Multiple Mechanisms for Pitx-1 Transactivation of a Luteinizing Hormone β Subunit Gene*

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The pituitary homeobox factor-1 (Pitx-1) transactivates a number of pituitary-specific genes through direct interaction with other specific transcription factors. We demonstrate here that Pitx-1 plays a crucial role in the regulation of the Chinook salmon luteinizing hormone β gene promoter through a number of novel mechanisms. On the proximal promoter its action involves a synergistic effect with steroidogenic factor-1 (SF-1) alone or in combination with the estrogen receptor; promoter activity being induced by 9- or 35-fold over controls, respectively. Further upstream, a series of four Pitx-1 response elements (located between 1366 and 1506 bp from the transcriptional start site) is also involved in regulating the promoter activity. The two distal sequences have the greatest effect on the basal activity and are also essential for the gonadotropin-releasing hormone (GnRH) response. Mammalian two-hybrid assays revealed that Pitx-1 can homodimerize. Moreover, circular permutation assays indicate that binding of Pitx-1 to more than one response element induces conformational changes of the target DNA. This constitutes an additional mechanism through which Pitx-1 can mediate transactivation of this gene, allowing the demonstrated interaction of proximal response elements and distal enhancers, thus facilitating the maximal GnRH response that was seen in the longer promoter constructs. Our research also indicates that Pitx-1 is phosphorylated on three residues when bound to the DNA.

The pituitary homeobox factor-1 (Pitx-1) is expressed in all pituitary cell types and is essential in the development and synthesis of pituitary hormones such as prolactin, pro-opiomelanocortin, and the gonadotropins (1–5). Although first discovered in the pituitary, Pitx-1 is also expressed in tissues derived from the first branchial arch, in the lateral mesenchyme, and in the developing hindlimb. Its role in development includes regulating growth, morphogenesis, and hindlimb patterning (2, 6). Previous studies have shown that it interacts with a number of different protein partners including Pit-1, steroidogenic factor-1 (SF-1), and basic helix-loop-helix factors such as NeuroD/Pan1 (3, 7). It has further been suggested that the cell-specific actions of this transcription factor are a function of these interacting proteins (2, 3, 8, 9).

A consensus of research has suggested that Pitx-1 interacts with SF-1 and early growth response factor-1 (Egr-1) to form a tripartite complex on the mammalian luteinizing hormone β (LHβ) subunit gene promoter, which mediates the gonadotropin-releasing hormone (GnRH)-regulated transcription of this gene (10–13). The very similar structure of the proximal promoter across species further indicates that the role of this tripartite element has been highly conserved through evolution (for example, see Refs. 14–16). Egr-1 appears to be the main activator, because it is transcriptionally up-regulated and probably also phosphorylated within minutes of GnRH exposure (10, 11). SF-1 may also be phosphorylated as a result of activation of the MAPK pathway by GnRH (17). Pitx-1 acts on the LHβ gene in synergy with these two factors, although the molecular mechanism of its actions has yet to be elucidated.

The LHβ promoter of a fish, the Chinook salmon (csLHβ or GtHIIβ), shows only partial structural similarity with its mammalian homolog, although it is highly responsive to stimulation by GnRH. It contains putative Pitx-1 REs at 232 and 586 bp from the transcriptional start site but does not contain a functional Egr-1 response element (RE) and is completely unresponsive to Egr-1 (Fig. 1A) (18). Previous studies on the csLHβ promoter have demonstrated that SF-1 binding the proximal RE acts synergistically with ER, binding a proximalERE at 260 bp (18, 19). Moreover, the ER binding this proximal RE interacts with ER binding a distal RE at 2659 bp (20). Our recent studies have indicated that both of these EREs are involved in mediating the GnRH effect.

Apart from the potentially important role of the proximal promoter Pitx-1 REs, further upstream on the csLHβ promoter there is a series of four Pitx-1 REs located within 150 bp, between nucleotides 1366 and 1506. The aim of this study was to verify which of these proximal or distal Pitx-1 REs are responsible for regulating basal or GnRH-mediated transcription of this gene, and to understand some of the molecular mechanisms.

EXPERIMENTAL PROCEDURES

**Plasmid Constructs**—The csLHβ (sGtHIIβ)-chloramphenicol acetyl transferase (CAT) constructs have been previously described (21). Additional constructs were created by introducing a SalI restriction site upstream of the desired cutting site, and an XhoI restriction site on the
CAT 3 vector multiple cloning site (MCS) that was removed in the original cloning of the 3.3 construct. This SfiI-Xho fragment was then cloned into CAT 3 linearized with the same enzymes. Site-directed deletions were carried out using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the 3.3-kbp construct as a template, according to the manufacturer’s instructions. Primers used for these deletions were comprised of 12–22 bp of the region flanking the RE on both sides and its complementary sequence. All constructs were sequenced to verify correct deletions.

Expression vectors for Pitx-1, SF-1, ER, CBP, and P-300 were gifts from J. Drouin (Montreal), K. Parker (Durham, NC), Y. Valotaire (Rennes, France), and H. Zimmermann and H.U. Bernard (Singapore), respectively.

Constructs for the two-hybrid assay were prepared using the Gal4 activation domain (AD) and Gal4 DNA binding domain (BD) vectors, pM and pVP16 (CLONTECH, Palo Alto, CA). Sequence-specific primers containing restriction enzyme recognition sites at the 5’-ends were used to PCR amplify the DNA fragment from Pitx-1, CBP, and P300 1–5 expression vectors. This CBP expression vector contains the transcriptional adaptor motif (TRAM) and the entire cysteine-rich region (C/H3 domain) with L-galactosidase, a reporter gene used was pG5CAT (CLONTECH).

Promoter DNA (csLHβ 1377–1512 bp) or a single Pitx-1 response element was end-labeled with biotin (biotin-21-dUTP; CLONTECH) before incubation (0.15 μg of DNA) with LβT2 cell nuclear extract (0.3 μg; 3 h in humidity chamber at room temperature). The DNA was then attached to CiphergenTM preactivated surface chips through streptavidin (0.2 μg; Pierce Research Instruments). The bound DNA complexes were rinsed with phosphate-buffered saline before dissociation (saturated sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid) and analysis.

Bound peptides were labeled with [α-32P]dGTP (Amersham Biosciences), using Klenow fragment (Invitrogen). Excess unincorporated radioisotopes were removed using MicroSpin™ G-50 columns (Amersham Biosciences). The probe (0.2–0.4 ng at 3–4 × 10⁵ cpm) was added to 2 μg of the nuclear extract (binding buffer: 20 mM HEPES, pH 7.8; 4 mM Tris-HCl, pH 8.0; 10% glycerol; 1 mM dithiothreitol; 50 mM KCl; 10 mM MgCl₂ with 2 μg poly dIdC) and incubated for 20 min on ice. The mass of the bound proteins was detected by the attached laser desorption/ionization time-of-flight mass spectrometer to an accuracy of about 0.02%. The mass of the bound proteins was used as a basis for its identification in the mouse nuclear protein data base, through TagIdent (www.expasy.ch/tools/tagident.html). For the production of peptide fingerprints, the bound protein was reduced (dithiothreitol, 4.5 μg/μl; NH₄HCO₃, 100 mM; 30 min at room temperature) then alkylated (iodoacetamide, 54 μg/μl; NH₄HCO₃, 100 mM; 30 min at room temperature) before digestion with trypsin (3 μg/μl; NH₄HCO₃, 100 mM; 24 h at room temperature; sequencing grade, modified, Promega). The bound peptides were dissociated (20% α-cyano-4-hydroxy cinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid) and their masses detected as above.

**Cell Culture and Transfections**—COS1 cells were cultured in modified Eagle’s medium supplemented with 10% fetal calf serum, 10 mM HEPES, 0.1 mM modified Eagle’s medium non-essential amino acids, 1 mM sodium pyruvate solution, 100 units/ml penicillin, and 100 μg/ml streptomycin, (Invitrogen). LβT2 cells were cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose with 10% certified fetal calf serum, 10 mM HEPES, and the same antibiotics (Invitrogen). Cells were exposed to GnRH-A (des-Gly10, [D-Ala6]-LHRH; Sigma), this was added at a volume of 0.1% of culture media to give a final concentration of 10 nm. When cells were cotransfected with ER, E₂ (Sigma) was added to the media (0.1% volume) to give a final concentration of 10 nm.

CAT and β-galactosidase assays were performed as described previously (19). The CAT/β-galactosidase activity was calculated as a ratio to the basal levels of activity in the control (unstimulated and unmutated) csLHβ-CAT construct for most experiments or the negative control for the two-hybrid assay.

**Protein Chips and DNA-binding Proteins**—Promoter DNA (csLHβ 1377–1512 bp) or a single Pitx-1 response element was end-labeled with biotin (biotin-21-dUTP; CLONTECH) before incubation (0.15 μg of DNA) with LβT2 cell nuclear extract (0.3 μg; 3 h in humidity chamber at room temperature). The DNA was then attached to Ciphergen™ preactivated surface chips through streptavidin (0.2 μg; Pierce Research Instruments). The bound DNA complexes were rinsed with phosphate-buffered saline before dissociation (saturated sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid). As required, the bound proteins were treated with alkaline phosphatase (4 ng, 1 h at room temperature). The mass of the bound proteins was detected by the attached laser desorption/ionization time-of-flight mass spectrometer to an accuracy of about 0.02%. The mass of the bound proteins was used as a basis for its identification in the mouse nuclear protein data base, through TagIdent (www.expasy.ch/tools/tagident.html).

**Multiple Mechanisms of Pitx-1 Action**

**FIG. 1. GnRH responsiveness of the csLHβ gene promoter is conferred by the proximal promoter, but the longer 3.3-kbp construct exhibits the greatest response.** A, the proximal response elements for pSil, SF-1 (at 154 and 346 bp), and the two EREs have previously been shown to be functional (19, 20, 21). The proximal (at 232 bp) and four distal Pitx-1 REs (between 1366 and 1512 bp) are shown to be functional in the present study. The functions of the other REs marked remain putative. B, truncated versions of the csLHβ promoter were ligated to the CAT reporter and transfected into LβT2 cells for 48 h. Half of these cells were exposed to GnRH (10 nm) for the last 8 h. CAT levels in cell extracts were assayed, as were those of β-galactosidase, LacZ, which was added to the media (0.1% volume) to give a final concentration of 10 nm.
samples were loaded onto an 8% non-denaturing polyacrylamide gel (acrylamide/bisacrylamide, 29:1) and run with 0.5 TBE running buffer (0.045 M Tris borate, 1 mM EDTA) at 16 mA constant current at 4 °C. The gel was dried and exposed to Kodak BioMax film at −70 °C.

Construction of Bending Vectors and Bending Assays—Bending assays were carried out essentially according to the method described by Wu and Crothers (24). Sequence-specific primers, one of which contained a Pitx-1 binding site at the 5′-end, were used to PCR amplify the MCS of pT7 Blue-2 vector (Novagen). The amplified fragment was ligated to a linearized pT7 Blue-2 vector to produce a construct (Pitx Bend 1) with the Pitx-1 RE encompassed by two identical MCSs in the same direction. An additional vector containing three Pitx-1 response elements (Pitx Bend 3) was constructed by ligating a 3.1-kb fragment from Pitx Bend 1 (lacking the Pitx-1 RE and one MCS); an additional identical MCS, and a fragment of the csLHβ promoter containing three Pitx-1 REs (1512–1377 bp). All constructs were verified by sequencing.

Pitx Bend 1 and Pitx Bend 3 were digested with a number of restriction enzymes to generate a series of 230-bp (the former) and 370-bp (the latter) linearized DNA oligos containing Pitx-1 REs at various positions from the middle to the end of the fragment flanked by fragments of the MCSs. The overhangs of the oligos were labeled, and gel shift assays were carried out as described above.

Statistical Analysis—One way analysis of variance (ANOVA) followed by the Bonferroni t test was employed to determine means that were statistically different. Differences were considered significant when p < 0.05. All experiments were repeated at least three times. Representative results are presented, except for Fig. 1B, which was the compilation of data from more than one experiment; in each of these experiments activity of the 3.3 construct was induced by GnRH 3.5–6-fold over controls.

RESULTS

Maximal Induction of Promoter Activity by GnRH Occurs in the Longest 3.3-kbp Promoter-CAT Construct—A number of CAT 3 constructs containing truncated variants of the csLHβ promoter were transfected into LβT2 cells, and half of these cells were exposed to 10 nM GnRH for 8 h. The GnRH resulted in an increase in the promoter-driven CAT activity for the 289-bp promoter construct by more than 2-fold, although this was depressed by a putative repressor located between 289 and 447 bp, whose identity is not known. Of the longer constructs, induction of reporter activity was seen only by the full-length 3.3-kbp promoter, the GnRH increasing CAT levels ~5-fold over the controls (Fig. 1B).

A Proximal Pitx-1 Response Element Is Crucial for the GnRH Effect on Transcription—The full-length 3.3-kbp promoter-CAT construct, transfected into LβT2 cells, responded to GnRH exposure with an increase in CAT activity of nearly 4-fold. Deletion of the proximal Pitx-1 RE, located at 232 bp, abolished this effect. In contrast, deletion of a more distal Pitx-1 RE, located at 586 bp, had less of an effect, and the GnRH induced an increase in promoter activity that was still significantly higher than in controls (p < 0.0001). Deletion of both REs reduced basal promoter activity to around a third of that of the full-length 3.3-kbp control (Fig. 2).

Pitx-1 Activates the csLHβ Promoter by Acting in Synergy with Sf-1 and ER—Transfection of the Pitx-1 expression vector into COS1 cells over a range spanning 0.01–2 μg failed to stimulate activity of the csLHβ promoter. Moreover, the higher doses appeared to depress CAT activity (Fig. 3A). Transfection of 0.5–1.5 μg of the SF-1 expression vector increased CAT levels by as much as twenty-fold, whereas co-transfection of the Pitx-1 (0.1 μg) with 0.5–1 μg of the SF-1 expression vector more than doubled the effect of the lower doses of SF-1 alone (Fig. 3B). In contrast, co-transfection of Pitx-1 with ER (0.1–1.5 μg) showed no synergistic effect (Fig. 3C). Co-transfection of all three expression vectors (SF-1 and ER at 0.75 μg each) revealed that the addition of 0.05–0.1 μg Pitx-1 expression vector increased promoter activity by as much as 35-fold over the basal level of promoter activity and over 5× the already synergistic effect of ER and SF-1 (Fig. 3D).

Multiple Pitx-1 REs on the csLHβ Promoter Bind Pitx-1, Which Is Phosphorylated on Three Residues—In addition to the two Pitx-1 REs located on the proximal csLHβ promoter, four putative Pitx-1 REs are located further upstream, between 1366 and 1506 bp. To test whether all bind a single protein of similar mobility, gel shift assays were carried out using the various putative RE sequences, and nuclear extract from LβT2 cells. All of the oligonucleotides, including the consensus sequence (at 232 bp), bound a protein of similar mobility (Fig. 4A). The band was not seen when the same unlabelled oligonucleotide was preincubated with the nuclear extract but was apparent when a nonspecific oligonucleotide was used (not shown). The identity of the protein binding these sequences was further examined by mass spectrometry using DNA bound to a protein chip. A single-sized protein bound the DNA, whether using a single Pitx-1 RE oligo or using the 1377–1512 bp sequence of the csLHβ promoter. Its molecular mass was 34,313 ± 3.8 Da or 34,319 ± 4 Da (see Fig. 4, B and C) as compared with the predicted sequence from the cDNA sequence of 34,075 Da. Given that Pitx-1 contains a number of putative phosphorylation sites, we hypothesized that the bound protein could be Pitx-1 phosphorylated on three residues, explaining the 288–244 Da deviation from the predicted mass. This was confirmed by treatment of the bound protein with alkaline phosphatase (4 ng/chip for 1 h), which reduced the mass of the bound protein to 34,075 ± 0.5 Da or 34,076 ± 0.5 Da (see Fig. 4D).

Final confirmation of the identity of this protein as Pitx-1 was obtained by tryptic digestion of the bound protein, which produced five clear peptide peaks whose masses (12207, 9092, 5221, 3246, and 3028 Da) matched predicted peaks within 0.08% and were a result of 0–2 missed cleavages. These fragments covered 55% of the protein.
Pitx-1 REs on the Distal Promoter Work in Tandem to Regulate Basal and GnRH Responsiveness of the csLHβ/H9252 Promoter—Deletion of individual putative distal REs had no apparent effect on the GnRH response, which remained at a 3–5-fold increase in CAT activity (Fig. 5A). Deletions of any of the more proximal REs paired with another RE reduced basal levels of CAT activity to about 70% that of the full construct, although the fold increase by GnRH-stimulation remained at 3–3.5-fold. However, deletion of the two most distal REs (at 1446 and 1506 bp) caused basal levels of CAT activity to drop to around a quarter of those in the intact controls, and the GnRH no longer had an effect. Deletion of a third RE reduced basal levels further (to 30–40% of intact controls) for most of the constructs, although for the construct with the two most distal deletions, deletion of the RE at 1366 or 1386 bp did not reduce the activity further. Deletion of all four REs reduced CAT activity to undetectable levels (Fig. 5B).

Pitx-1 Homodimerizes but Does Not Appear to Interact with the Cofactors CBP or p300—Because the Pitx-1 molecules appeared to be working in tandem to activate the upstream promoter, mammalian two-hybrid assays were carried out to test the ability of Pitx-1 to homodimerize. The cDNA of Pitx-1 was ligated into both the pM and pVP16 vectors to create two fusion proteins: one (pM Pitx-1) comprised of the Pitx-1 protein and the Gal4 DBD, and the other (pVP16 Pitx-1) contained the Pitx-1 protein and AD. Transfection of both Pitx-1-containing vectors revealed an induction of the reporter gene CAT activity to 35-fold that of controls, whereas transfection of only one of the Pitx-1 containing constructs, together with the empty DBD (pM) or AD (pVP16) vector failed to stimulate CAT activity (Fig. 6).

Similar two-hybrid assays were carried out to test the ability of Pitx-1 to interact with the cofactors CBP or p300. For these, expression vectors containing various fragments of CBP or p300 cDNA were ligated into the pVP16 vector and transfected together with the pM Pitx-1 fusion vector. For all constructs co-transfected, the levels of CAT activity remained low and did not differ from controls (Fig. 6).

Pitx-1 Induces Conformational Changes in DNA Only When Binding to Multiple Sites—Gel shift assays were carried out using a labeled oligonucleotide containing a single Pitx-1 RE at different positions within the DNA fragment. Regardless of the
position of the RE, the oligos formed a single complex that was of similar mobility (Fig. 7A). In contrast, when different constructs were used containing the sequence at 1377–1512 bp of the csLHβ promoter, which includes the three more distal Pitx-1 REs, the position of the RE determined the relative mobility of the DNA-protein complex. Specifically, when the REs were located in the center of the DNA fragment, the mobility of the DNA-protein complex was consistently slower than when located at either end (Fig. 7B).

**DISCUSSION**

In the present studies we show that Pitx-1 is able to activate the csLHβ gene through novel mechanisms, including interaction with Sf-1–ER complex, and also through binding to multiple sites, thus inducing conformational changes in the DNA. We have also shown that Pitx-1 can homodimerize. Further, our results indicate that Pitx-1 is phosphorylated on three residues on binding to its DNA target.

Co-transfection of Pitx-1 with Sf-1 and ER expression vectors increased promoter activity by 35-fold. Although Pitx-1 has previously been shown to interact synergistically with Sf-1, our present results indicate considerable further synergistic action with the Sf-1–ER complex, although no independent interaction with ER alone was apparent. The magnitude of stimulation by cotransfection of all three expression vectors is remarkably similar to that seen on the mammalian LHβ promoter when Pitx-1, Sf-1, and Egr-1 were overexpressed (10), and we were not able to demonstrate any interaction between Pitx-1 and CBP or P300 in the present study.

The lack of interaction between Pitx-1 and CBP/P300 contrasts with the recent report that HOX homeodomain factors interact with CBP and P300 through the homeodomain. The result of this interaction is a block of the cofactor’s histone acetyl transferase activity and also prevention of the protein binding its DNA target (31), implicating an inhibitory role for HOX transcription factors on transcription of chromatin DNA. In that study, the homeodomain proteins were synthesized and interactions examined using largely in vitro systems. The only demonstration of the interaction in vivo was in transfected 292T cells. The present experiments revealed that all Pitx-1 bound to its target are phosphorylated on three residues, which could indicate the constitutive phosphorylation of Pitx-1. Alternatively, phosphorylation may be a requirement of DNA binding. The fact that the Pitx-1 did not become detached from the DNA as a result of dephosphorylation does not negate this possibility, as the protein was already bound to the DNA when the phosphates were removed.
The Pitx-1 contains a number of putative phosphorylation sites, including three for each of the kinases, MAPK, and protein kinase C, both of which are activated by GnRH (32). It could be speculated, therefore, that the phosphorylation not only allows binding of the Pitx-1 to DNA, but also prevents interaction with CBP/P300, which would preclude transcriptional activation.

No other information is available on the coactivators bound by Pitx-1, but the related protein, bicoid, appears to have two mechanisms of action, one of which is by binding TFIID through which it recruits the TBP/H18528TAF complex to the promoter and so directs transcriptional activation (33), whereas the other mechanism is independent of these activation domains but has not yet been fully defined (34). The reduction in csLH/H9252 promoter activity seen here when large amounts of Pitx-1 expression vector were co-transfected further suggests that Pitx-1 does indeed compete either for coactivators common to factors that regulate basal transcription of this gene or for components of the preinitiation complex.

Upstream on the distal part of the csLHβ promoter, four additional Pitx-1 REs (between 1366 and 1506 bp) also mediate both basal and GnRH-stimulated transcription of this gene. The fact that deletion of individual REs has no apparent effect on promoter activity suggests that some of the REs are redundant and indicates that Pitx-1 proteins interact with each other to elicit this effect. Notably, however, deletion of the distal two Pitx-1 REs (at 1442 and 1506 bp) has a more dramatic effect on the basal levels and also abolished GnRH responsiveness. Results from the two-hybrid assay study show that Pitx-1 can homodimerize. Thus it appears that binding to at least one of the distal Pitx-1 REs (at 1442 or 1506 bp) is crucial to allow homodimerization of Pitx-1. The distance between the proximal REs, is presumably too short to allow interaction of Pitx-1s binding these two sites.

The present study, suggesting that Pitx-1 binds as a homodimer to the distal region of the csLHβ promoter and so induces a conformational change in the promoter, is in line with previous findings. The specific DNA sequence recognized by homeobox proteins is only 5–6 bp long, and the precise targeting to a variety of promoters often occurs through interactions with other specific proteins or as a homodimer (2, 3, 7, 25). Examples are the paired (Pax-6) class homodimer or the MATa1 and MATa2 heterodimer (35–37), both of which induce considerable bending of the target DNA. Dimerization accompanied by conformational changes in the target DNA occurs also in many other groups of DNA binding proteins, such as leucine zippers (38), helix-loop-helix motifs (39), and zinc fingers (40, 41).

The Pitx-1-induced conformational change in the promoter of csLHβ would be facilitated further by intrinsic curvature arising from a poly(A/T) tract (T2A10GA4TA2T2A5) located just over 100-bp upstream, at 1635 bp (42). DNA bending can augment transcription by facilitating interactions between distal enhancers and proximal binding factors (43, 44), and distal REs.

![Figure 5](http://www.jbc.org/content/26205/10/26205/F5)

**Figure 5.** Distal Pitx-1 REs work in tandem to mediate both basal and GnRH-activated transcription. L6T2 cells were transfected with either the full-length 3.3-kbp csLHβ-CAT construct or the same construct in which the various distal Pitx-1 REs had been deleted, either alone (A) or in various combinations (B), as marked. Promoter activity is expressed as ratio to full-length promoter activity in unexposed cells. Mean ± S.E., n = 4. Statistical analysis in B was carried out separately for control (lowercase) or GnRH-exposed cells (uppercase); ND, non-detectable. NS, non-significant (p > 0.05). For all other constructs the mean in GnRH exposed cells was significantly higher than in control cells transfected with the same construct.
Pitx-1 in its transactivation or interaction with cofactors has homodimerize to induce DNA bend. The role of phosphorylation of Pitx-1, which contains its own intrinsic transactivation capability, is not able to facilitate the promoter response to GnRH, suggesting that the Pitx-1 binding these sites does not yet to be elucidated and may constitute an additional mechanism regulating LHβ3 transcription.

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3 P. Melamed, J. Seah, and C. Hew, unpublished results.

**FIG. 6.** Pitx-1 homodimerizes but does not interact with CBP or P300. Mammalian two-hybrid assays were carried out to test the ability of Pitx-1 to homodimerize or to interact with CBP or P300. Pitx-1 cDNA was ligated into pM and pVP16 vectors, and CBP or P300 fragments were ligated into the pVP16 vector. The various pM and pVP16 constructs were co-transfected together with the pG5CAT reporter gene, and CAT activity was measured 72 h later. CAT activities were normalized with p-galactosidase levels in the same sample and were expressed as a percentage of the negative control pM-pVP16-activated CAT levels. Mean ± S.E., n = 4. Statistical analysis as described in the legend to Fig. 1.

**FIG. 7.** Binding of Pitx-1 to more than one RE causes conformational change in the DNA target. A, a single Pitx-1 RE or 1377–1512 bp of the csLHβ promoter containing three Pitx-1 REs (B) was inserted between two identical MCSs and cut with three enzymes to produce a number of fragments differing only in the location of the REs. These fragments were labeled, and gel shift assays were carried out with LβT2 nuclear extract as described in the legend to Fig. 4.
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