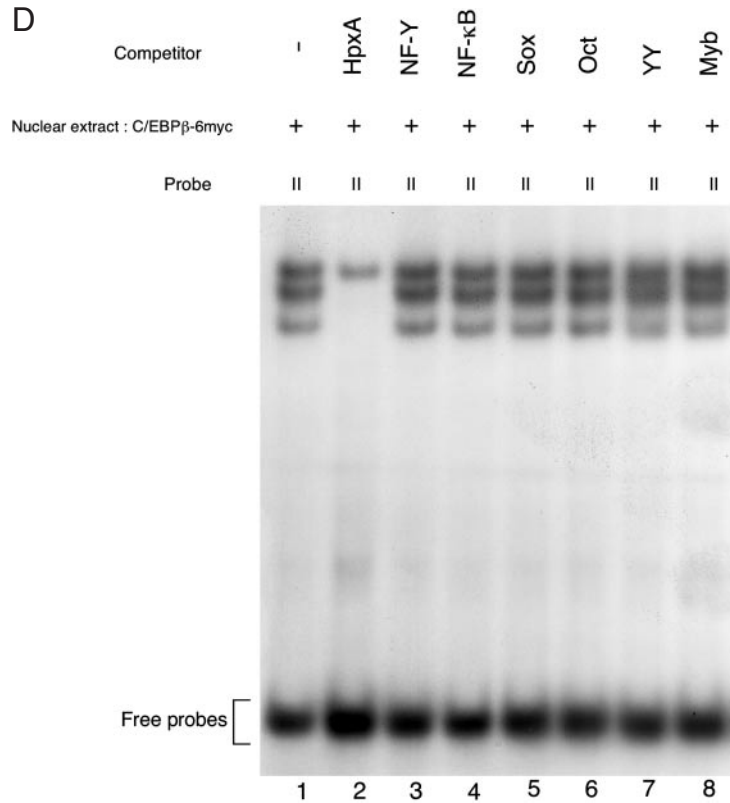
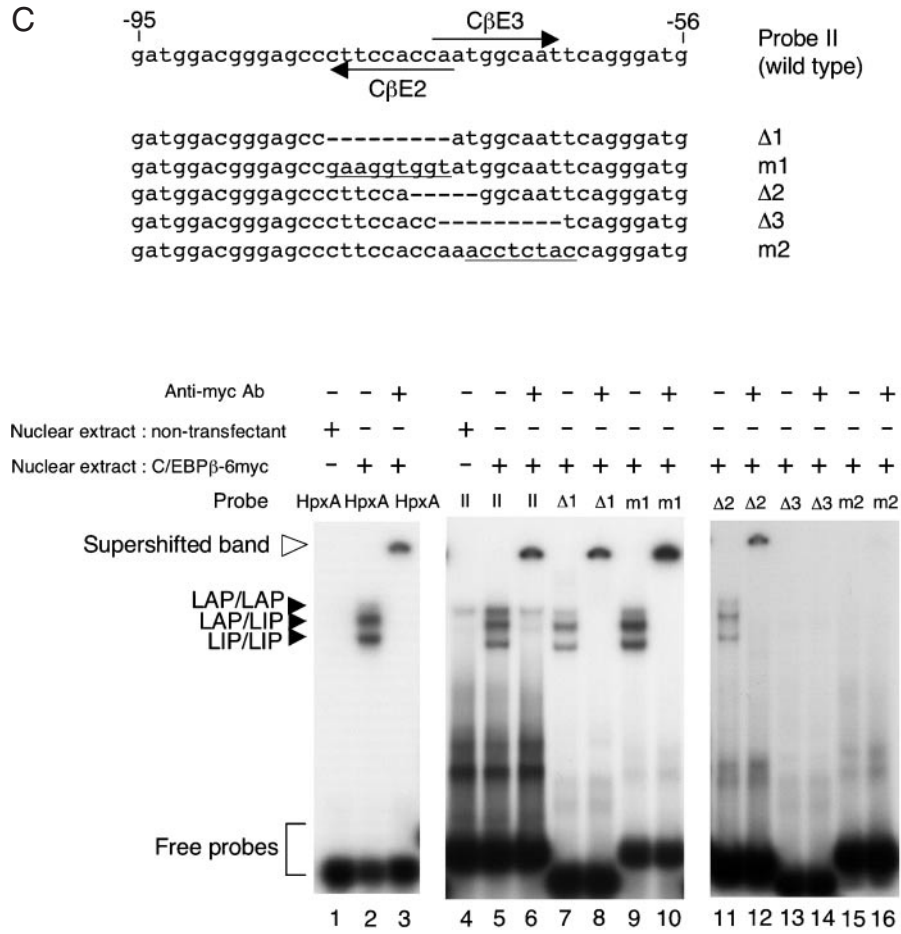


**Figure 2.** Transactivation of the *Id2* promoter by C/EBPβ. In the *Id2* promoter region spanning positions -2248 to +84 from the transcription initiation site, there are three potential C/EBPβ binding sites, named CβE1 (-445 to -436), CβE2 (-81 to -73) and CβE3 (-73 to -65), as indicated by arrows in the schematic representation of pGL2-*Id2*/A. The numbers shown on the left of the respective reporter constructs indicate the positions from the transcription initiation site. The sequence comparison of these sites with the consensus binding site (8), TT/GNNGNAAT/G, is shown in the inset. In the reporter assay, each of the reporter plasmids, which contained various lengths of the *Id2* promoter, was co-transfected into NIH3T3 cells with pPGK-renilla, together with pMSV-C/EBPβ or pMSV-mock, as indicated by + and -, respectively, in the middle, and luciferase activities were determined after 48 h. Luciferase activities of the respective reporter constructs were determined by normalizing by the respective renilla luciferase activity. Fold induction of luciferase activity by C/EBPβ is indicated on the right. The mean and standard error of the mean values of three independent experiments are shown.

mutations in the domain related to C $\beta$ E2 and/or C $\beta$ E3, and performed EMSA. As shown in Figure 3C, C/EBP $\beta$  bound normally to oligonucleotides  $\Delta$ 1 and m1 (lanes 7–10), which had a deletion and base substitutions in C $\beta$ E2, respectively. In addition, C/EBP $\beta$  formed a complex with oligonucleotide  $\Delta$ 2

(lanes 11 and 12 in Figure 3C), in which the spacer between C $\beta$ E2 and C $\beta$ E3 was deleted. On the other hand, deletion and base substitutions in C $\beta$ E2 (oligonucleotides  $\Delta$ 3 and m2, respectively) failed to form a retarded complex with C/EBP $\beta$  (lanes 13–16 in Figure 3C). These results strongly suggest





that C/EBP $\beta$  binds to C $\beta$ E3, although C $\beta$ E3 displays relatively low sequence conservation with the C/EBP $\beta$  consensus binding site (inset in Figure 2).

C $\beta$ E3 contains sequences that are similar to the consensus binding sites of other transcription factors including NF-Y, NF- $\kappa$ B, Sox, Oct, YY and Myb. To exclude the possibility that the retarded bands shown in Figure 3A and C contain these transcription factors, we performed competitive EMSA using the oligonucleotides that contain the consensus binding sites of these transcription factors. As shown in Figure 3D, none of the oligonucleotides competed for nor altered the retarded bands generated by probe II and the nuclear extract of transfectants of pC/EBP $\beta$ -6Myc (lanes 1 and 3–8), while the HpxA oligonucleotide did (lane 2). Therefore, it is unlikely that transcription factors other than C/EBP $\beta$  bind C $\beta$ E3 directly or indirectly.

### Transactivation by C/EBP $\beta$ depends on C $\beta$ E3

To confirm that C $\beta$ E3 is responsible for the transactivation of the *Id2* promoter by C/EBP $\beta$ , we constructed three reporter plasmids, pGL2-*Id2*/E $\Delta$ 1, pGL2-*Id2*/E $\Delta$ 2 and pGL2-*Id2*/E $\Delta$ 3, with deletion of both C $\beta$ E2 and C $\beta$ E3, C $\beta$ E2 or C $\beta$ E3, respectively, and transfected them with or without a C/EBP $\beta$  expression vector into NIH3T3 cells. As shown in Figure 4, pGL2-*Id2*/E $\Delta$ 2 expressed luciferase activity comparable to that of the wild-type pGL2-*Id2*/E plasmid, whereas no appreciable response to C/EBP $\beta$  was observed with pGL2-*Id2*/E $\Delta$ 1 or pGL2-*Id2*/E $\Delta$ 3. These results, together with the data shown in Figure 3B, indicate that the regulation of *Id2* expression by C/EBP $\beta$  occurs via the most proximal C $\beta$ E in the *Id2* promoter region.

### Direct binding of C/EBP $\beta$ to the endogenous *Id2* promoter

We next performed the chromatin immunoprecipitation (ChIP) assay to demonstrate that C/EBP $\beta$  is present on the *Id2* promoter region containing C $\beta$ E2 and C $\beta$ E3. To do this, NIH3T3 cells were transfected with the C/EBP $\beta$ -6xMyc expression vector and the cell lysate prepared after fixation was precipitated with an Ab against Myc or C/EBP $\beta$ , followed by PCR with primers for the *Id2* promoter. As shown in Figure 5 (lanes 9 and 10), both antibodies enriched the chromatin-containing DNA of the *Id2* promoter region compared to incubation with normal rabbit IgG or without Ab (lanes 7 and 8), similar to the case of the insulin-like growth

factor-1 (*Igf1*) gene, which is known to be a direct downstream target of C/EBP $\beta$  (26). On the other hand, no such enrichment was detected in cells transfected with the parental expression vector (Figure 5, lanes 2–5). These data indicate that C/EBP $\beta$  is bound to the endogenous *Id2* promoter.

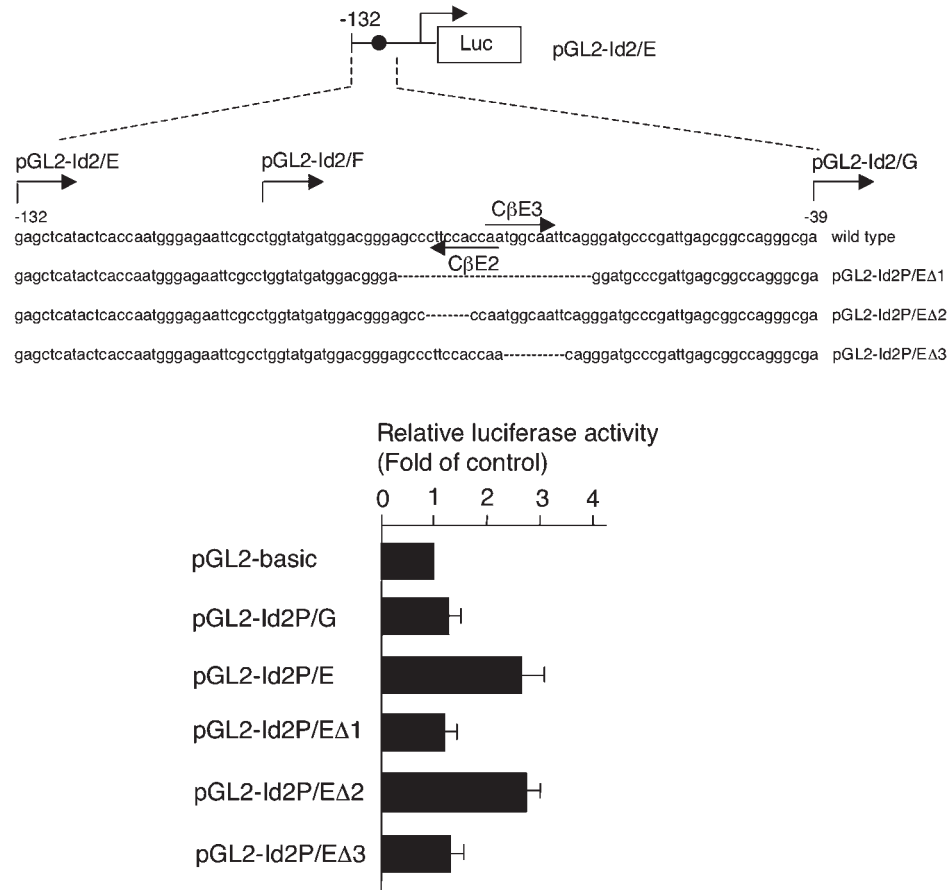
### Reduced expression of *Id2* in C/EBP $\beta$ -deficient mammary glands during pregnancy

We finally examined *Id2* gene expression in pregnant mammary glands of C/EBP $\beta$ -null mice during pregnancy. Since C/EBP $\beta$ -null mice are infertile, we transplanted mammary epithelial cells into the inguinal fat pads of *Rag2*-null mice in which mammary glands had been precleared by removing mammary glands of the host at the age of 3 weeks (4). After 7 weeks of transplantation, mice were mated with male mice and RNA was purified from the mammary glands at 10 days post coitus (d.p.c.). In morphological examination, mammary glands were successfully reconstituted in all recipients and the impaired proliferation of mammary epithelial cells was observed in the gland reconstituted with C/EBP $\beta$ -null mammary epithelial cells, as expected (Figure 6A). Northern blot analysis demonstrated that, in the non-pregnant state, there was no significant difference in the basal expression level of *Id2* between mammary glands reconstituted with mammary epithelial cells of C/EBP $\beta$ -null mice and those reconstituted with cells of wild-type mice. In contrast, mammary glands reconstituted with C/EBP $\beta$ -null mammary epithelial cells were devoid of the pregnancy-induced elevation of *Id2* mRNA expression. Thus, it was suggested that *Id2* expression is under the influence of C/EBP $\beta$  in mammary glands during pregnancy, but not in the non-pregnant state.

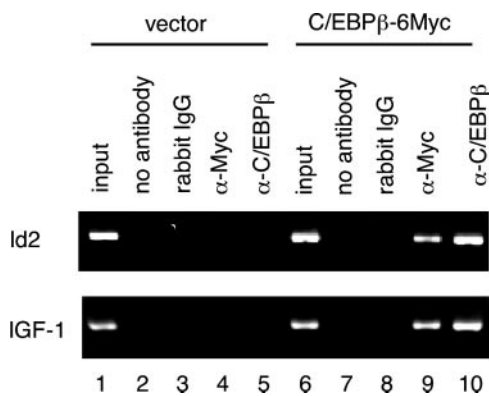
## DISCUSSION

Mammary gland development is divided into several stages that are related to sexual development and reproduction: the embryonic, prepubertal, pubertal, pregnancy, lactation and involution stages (2). Each stage shows distinct requirements for specific genes for completion of the stage and/or progression to the next stage, as revealed or confirmed by gene inactivation studies in mice (2). Among these stages, the pregnancy stage, where robust proliferation and differentiation of mammary epithelial cells lead to lobuloalveolar development, is controlled by various molecules including the prolactin receptor, Stat5, receptor activator of NF- $\kappa$ B (RANK), receptor activator of NF- $\kappa$ B ligand (RANKL), cyclin D1, C/EBP $\beta$  and

**Figure 3.** EMSA for C/EBP $\beta$  with the *Id2* promoter. EMSA was performed with  $^{32}$ P-labeled probes and nuclear extracts obtained from NIH3T3 cells transiently transfected with pC/EBP $\beta$ -6Myc or parental NIH3T3 cells. Oligonucleotide HpxA was used as a positive control for C/EBP $\beta$  binding. DNA–protein complexes were separated on a 6% polyacrylamide gel, and the gel was dried and exposed to X-ray film. Three retarded bands derived from dimers of C/EBP $\beta$  isoforms are indicated by filled arrowheads (LAP/LAP, LAP/LIP and LIP/LIP). The supershifted band formed with anti-Myc Ab is indicated by an open arrowhead. (A) On the top, probes used in EMSA are aligned with their positions in the *Id2* promoter sequence. Arrows indicate the positions of C $\beta$ E2 and C $\beta$ E3. The probes used were HpxA (lanes 1–3), probe I (lanes 4–6), probe II (lanes 7–9), probe III (lanes 10–12) and probe IV (lanes 13–15). Nuclear extracts were prepared from NIH3T3 cells transfected with pC/EBP $\beta$ -6Myc (lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, 15) or the parental NIH3T3 cells (lanes 1, 4, 7, 10, 13). For the detection of a supershifted band, anti-Myc Ab was included in the incubation mixture (lanes 3, 6, 9, 12, 15). In lanes 16 and 17, a 200-fold molar excess of the HpxA oligonucleotide was added as cold competitor. (B) Expression of C/EBP $\beta$  isoforms in NIH3T3 cells. Left and right panels show the results with anti-C/EBP $\beta$  and anti-Myc Abs, respectively. Origins of nuclear extracts are shown on the top. Positions of the respective C/EBP $\beta$  isoforms are indicated by arrowheads (untagged-C/EBP $\beta$ ) and arrows (6Myc-tagged-C/EBP $\beta$ ) on the right of each panel. Positions of size markers are indicated on the left of each panel. (C) Mutant oligonucleotides designed based on probe II were used in EMSA. The positions of C $\beta$ E2 and C $\beta$ E3 are indicated by arrows. Probes  $\Delta$ 1 and  $\Delta$ 3 are C $\beta$ E2 and C $\beta$ E3 deletion mutants of probe II, respectively. Deleted regions are indicated by dashes. Base-substituted regions are underlined. The probes used were HpxA (lanes 1–3), probe II (lanes 4–6),  $\Delta$ 1 (lanes 7 and 8), m1 (lanes 9 and 10),  $\Delta$ 2 (lanes 11 and 12),  $\Delta$ 3 (lanes 13 and 14) and m2 (lanes 15 and 16). Nuclear extracts were prepared from NIH3T3 cells transfected with pC/EBP $\beta$ -6Myc (lanes 2, 3, 5–16) or the parental NIH3T3 cells (lanes 1 and 4). For the detection of a supershifted band, anti-Myc Ab was included in the incubation mixture (lanes 3, 6, 8, 12, 14 and 16). (D)  $^{32}$ P-labeled probe II was incubated with nuclear extracts containing C/EBP $\beta$ -6Myc, together with 200-fold molar excess of the cold competitors that contain the binding sites of the transcription factors as indicated on the top (see Materials and Methods).



**Figure 4.** Transactivation of the *Id2* promoter by C/EBPβ is via CβE3. Mutant reporter plasmids were constructed based on pGL2-Id2/E. pGL2-Id2/EΔ1 lacks the region containing both CβE2 and CβE3. Core sequences of CβE2 and CβE3 are deleted in pGL2-Id2/EΔ2 and pGL2-Id2/EΔ3, respectively. Deleted regions are indicated by dashes. NIH3T3 cells were co-transfected with reporter plasmids and an internal control vector, pGL-renilla, together with pMSV-C/EBPβ or pMSV-mock. Luciferase activity was determined as in the experiments shown in Figure 2. Transactivation of the respective reporter plasmids by C/EBPβ is presented as fold induction of transactivation of pGL2-basic by C/EBPβ. The mean and standard errors of the mean values from three separate assays are shown in the histograms.

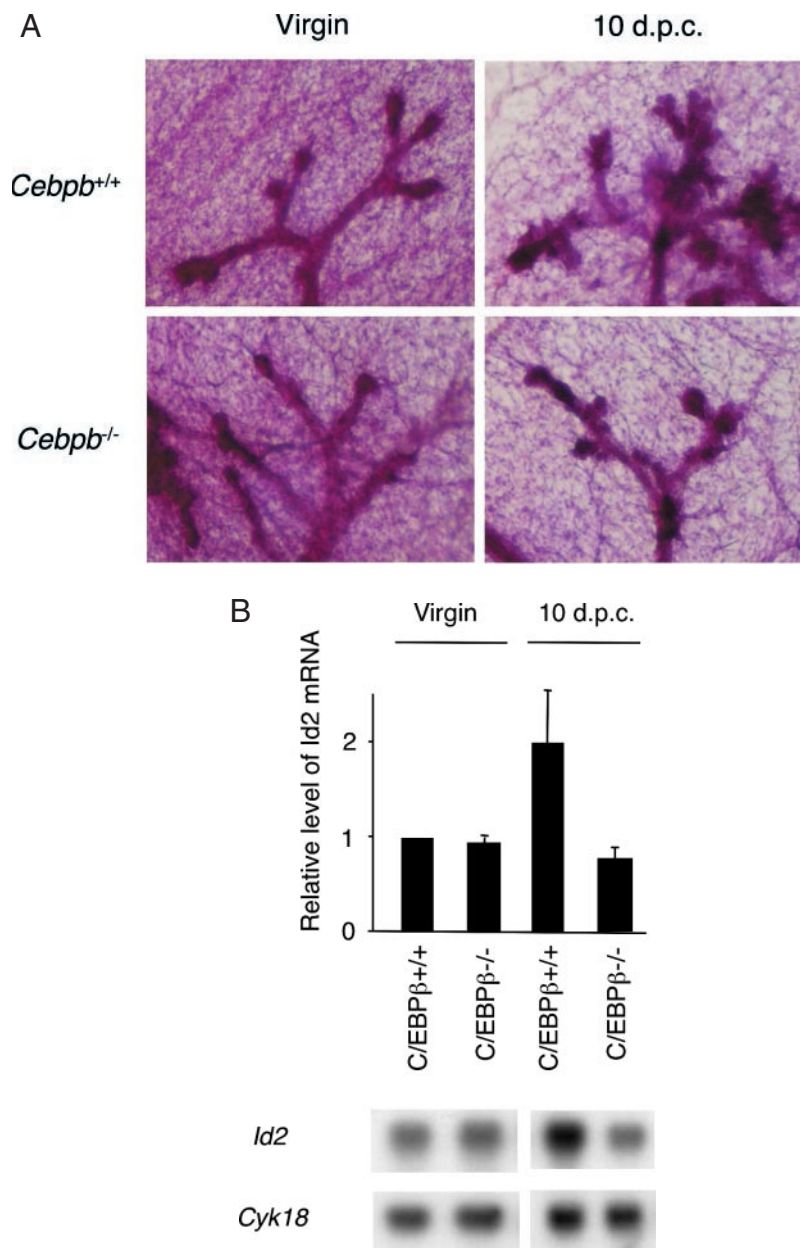


**Figure 5.** Binding of C/EBPβ to the *Id2* promoter *in vivo*. The ChIP assay was performed using NIH3T3 cells transiently transfected with empty vector (lanes 1–5) or pC/EBPβ-6Myc (lanes 6–10). The chromatin-associated DNA was incubated without Ab (lanes 2 and 7) or with normal rabbit IgG (lanes 3 and 8), anti-Myc Ab (lanes 4 and 9), or anti-C/EBPβ Ab (lanes 5 and 10). An aliquot (2.5%) of the total chromatin DNA was used for input (lanes 1 and 6). Immunoprecipitates were subjected to PCR with a primer-pair specific to the *Id2* promoter that amplified a 311 bp fragment (upper panel). As a positive control, PCR was carried out with a primer-pair specific to the *Igf-1* promoter, which amplified a 287 bp DNA fragment (lower panel). After 32 cycles of amplification, PCR products were electrophoresed through a 3% agarose gel and visualized by ethidium bromide staining.

*Id2* (2). The present study provides evidence linking C/EBPβ and *Id2* in a gene network that is involved in lobuloalveolar development at the pregnancy stage of the mammary gland. Furthermore, RANKL, also known as osteoprotegerin ligand (OPGL), has been shown to induce rapid nuclear translocation of C/EBPβ from the cytosol in mammary epithelial cells and to regulate gene expression of β-casein (*Csnb*) gene, one of the most abundant milk proteins (27). Therefore, it is conceivable that an intracellular signaling cascade provoked by RANKL/OPGL induces *Id2* gene expression via C/EBPβ during lobuloalveolar development of the mammary gland during pregnancy.

Besides a lactation defect, mice deficient for *Id2* display multiple defects, mainly in the immune system, including agenesis of lymph nodes and Peyer's patches, impaired natural killer cell development (14), selective loss of CD8α<sup>+</sup> dendritic cells (28,29), Th2 dominance with an increased serum IgE level (28,30) and reduction of intestinal lymphocytes (31). These defects are not observed in C/EBPβ-null mice. On the contrary, C/EBPβ-null mice exhibit impaired macrophage function against bacteria and tumors (32,33), and are sterile as a result of defective differentiation of ovarian granulosa cells (34). These phenotypes are not shared with *Id2*-null mice, although a fraction of *Id2*-null female mice show sterility





**Figure 6.** *Id2* expression in mammary glands deficient for C/EBP $\beta$ . Epithelial cells of wild-type or C/EBP $\beta$ -null mammary glands were transplanted into precleared mammary glands of female *Rag2*-null mice. After 7 weeks, reconstituted mammary glands of non-pregnant and pregnant (10 d.p.c.) mice were subjected to whole mount analysis and RNA purification. (A) Morphology of reconstituted mammary glands in the non-pregnant and pregnant mice. Reconstituted mammary glands were dissected out, mounted on a slide glass and stained with carmine. The genotype of a donor and the pregnant state of a recipient are indicated on the left and the top, respectively. Both wild-type and C/EBP $\beta$ -null mammary epithelial cells reconstituted the mammary gland of a recipient successfully and no significant difference was observed in the non-pregnant state. In the mammary gland at 10 d.p.c., disturbed epithelial proliferation is evident in the gland reconstituted with C/EBP $\beta$ -null mammary epithelial cells. (B) Northern blot analysis of *Id2* mRNA expression. RNA was purified from reconstituted mammary glands. Genotypes of transplanted mammary epithelial cells are indicated on the top. The lower panel shows the representative data. The probes used are indicated on the left of the lower panels. The upper panel shows the relative *Id2* expression normalized by *Cyk18* expression. The mean and standard error of the mean values obtained from three independent samples are shown in the histograms.

for unknown reasons (Y. Yokota, unpublished data). Currently, therefore, the regulation of *Id2* expression by C/EBP $\beta$  seems to be specific to mammary epithelial cells during lobuloalveolar development. However, we cannot exclude the possibility that redundancy within the respective gene family compensates for the function of the mutant genes and prevents the mice from developing a defect in other cell types.

It has been shown that *Id1*, a founding member of the *Id* gene family, is regulated by C/EBP $\beta$  in pro-B cells (35,36). Interestingly, IL-3-dependent expression of *Id1* relies on C/EBP $\beta$  and Stat5 via the pro-B-cell enhancer element, and deacetylation of C/EBP $\beta$  by a histone deacetylase recruited by Stat5 is required for transcription of *Id1* (36). Since Stat5 is an important transcription factor that is activated by prolactin

signaling, it will be interesting to know whether a similar mechanism operates in the regulation of *Id2* expression.

Among the molecules that are required for lobuloalveolar development, some have been found to be involved in mammary carcinogenesis, as might be predicted based on the fact that robust cell proliferation occurs during lobuloalveolar development. Cyclin D1 is a typical example: overexpression of cyclin D1 protein is observed in more than half of human breast cancers (37–39) and amplification of the gene locus is detected in 15–20% of such cancers (40). Through computational analysis of the expression patterns of genes across tumor specimens, a recent study demonstrated that C/EBP $\beta$  plays a role in the consequences of cyclin D1 overexpression (41). It is therefore tempting to speculate that the cyclin D1-C/EBP $\beta$ -Id2 axis is involved in the development of mammary tumors.

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