COUP-TF Plays a Dual Role in the Regulation of the Ovalbumin Gene

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ABSTRACT: The ovalbumin (Ov) gene contains a number of regulatory elements that control its transcriptional activity and restrict expression to avian oviduct. One major regulatory region, the steroid-dependent regulatory element (SDRE), is required for induction by estrogen and corticosterone. Another region, the negative regulatory element (NRE), downstream of the SDRE, acts primarily to repress gene expression. In addition, experiments within indicate that the binding site for the COUP transcription factor (COUP-TF) is also required for Ov gene transcription. To examine the interactions involving the SDRE, the NRE, and the COUP binding sites on Ov gene transcription, mutations in these regions were made and transfected into primary oviduct cell cultures. These experiments show that without the NRE, the SDRE is sufficient for induction by estrogen and corticosterone, irrespective of the COUP site. However, with the NRE intact, the COUP site is required for steroid induction, although without the NRE, the COUP site attenuates transcriptional activity. More interestingly, overexpression of COUP-TF1 with the Ov wild-type reporter construct alleviates the requirement for steroid hormones. These results demonstrate that the COUP site is essential and has a dual role in Ov gene transcription and that steroid hormones might directly or indirectly regulate the activity of COUP-TF1.

Eukaryotic gene regulation often requires a complex set of cis-acting DNA elements, both positive and negative, to maintain appropriate gene expression. These elements serve as recognition sequences for specific DNA-binding proteins, which facilitate the assembly of transcription complexes to elicit either selective activation or repression of the particular gene. Initiation of transcription is a primary control point in the regulation of differential gene expression and depends ultimately on proteins that interact with specific elements in gene promoters (*1*). The varied nature of these interactions provides virtually unlimited possibilities for regulation and results in an elaborate mechanism for controlling gene expression (*2*).

But how can a relatively small number of transcription factors achieve the high level of specificity required to regulate the complicated patterns of gene expression observed in higher eukaryotes? Part of the answer lies in the fact that transcription factors and DNA elements are composed of modular units. For example, DNA elements often contain distinct sets of transcription factor binding sites, and variations in the arrangement of the binding sites provide the potential to create unique nucleoprotein complexes by forming heterodimers within and among families of transcription factors (*2*). Synergistic interactions within these complexes that may be dependent on developmental stage, cell-type, or environmental cues can result in specificity, a potential for multiple regulatory controls, and a high level of transcription. The complexity of transcriptional regulation has been further augmented through the use of transcription factor families whose members possess related structural motifs and are often able to bind to common target DNA sequences (*3*).

For three decades, the chicken oviduct has been exploited as a model system for studying the regulation of eukaryotic gene expression by steroid hormones. As a result, the biology of the system is well-defined (*4*). Estrogen promotes differentiation of the tubular gland cells and induces expression of the genes encoding the four major egg white proteins: ovalbumin (Ov), transferrin, lysozyme, and ovomucoid (*4*). The production of these egg white proteins comprises nearly 80% of the total protein synthesis of the oviduct tubular gland cells. Ov is the major egg white protein, accounting for 3×10^{19} molecules/day synthesized in the oviduct in response to estrogen. After primary exposure to estrogen, secondary exposure to three other classes of steroids (androgens, glucocorticoids, and progestins) results in induction of the Ov gene (*4*). This massive production of Ov in response to estrogen derives from a 20-fold increase in the transcription rate of the gene and a 10-fold increase in the stability of the resultant mRNA (*5*, *6*). Furthermore, cell- and tissue-culture experiments have shown that, in addition to estrogen, insulin (*7*) and corticosterone (*8*) are required for maximal expression of the Ov gene. Finally, when the estrogen stimulus is removed, the production of the egg white proteins is abolished, and the number of oviduct tubular gland cells decreases through apoptosis initiated by BMP-7 (*9*).

The expression of the Ov gene is controlled by a complex array of 5′-flanking elements (Figure 1). Deletional analysis of the Ov 5′-flanking region using OvCAT fusion genes revealed a steroid-dependent regulatory element (SDRE) from −892 to −793 and a negative regulatory element (NRE) from −350 to −100 (*10*, *11*). Although steroid receptors do not appear to bind directly to the SDRE, responsiveness to

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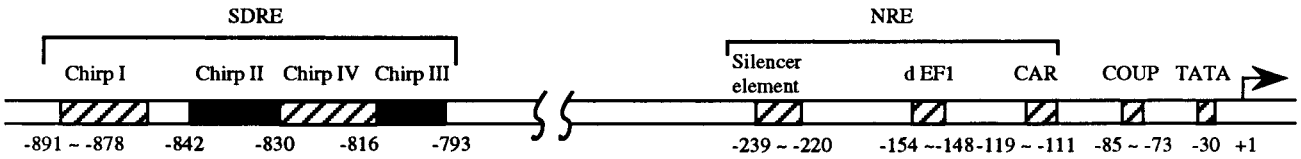

FIGURE 1: Regulatory elements in the 5′-flanking region of the chicken ovalbumin gene. Abbreviations used: SDRE, steroid-dependent regulatory element; Chirp, chicken ovalbumin-induced regulatory-protein binding site; NRE, negative regulatory element; dEF1, δ-crystallin/E2-box-factor binding site; CAR, chicken ovalbumin upstream promoter-adjacent repressor; COUP, chicken ovalbumin upstream promoter; TATA, TATA box. This is not drawn to scale.

all four classes of steroids that regulate this gene maps within this region (*11*). Our recent work showed that *ch*ick Ov-*i*nduced *r*egulatory *p*rotein-I (Chirp-I), which is a member of the winged-helix family of transcription factors, binds from −892 to −878 (*12*, *13*). Also, linker scanner mutations in the NRE identified three separate regulatory elements (−204 to −175, −153 to −148, and −119 to −111) (*14*). The transcription factor δ-crystallin/E2-box factor (δEF1) binds to the site from −153 to −148 and enhances induction of the Ov gene by estrogen (*15*). In addition, the site between −119 and −111 is a strong repressor site, designated the *C*OUP-*a*djacent *r*epressor (CAR) site (*14*). The distal site between −204 and −175 may bind a transcriptional activator, because mutation of that site appears to attenuate the steroid-induced transcriptional activity, but not the basal activity (*14*).

The canonical TATA and CCAAT boxes have been characterized, as well as the binding site for the *c*hicken *O*v *u*pstream *p*romoter *t*ranscription *f*actors (COUP-TFs) (*16*). The COUP-TFs are orphan members of the nuclear receptor superfamily and function in the transcriptional regulation of a wide variety of genes (*17*). COUP-TFI was first identified in HeLa and chick oviduct protein extracts as a factor that stimulates transcription from the Ov promoter in vitro (*18*, *19*). COUP-TF was found to bind to an element (COUP) from −85 to −73 in the Ov promoter and, along with the non-DNA-binding transcription factor p300, to be essential for in vitro transcription of the Ov gene (*16*, *20*). Although the original data showed that COUP-TF was involved in the transcriptional activation of the Ov gene in these in vitro transcription reactions, since that time the majority of reports indicate that COUP-TF is involved in the repression of its target genes (*21*−*31*).

Although the number is relatively small, a few reports support the contention that COUP-TF can activate gene expression upon binding to various regulatory elements (*32*−*34*). Moreover, COUP-TF may play a dual regulatory role, depending on the promoter context. For example, when the COUP site is in its natural context in the ornithine transcarbamylase (OTC) promoter, transcription is repressed (*3*). However, when that same site is placed out of context and upstream of heterologous promoters, transcription is activated. Thus, COUP-TF appears to be a promiscuous and versatile transcription factor because it recognizes a number of diverse binding sites and has opposing effects on transcription, depending on the DNA context of its binding site.

Although considerable effort has explained some of the regulatory elements in the Ov gene, little is known about how the SDRE, NRE, and COUP binding sites function to coordinately regulate appropriate steroid-dependent gene expression. Here, we report that the SDRE and COUP binding sites are required for the induction of the Ov gene in response to steroid hormones. Moreover, the COUP-TF plays a dual role in the regulation of the Ov gene. It represses basal expression in the absence of steroids, and it is required for induction by steroids. This work sheds light on the functional relationship between the SDRE and the NRE as well as on the role of the COUP binding site. More importantly, overexpression of COUP-TF can partially alleviate the requirement for steroid hormones, suggesting that COUP-TF is one of the limiting factors that keeps the gene silent in the absence of steroids.

MATERIALS AND METHODS

Animals. Sexually immature White Leghorn chickens were treated with the synthetic estrogen diethylstilbestrol (DES) by subcutaneous implantation of two 20-mg pellets (Hormone Pellet Press, Leawood, KS) for 14 days, as previously described (*8*). The pellets were withdrawn 48 h before the birds were sacrificed in order to ensure that endogenous Ov mRNA amounts had returned to basal (uninduced) levels.

Plasmids. (a) SDRE Constructs. All OvCAT constructions contained the indicated number of 5′-flanking and transcribed nucleotides to +9. To create the OvCAT fusion genes containing the SDRE at various locations, the pOvCAT-900H (*35*) was digested with *Hin*dIII to generate a fragment that contained the SDRE (−900 to −732) flanked by *Hin*dIII linkers (H-SDRE-H). This resultant fragment was then subcloned into the *Hin*dIII site of the pOvCAT-087C in the forward orientation to create pOvCAT-087M. To insert the SDRE downstream from the CAT structural gene, the H-SDRE-H was blunt-ended using Klenow and was inserted into the *Sma*I site of the pOvCAT-308N (*35*) to generate pOvCAT-308I. To create constructs in which the SDRE was directly linked to the NRE, fragment H-SDRE-H was subcloned in the forward and reverse orientations into the *Hin*dIII site of the pOvCAT-308N to create pOvCAT-308F and -308H, respectively. To create constructs containing the SDRE, but not the NRE or the COUP sites, fragment H-SDRE-H was subcloned in the forward and reverse orientations into the *Hin*dIII site of the pOvCAT-058 to create pOvCAT-058E and -058F, respectively.

(b) Linker Scanning Mutation Constructs. A two-step polymerase-chain-reaction (PCR) method was used to create the linker scanner (LS) mutations in the Ov 5′-flanking region essentially as described (*12*, *36*). A pair of synthetic oligonucleotides was used to make each LS mutation, and these primers contained the restriction site that replaces the wild-type sequence. Two additional primers were used to make each LS mutation, an antisense oligonucleotide from within the CAT gene (CTGAAAATCTCGCCAAG) and the sense universal primer (GTAAACGACGGCCAGT). Standard conditions were used for the PCR reactions: 95 °C (1

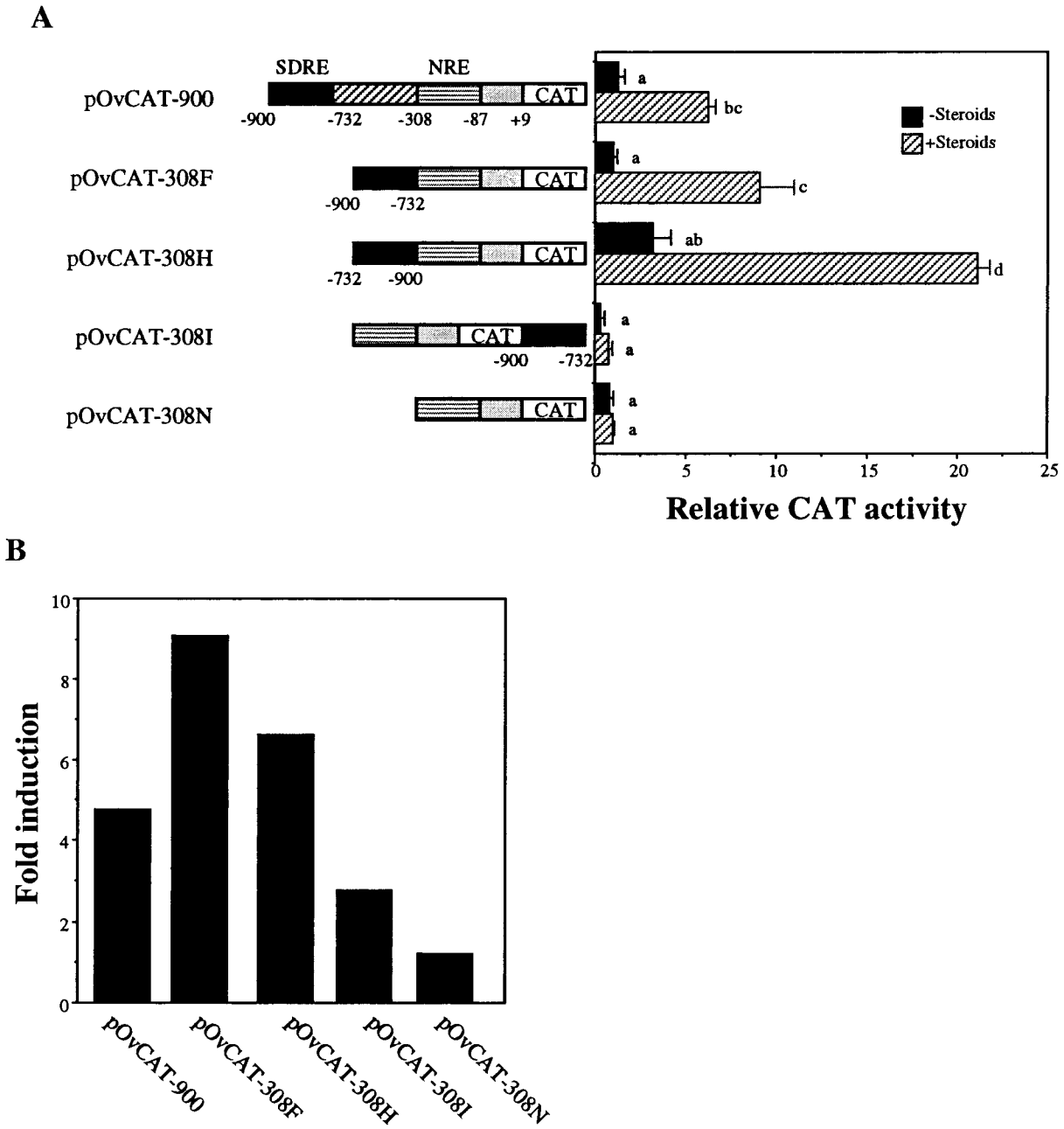

FIGURE 2: Linker sequence between the SDRE and the NRE is not necessary for the steroidal induction of the Ov gene. (A) OvCAT fusion genes were constructed that contain the indicated sequences from the Ov gene inserted into pOvCAT-308N to create pOvCAT-308F, pOvCAT-308H, and pOvCAT-308I. The fusion genes were transfected into oviduct tubular gland cells by $CaPO_4$ coprecipitation. After transfection, cells were cultured in either a serum-free medium containing insulin (50 ng/mL) or a serum-free medium containing estrogen (1×10^{-7} M) and corticosterone (1×10^{-6} M). The cells were harvested 36 h later and assayed for CAT activity. CAT activity is expressed as relative to that achieved with pOvCAT-308N in the absence of steroids. This is a representative experiment with two replicates for each treatment. The error bars represent the standard error of the mean. Means not sharing a common letter (a−d) are significantly different at $p < 0.05$ by ANOVA. (B) To calculate the extent of induction by estrogen and corticosterone, CAT assay values with steroids were divided by those without steroids and are shown in terms of fold induction.

min), 50 °C (1 min), 72 °C (1.5 min), 25 cycles, 72 °C (4 min), 4 °C (hold). pOvCAT-LS-COUP, LS-FF, LS-GG, LS-HH, and LS-II were generated from pOvCAT-900 by replacing the indicated Ov nucleotides with a synthetic *Xba*I restriction site (Figure 4A). All plasmids were sequenced using dideoxy sequencing (*37*). pRSVCOUP-TFI contains the chicken COUP-TFI cDNA driven by the RSV promoter and was kindly provided by Dr. M.-J. Tsai.

Tubular Gland Cell Culture and Transfection. Primary oviduct tubular gland cells from estrogen-withdrawn immature chickens were isolated and transfected as previously described (*8*, *10*). In brief, the cells were prepared by collagenase dissociation, and equal moles of DNA were transfected at the time of cell isolation by $CaPO_4$ coprecipitation. To reduce variations in transfection efficiency, all cells transfected with a particular plasmid were pooled after the transfection and were then aliquoted into the appropriate serum-free culture medium as described in the legend for Figure 2. Each plasmid was transfected in at least two experiments, using different DNA preparations.

CAT Assays. The oviduct cells used for the CAT assays were harvested 24−36 h after transfection. Total protein

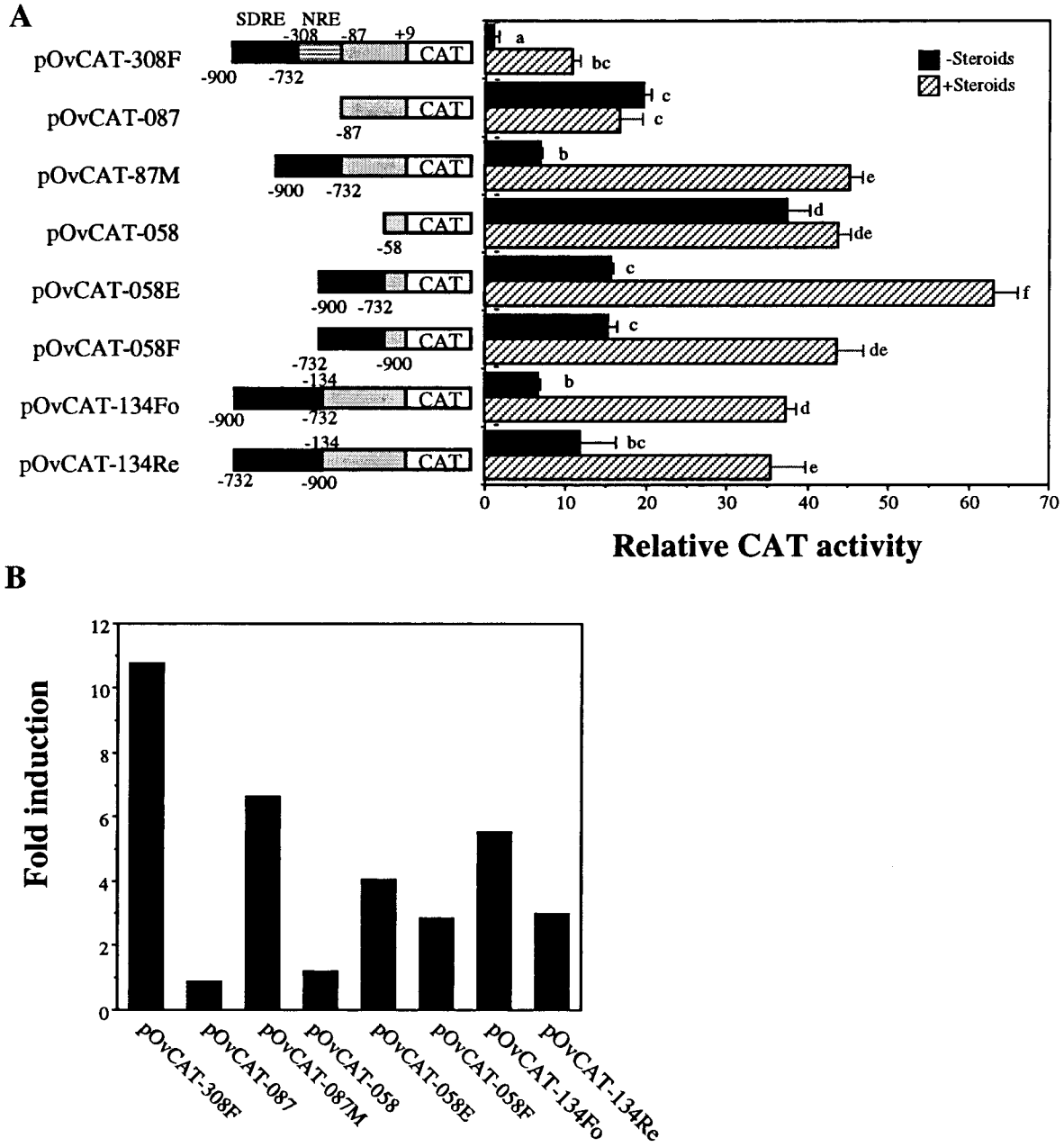

FIGURE 3: Deletion of the SDRE abolishes steroid induction, but deletion of the COUP site does not affect steroid induction in the absence of the NRE. (A) The plasmids were created as described in Materials and Methods. These constructs were transfected into oviduct tubular gland cells as described in the Figure 2 legend. CAT activity is expressed as relative to that achieved with pOvCAT-308F in the absence of steroids. These data are from two experiments with four replicates for each treatment. The error bars represent the standard error of the mean. Means not sharing a common letter (a−f) are significantly different at $p < 0.05$ by ANOVA. (B) To calculate the extent of estrogen and corticosterone induction, CAT values with steroids were divided by those without steroids and are shown in terms of fold induction.

concentration was determined in duplicate using the Bradford protein assay, with bovine γ-globulin as a standard (*38*). All CAT assay reactions contained 0.2 μCi of [^{14}C]-chloramphenicol (50 mCi/mmol) and 100 μg of protein and were performed as described, with certain modifications (*39*, *40*). Reactions were incubated for 16 h at 37 °C with 4.4 mM acetyl coenzyme A. These reaction conditions for the oviduct extracts were shown to be linear. Percent conversion of acetylated end product was calculated by cutting out the radioactive spots on the TLC plates and by counting the unacetylated chloramphenicol and the acetylated (both mono- and di-) chloramphenicol in separate liquid scintillation vials. The data were averaged and are plotted in all cases as standard error of the mean. The data were analyzed using ANOVA to determine statistical differences as noted in the figure legends.

RESULTS

The SDRE Functions as an Enhancer. Because both the SDRE and the NRE are required for steroid-dependent regulation of the heterologous thymidine kinase promoter (*11*), these two elements appear to act as a single functional entity, despite a physical separation of 450 bp. Critical to this hypothesis is whether the region between the two elements (−731 to −309, the "linker" sequence) has any functional relevance in the induction of the gene in response to steroid hormones. The experiment depicted in Figure 2 was designed to address that question. Although earlier

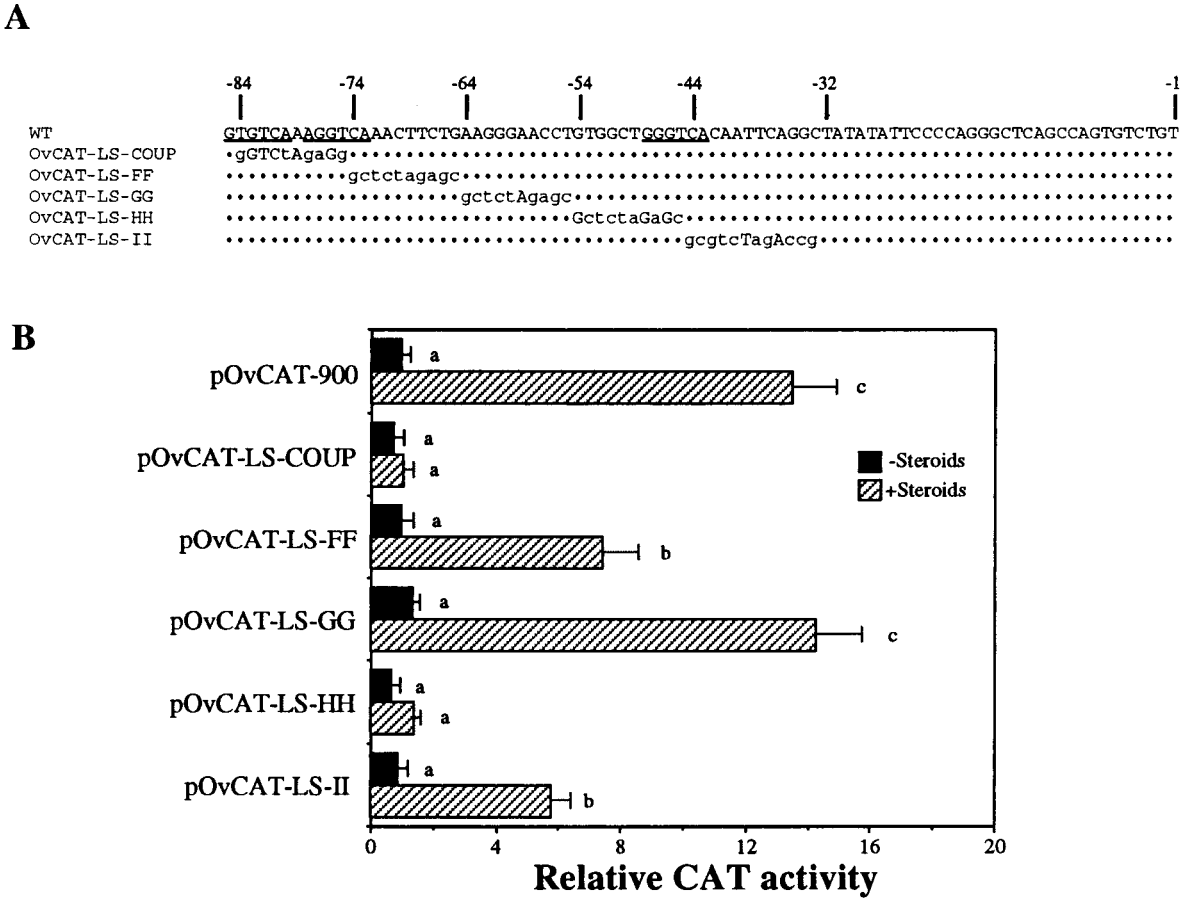

FIGURE 4: Induction by steroid hormones requires the COUP binding site and the region from −54 to −45 when the NRE is present. (A) Sequence of the wild-type Ov proximal promoter region and of the LS mutations that were made in this region. Wild-type nucleotides are indicated by capital letters, whereas lowercase letters show mutated bases. Nucleotides that are represented by dots are the same as wild type. The COUP binding site and half-site are underlined. (B) The LS mutations were transfected into oviduct tubular gland cells as described in the Figure 2 legend. CAT activity is expressed as relative to that achieved with pOvCAT-900 in the absence of steroids. These data are from three experiments with six replicates for each treatment. The error bars represent the standard error of the mean. Means not sharing a common letter (a, b) are significantly different at $p < 0.05$ by ANOVA.

experiments defined the SDRE as the region from −900 to −521 and the NRE as the region from −308 to −132 (*10*, *11*), more recent data limited the SDRE to the region from −892 to −793 (*41*) and the NRE from −308 to −88 (*35*). To determine whether the smaller SDRE requires the NRE for induction by steroid hormones, the SDRE was subcloned in both the forward and the reverse orientations adjacent to the 5′-end of the NRE (Figure 2A: pOvCAT-308F and pOvCAT-308H, respectively). As expected, both of these plasmids without the Ov linker sequence retained functional induction by estrogen and corticosterone at least as great as that of the wild-type, parent construct, pOvCAT-900 (Figure 2). Clearly, this shows that the linker sequence between the SDRE and the NRE is not necessary for induction of the gene, at least in this context. In addition, the SDRE was subcloned far downstream of the Ov promoter, but this construct retained a functional NRE in its wild-type position (Figure 2A: pOvCAT-308I). Convincingly, this construct was not induced by steroid hormones when compared to pOvCAT-900 but was instead similar to the construct lacking an SDRE, pOvCAT-308N (Figure 2). These data show that the SDRE does not function when placed downstream of the promoter. This raises the possibility that SDRE must be located upstream of the NRE, as in the wild-type context. This result further supports the contention that the SDRE and the NRE act as a single functional entity to initiate transcription of the Ov gene, because constructs containing just these two functional elements behave similarly to wild-type.

Deletion of the SDRE Abolishes Steroid Induction but Deletion of the COUP Binding Site Increases Transcriptional Activity in the Absence of the NRE. Although the COUP-TFs were originally identified as regulating the Ov gene in in vitro transcription reactions, this has not been verified in an in vivo situation. Because the NRE contains multiple regulatory elements, the first logical step was to investigate the function of the COUP binding site (−85 to −73) in conjunction with the SDRE but without the NRE. The SDRE was subcloned in the forward orientation upstream of −87 (pOvCAT-087M), which contains the COUP site, or in the forward and the reverse orientations upstream of −58 (pOvCAT-058E and pOvCAT-058F, respectively), which lacks the COUP site. These constructs were transfected into primary oviduct cell cultures. The results are depicted in Figure 3. Both constructs lacking the SDRE, pOvCAT-087 and -058D, exhibit no induction by estrogen and corticosterone. However, with the SDRE (pOvCAT-087M, -058E, and -058F) transcriptional activity was repressed without steroids, yet induction by steroids was reconstituted (Figure 3B). These data clearly show that, without the NRE, the SDRE can support induction by steroids when juxtaposed next to the promoter. Interestingly, induction was observed

regardless of whether the COUP site was present. However, without the COUP site, induction by steroids was reduced by half (Figure 3B), because transcriptional activity in the absence of steroids is higher (Figure 3A). Correspondingly, the basal activity of pOvCAT-058 is also higher than the basal activity of pOvCAT-087. These data suggest that COUP-TF plays a repressive role in the expression of the *Ov* gene in the absence of steroids. Concern exists, however, that this negative effect could be artifactual because the COUP site is just downstream from the SDRE. To address this question, the 5′-flanking region was extended to −134, and the SDRE was inserted. Both pOvCAT-134Fo and pOvCAT-134Re showed activity comparable to pOvCAT-087M, indicating that the results with the shorter constructs are valid. Thus, these data show that, in the absence of steroid hormones, the COUP site represses the transcriptional activity of the Ov gene.

Induction by Steroid Hormones Requires the COUP Binding Site and the Region from −54 to −45 when the NRE Is Present. To further address the function of the COUP site on the induction by steroids in the context of the wild-type gene, five linker scanning (LS) mutations that cover the region from −84 to −33 were constructed (Figure 4A). Thus, the sequence from −900 to +9 was left intact except for the LS mutations. These constructs were transfected into primary oviduct cell cultures. The results are depicted in Figure 4B. A mutation in the COUP site (OvCAT-LS−COUP) totally abolished steroid-responsiveness. Because the COUP site in the *Ov* gene has been identified as the 12-bp direct repeat, −85 *GTGTCAAAGGTCA* −73 *(16, 42)*, OvCAT-LS−COUP has 11 out of 12 of the bases mutated. The steroid responsiveness of OvCAT-LS−FF, which partially mutates the 3′ COUP half-site, decreased responsiveness to steroid hormones to 50% of the wild-type. Interestingly, all induction was lost with OvCAT-LS−HH, which mutates four out of the six base pairs of a single putative COUP-TF half-site (−48 to −43) downstream of the COUP site. Transcriptional activity of OvCAT-LS−II, which mutates two of the bases in this downstream half-site, was also reduced to about 40% of that of the wild type (Figure 4B). These data clearly show that the COUP site, which has no effect on regulation without the NRE, is essential in the wild-type context. Interestingly, these data also demonstrate that a potential downstream COUP half-site contributes to Ov gene regulation.

Overexpression of COUP-TF I Alleviates the Requirement for Steroid Hormones. To directly test the hypothesis that steroid induction of the Ov promoter involves COUP-TFI, pRSV−COUP-TFI was cotransfected with wild-type pOvCAT-900 or with OvCAT-LS−COUP, which contains an LS mutation in the COUP site (Figure 5A). To hold the total amount of transfected DNA constant, pRSV-βGal was used such that the total amount of DNA was 1.2 μg. With and without steroid hormones, the expression of the wild-type Ov promoter (pOvCAT-900) was higher with increasing amounts of COUP-TFI (Figure 5A). As a consequence of the increase in the basal activity, the fold induction by steroid hormones was attenuated (Figure 5B). In contrast, overexpression of COUP-TFI had no effect on expression of OvCAT-LS−COUP (Figure 5). To more easily assess the effects of the COUP-TF overexpression on transcription in the absence of steroids, basal expression was plotted in Figure 5C. Those data indicate that 1.2 μg of COUP-TF increases transcription about 12-fold in the absence of steroids. Thus, overexpression of COUP-TFI can partially alleviate the requirement for estrogen and corticosterone. These results imply that COUP-TFI is one of the limiting factors that causes low transcriptional activity of the Ov gene in the absence of steroid hormones.

DISCUSSION

Because the role of the COUP binding site had never been previously addressed in an in vivo system and because the COUP site lies adjacent to the NRE element, experiments were conducted to determine the functional role of this site. The results from this paper imply that the COUP binding site has a vital role in the induction of the gene, because there is a total loss of activity when the site is mutated (Figure 5). Moreover, the COUP site plays a dual role in the regulation of the Ov gene because it represses basal expression in the absence of steroids (Figure 3).

To date, much of the literature indicates that COUP-TF represses the genes to which it binds. Mechanistically, COUP-TF functions through (1) competition with other nuclear receptors for occupancy of target sites, (2) competition for retinoid × receptors, (3) active repression of basal transcription, and/or (4) trans-repression by forming heterodimers with retinoic acid receptors or thyroid hormone receptors (*17*). In a few cases, such as with the mouse NGFI-A (*32*), rainbow trout estrogen receptor (*33*), and human immunodeficiency virus type-I long-terminal-repeat (*34*) genes, COUP-TF activates transcription. In another example, two elements in the promoter and enhancer of the OTC gene are recognized by both COUP-TF and HNF-4, showing that these two factors have closely related binding specificities (*3*). While the HNF-4 activates expression from the OTC promoter, the COUP-TF represses expression. However, when this HNF-4/COUP site was ligated to a heterologous promoter, COUP-TF activated transcription. Importantly, though, this study showed that COUP-TF is able to induce transcription, depending on the promoter context, suggesting a dual regulatory role for this transcription factor. Similarly, the COUP site exhibits dual roles in Ov gene regulation. Although the OTC gene demonstrated that COUP-TF can have opposing roles, depending on promoter context, the Ov gene may be the first natural promoter regulated both positively and negatively by COUP-TF, depending on the environmental signals. However, as discussed below, the COUP site is a common DNA-binding motif for members of the nuclear receptor family. Therefore, we cannot rule out the possibility that another family member is responsible for the repressive effects exerted through the COUP site in the absence of steroid hormones.

Interestingly, the LS mutation between −54 and −45, which contains a putative half-site for COUP-TF, totally abolished the induction by estrogen and corticosterone. COUP-TF was first identified by binding a direct repeat separated by one nucleotide (−85*GTGTCAAAGGTCA*-73) in the Ov gene (*16*, *42*). Although, the one nucleotide spacer with the AGGTCA motif site is the most common COUP site found in natural promoters (*17*), COUP-TFs also bind to the COUP direct repeat with different spacers. For example, all the following attest to the promiscuity of COUP-

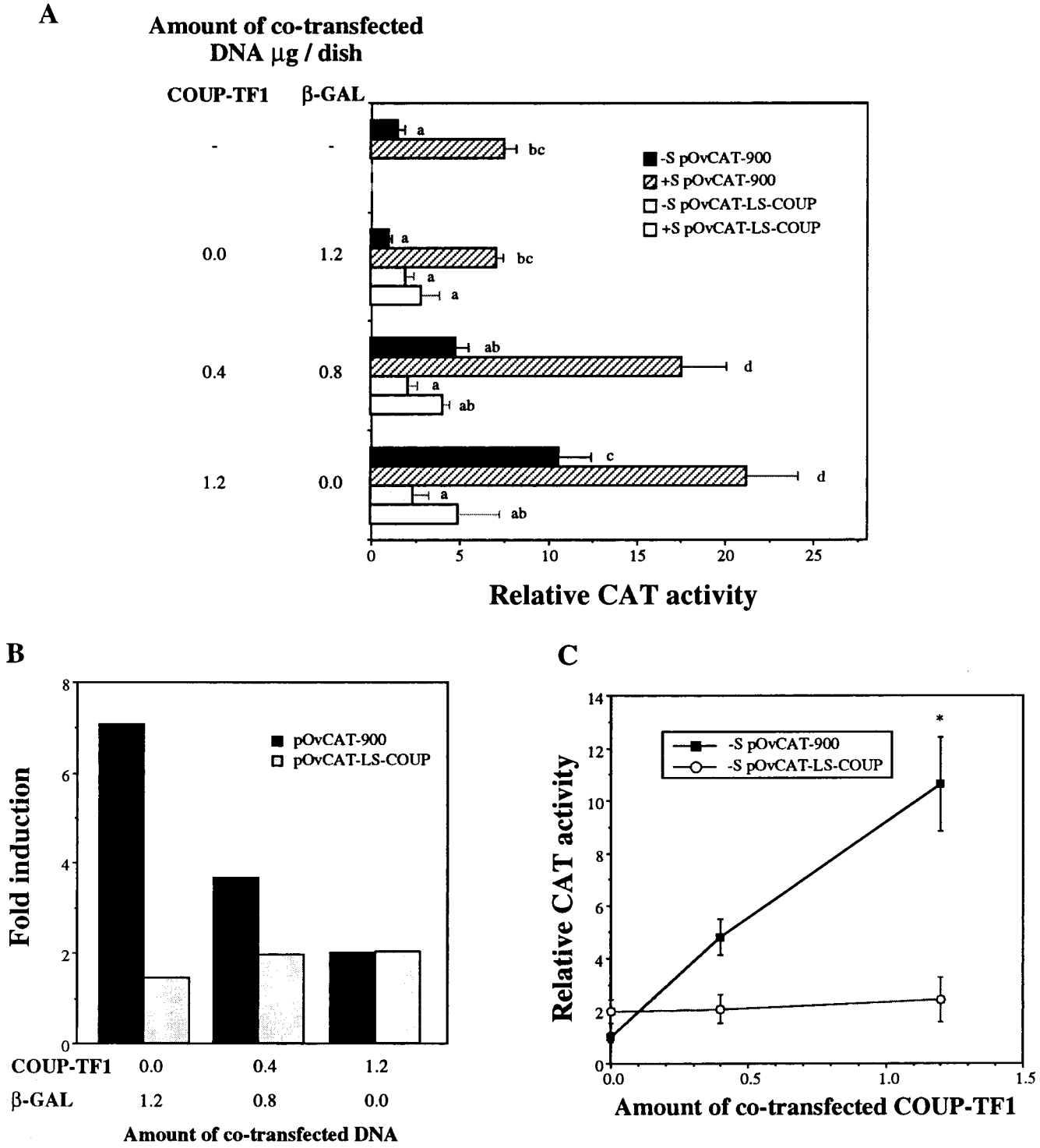

FIGURE 5: Overexpression of COUP-TFI alleviates the requirement for steroid hormones. (A) The wild-type pOvCAT-900 construct or the pOvCAT-LS−COUP construct were cotransfected with increasing amounts of pRSVCOUPTF as described in the Figure 2 legend. The amount of transfected DNA was held constant at 1.2 μg by adding pRSVβGal. CAT activity is expressed as relative to that achieved with pOvCAT-900 in the absence of steroids. These data are from two experiments with four replicates for each treatment. The error bars represent the standard error of the mean. Means not sharing a common letter (a−c) are significantly different at $p < 0.05$ by ANOVA. (B) To calculate the extent of induction by estrogen and corticosterone, CAT values with steroids were divided by those without steroids and are shown in terms of fold induction. (C) The effect of COUP-TFI cotransfection on constructs in cells cultured without steroids. An asterisk indicates significantly different compared with the corresponding same reporter plasmid at $p < 0.05$ by ANOVA.

TF: the 0-bp spacer in the oxytocin and hemopexin genes (*43*), the 2-bp spacer in the sea urchin actin III B gene (*44*), the 6-bp spacer in the RIPE−1 element of the rat insulin 2 gene (*45*), the 7-bp spacer in the arrestin gene (*46*), the 9-bp spacer in the HIV-LTR gene(*47*), and the everted repeats of 8- and 14- bp in the acyl-CoA dehydrogenase gene (*48*). Footprint analysis showed that COUP-TF can even bind with a 24-bp spacer (*49*). In the Ov gene, there is also a 24-bp spacer between the COUP site and the COUP half-site (−48 to −43). It might be possible that COUP-TF can work through this 24-bp spacer to bind both elements concomitantly.

Conversely, another possible explanation is that a different receptor binds to the gGGTCA half-site because the binding site for COUP-TF is recognized by a number of other nuclear receptors (*50*). In the human apolipoprotein CIII (apoCIII) gene, the C3P regulatory element located at −90 to −66 is necessary for maximal expression (*51*). Mietus−Snyder et al. (*51*) have demonstrated that three members of the nuclear receptor superfamily [hepatocyte nuclear factor-4 (HNF-4), apolipoprotein A1 regulatory protein (ARP-1), and Ear 3/COUP-TF] act at the C3P site. HNF-4 activates apoCIII gene expression, while ARP-1 and Ear3/COUP-TF repress its expression. Perhaps the Ov gene might be regulated in a similar fashion. Differential binding of a factor or set of factors at the Ov COUP site may regulate expression of the gene. Although it is unclear at this time which factor(s) is binding to the COUP core half-site, on the basis of sequence comparison, the binding site for both H4TF-1 (*52*) and v-ErbA (*53*) overlap the binding site for COUP. Furthermore, in vitro gel-mobility-shift experiments indicated that COUP-TF does not bind to the isolated half-site (*54*). Nonetheless, it is possible that this half-site, in conjunction with the whole COUP site, represents a tripartite COUP regulatory element.

Our observation that overexpression of COUP-TF obviates some of the requirement for steroid hormones raises the possibility that estrogen and/or corticosterone regulates COUP-TF. Although high levels of COUP-TF expression are induced by retinoids (*55*) or sonic hedgehog (*17*), no effects of steroids on the levels of COUP-TF have been reported as yet. However, evidence suggests that an indirect relationship exists between COUP-TF and steroids. Some reports indicate that COUP-TF down regulates the induction of target gene expression by some steroid receptors. For example, overexpression of COUP-TF blocked the estrogen-stimulated response of the mouse lactoferrin gene due to competition between *the* COUP-TF and estrogen receptor (*23*). In contrast, expression of the rainbow trout estrogen receptor, which is positively autoregulated by estrogen, is enhanced by COUP-TF (*33*). In the Ov gene, estrogen appears to modulate the transcriptional activity of COUP-TF. Whether this increase in transcriptional activity is the result of estrogen increasing the mass or the activity of COUP-TF remains to be determined.

Characterization of the 5′-flanking region of the Ov gene has revealed that regulation of the gene is dependent upon a number of DNA elements. Although the SDRE is absolutely required for the steroid-mediated response, steroid receptors do not directly bind to this region (*11*). In addition, ongoing protein synthesis is required for induction of the gene, which occurs only after a lag of 2 h (*6*). Additional experiments have demonstrated that several labile proteins bind to the Ov gene, both in the SDRE (*12*) and in the NRE (*15*). One of these, δEF1, appears to be directly induced by estrogen (*15*). Although it is difficult to definitively interpret experiments in which the normal contextual relationships between regulatory elements is disrupted by deletion or by moving them relative to each other, a number of conclusions can be reached about the experiments presented in Figures 2−5. First of all, without the NRE and COUP sites, the SDRE is sufficient for induction of the proximal promoter (−58 to +9) by steroids (Figure 3). Conversely, without the NRE the COUP site represses transcription, irrespective of steroids (Figure 3). However, with the NRE, the COUP site is essential for steroid induction (Figures 4 and 5). These data clearly show that the COUP site has a dual role in the regulation of the Ov gene by estrogen and corticosterone. It represses basal activity in the absence of steroids and is required for induction by steroids.

Clearly, the Ov gene is under complex positive and negative regulation. Experiments described herein have identified two regulatory sites, the COUP site and the −54 to −45 site, that are critical for the expression of the Ov gene in the wild-type context. While this manuscript begins to shed light on the molecular actions at these sites, a better understanding of the relationship of the individual regulatory elements to one another will be gained through further experimentation and characterization of the mechanisms by which COUP-TF is mobilized in response to steroid hormones. Identification of the DNA-binding factors and co-activators that interact at these regulatory sites will illuminate the exact mechanisms by which the Ov gene achieves its elegant tissue-specific and hormone-dependent expression.

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