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Chick Ovalbumin Upstream Promoter-Transcription Factors (COUP-TFs): Coming of Age*

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I. Introduction

THE steroid/thyroid hormone receptor (TR) superfamily of proteins consists of many ligand-activated transcriptional regulators and a rapidly growing number of orphan receptors (1–7). Among the orphan receptors, Chick Ovalbumin Upstream Promoter-Transcription Factor (COUP-TF) is one of the best characterized. COUP-TF was first identified as a homodimer that binds to a direct repeat regulatory element in the chicken ovalbumin promoter (8–10). This element contains an imperfect direct repeat of AGGTCA sequence, which has been shown to be essential for efficient *in vitro* transcription of the chicken ovalbumin promoter (8, 9, 11). In the late 1980s, our laboratory successfully purified COUP-TF from HeLa cell nuclear extracts using a combination of conventional and DNA affinity column chromatographic techniques (10, 12). A polyclonal antibody was then generated against the purified material and used to screen a HeLa cell cDNA library (10). A clone that cross-reacted with both the antibody and COUP-TF-binding site was obtained and designated as human COUP-TFI (hCOUP-TFI) (10). In 1988 hCOUP-TFI was also cloned independently by another group via homology to human erbA and named EAR-3 (13). Sequence analysis revealed that COUP-TFI is a member of the steroid/TR superfamily. Subsequently, hCOUP-TFII was cloned based on its high homology to hCOUP-TFI (14). hCOUP-TFII was also cloned independently by another group as an apolipoprotein AI-regulatory protein-1 (ARP-1) from a placental library (15). Through homology screening, homologs of COUP-TFs from many different species have been identified (16, 17), and their expression patterns have been analyzed.

In this review, we attempt to provide an overview of COUP-TFs with respect to their structural homology to members of the steroid receptor superfamily, their molecular and biochemical characteristics as putative negative regulators, their expression patterns during development, their regulation, and, most importantly, their possible physiological functions.

II. The COUP-TF Gene Family

A. Homology to other members of the steroid/thyroid hormone receptor superfamily

The amino acid sequence deduced from the hCOUP-TF cDNAs revealed significant similarities to members of the steroid/TR superfamily of genes (10, 14, 15) (Fig. 1). A 66-amino acid region in COUP-TF, which contains two conserved Zn-finger motifs, signified the presence of the DNA-binding domain (DBD) of a nuclear receptor. In COUP-TFs, all 20 invariant amino acids were conserved and 11 of 12 conserved residues were identical except that a conserved lysine in the second finger is replaced by a glutamine (10). Based on the P-box sequence, COUP-TFs can be classified as members of estrogen receptor (ER)/TR subfamily which bind to a Pu-GGTCA repeat. There is only one amino acid difference within the DBD of COUP-TFI and II, and it is a conservative change from Ser to Thr (10, 13–15). In addition

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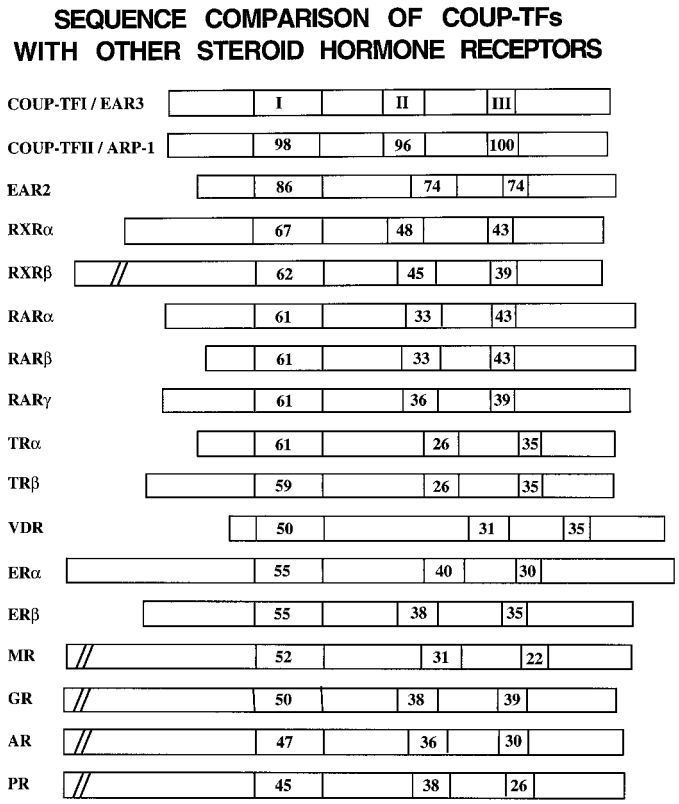

FIG. 1. Homology of the steroid receptor supergene family. There are three regions of homology, referred to as regions I, II, and III. Region I has been identified as the DBD. Regions II and III are important for the formation of the ligand-binding pocket (10).

to the Zn-finger DNA-binding motif, there are two regions, II and III, in the ligand-binding domain (LBD) that share significant homologies between various members of the steroid receptor superfamily (10, 16). These two regions have been shown to be important for the formation of a ligand-binding pocket (18). COUP-TFI and II are 96% and 100% identical in these two regions. COUP-TFs are most related to the retinoid X receptor (RXR)/retinoid acid receptor (RAR) subfamily members and share the highest homology with RXRα, having 67%, 48%, and 43% in the DBD, region II and region III, respectively (Fig. 1). The homology is slightly reduced in comparison to various members of the RAR subfamily, but is greatly decreased when compared with members of the steroid receptor family, such as mineralocorticoid receptor (MR), glucocorticoid receptor (GR), androgen receptor (AR), and progesterone receptor (PR) (Fig. 1).

Based on sequence homology alignment of the LBD, the evolutionary relationship between COUP-TFs and other members of the steroid/TR superfamily is represented in Fig. 2. It is clear that COUP-TFs are most closely related to EAR2 and members of the RXR family. The groups with the second highest percentage of homology are HNF4 and TLX. Further apart in sequential order are other orphan receptors, TR2, TR4, SF1, LRH1, ERR1, and ERR2, with ER as the only liganded receptor in these groupings.

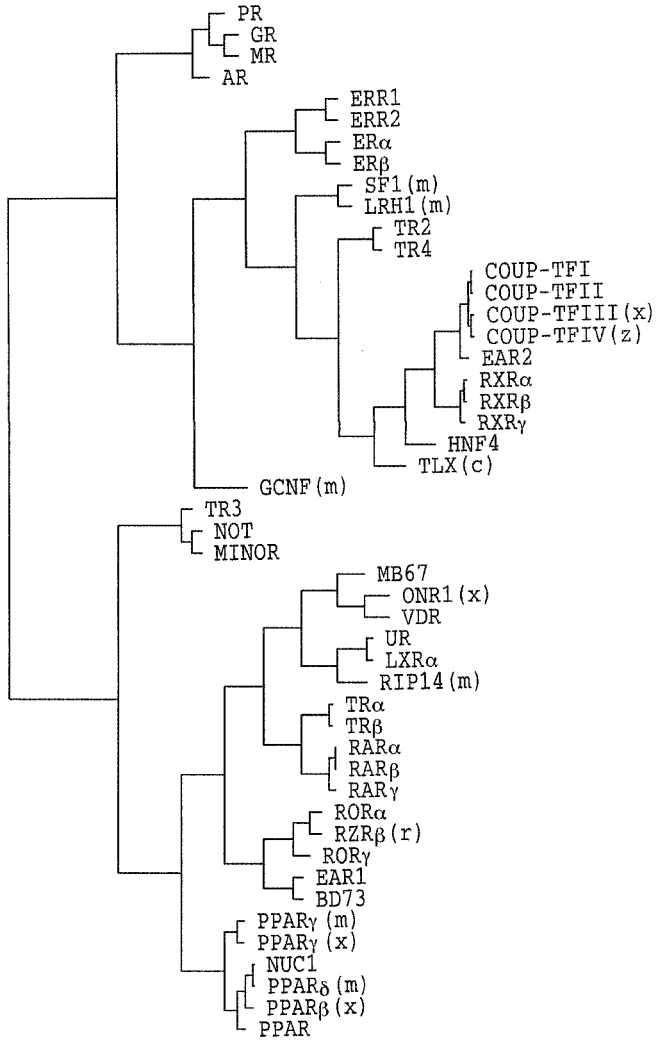

FIG. 2. Evolutionary tree for the vertebrate steroid hormone/thyroid hormone receptor superfamily. Because many genes, even from the same species, have several different names, and there is no consensus as to which name should be used, it is difficult to choose one name over the other. To avoid any controversy, where it is possible, human genes and their names are used in this evolutionary tree. If a human gene is not available, mouse (m), rat (r), chicken (c), *Xenopus* (x), or zebrafish (z) genes are used as indicated in the *parentheses*. GCG's multiple sequences alignment program "pileup" was used to align the hormone-binding domain peptide sequences from all steroid/thyroid receptors. The resulting alignment was then manipulated by the program "distances" (Kimura protein distance) followed by "growtree" (UPGMA method) to generate the phylogram. The accession numbers for the receptors are: PR (M 15716); GR (M10901); MR (M16801); AR (A34942); ERR1 (X51416,Y00290); ERR2 (X51417, Y00290); ERa (Y00102); ERb (U57439); SF1(m) (S65878); LRH1(m) (M81385); TR2 (M29960); TR4 (L27586); COUP-TFI (X16155, X58241); COUP-TFII (M64497, M62760); COUP-TFIII(x) (X06093); COUP-TFIV(z) (X70300); EAR2 (X12794); RXRa (X52773); RXRb (M84820); RXRg (U38480); HNF4 (X76930); TLX(c) (S72373); GCNF(m) (U14666); TR3 (L13740); NOT (X75918); MINOR (U12767); MB67 (Z30425, L29263); ONR1(x) (X75163); VDR (J03258); UR (U14534); LXRa (U22662); RIP14(m) (U09416); TRa (M24748); TRb (X04707); RARa (X06614); RARb (X06538); RARg (M24857); RORa (U04897); RZRb(r) (L14610); RORg (U16997); EAR1 (M24898); BD73 (L31785); PPARg(m) (U01664); PPARg(x) (M84163); NUC1 (A45360); PPARd(m) (U10375); PPARb(x) (M84162); PPAR (L02932).

B. Sequence homology within the COUP-TF gene family

Many hCOUP-TFs homologs have been cloned from other species. These include mCOUP-TFI and mCOUP-TFII from mouse (19, 20), cCOUP-TFII from chicken (21), rCOUP-TFI from rat (22), hamCOUP-TFI from hamster (M. J. Tsai, unpublished result), xCOUP-TFI, II, and III from *Xenopus* (23) (C. Queva and V. Laudet, personal communication), the seven-up gene (svp) from *Drosophila* (24), the svp[44], svp[46], and svp[40] genes from zebrafish (25, 26), and the sp-COUP-TF from sea urchin (27). In fact, COUP-TFs have been cloned from all species of metazoans (V. Laudet, personal communication). Because COUP-TF genes are conserved from humans to early metazoans, it is likely that COUP-TFs are a primordial member of the steroid/TR superfamily.

Sequence comparison between various COUP-TF genes clearly shows that they belong to a distinct subfamily within the steroid/TR superfamily. The DBDs within different members of COUP-TF minimally share 94% amino acid sequence identity, which is considerably greater than the conservation between other subfamily members. Based on homology alignments of the LBDs, vertebrate COUP-TFs can be subdivided into four groups (28) (Fig. 3). The first group includes hCOUP-TFI/EAR3, mCOUP-TFI, hamCOUP-TFI, rCOUP-TFI, xCOUP-TFI, and zsvp[44]. The second group includes hCOUP-TFII/ARP-1, mCOUP-TFII, cCOUP-TFII, xCOUP-TFII/xCOUP-TFB, and zsvp[40]. xCOUP-TFIII/xCOUP-TFA is classified as the third group because it is equally homologous (90%) to the subgroup of COUP-TFI and COUP-TFII. The zCOUP-TFIV/svp[46] is considered as group 4 because it shows similar homology (90%) to the other three groups. Invertebrate dsvp and suCOUP-TF are slightly more distantly related to other vertebrate COUP-TF members. Although EAR2 is the next closely related gene, its homology is much reduced in comparison to members of the COUP-TF subfamily (70% *vs.* >90%). In addition, the duplication between COUP-TFs and EAR2 was completed after the division of these early metazoans and the most modern ones such as arthropods and vertebrates (Laudet, personal communication). Therefore, EAR2 is not classified as a COUP-TF subfamily member and will not be discussed in this review.

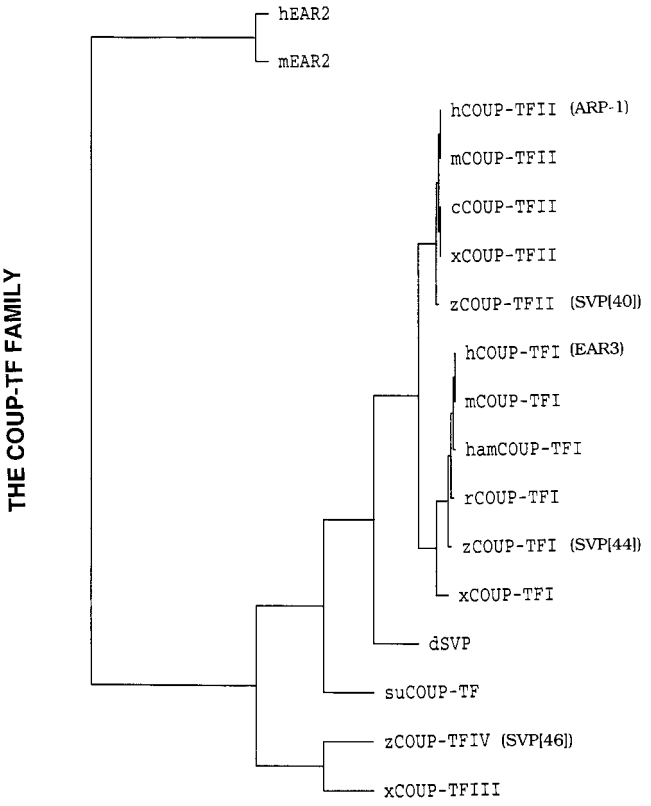

FIG. 3. Evolutionary tree for the COUP-TF family: h, human; m, mouse; r, rat; ham, hamster; x, *Xenopus*; z, zebrafish; d, *Drosophila*; su, sea urchin. hEAR2 (X12794); mEAR2 (X76654); hCOUP-TFII (ARP-1) (M64497, M62760); mCOUP-TFII (U07635); cCOUP-TFII (U00697); xCOUP-TFII (V. Laudet personal communication); zCOUP-TFII (SVP[40]) (S80986); hCOUP-TFI (EAR3) (X16155, X58241); mCOUP-TFI (X74134); hamCOUP-TFI (Tsai, *et al.*, unpublished result); rCOUP-TFI (U10995); zCOUP-TFI (SVP[44]) (X70299); xCOUP-TFI (V. Laudet, personal communication); dSVP (M28863); suCOUP-TF (L01104); zCOUP-TFIV (SVP[46]) (X70300); xCOUP-TFIII (X63092).

Within the same subgroup, the homology in both the DBD and the putative LBD are striking (Fig. 4). The DBDs of COUP-TFI or II in different species are identical, implying that they bind to a similar or an identical response element (16). Most surprisingly, the putative LBDs of COUP-TF I and II are 99.6% identical among vertebrates (16). Even svp from *Drosophila* shares 90% identity. Such a high degree of homology suggests that these domains are critical for the biological function of COUP-TFs, even though a ligand or a specific interacting protein unique for COUP-TFs has not been identified. In contrast, the N-terminal domain of COUP-TFI and II are significantly diverged, having only 45% identity, which may provide distinct functions for the two different members (10, 13–15). There are clusters of proline- and glutamine-rich regions that resemble the activation domains of transcription factors such as CTF and SP1, respectively. These domains also contain multiple consensus phosphorylation sites for protein kinase A and proline-directed kinase, consistent with the finding that COUP-TFs are phosphorylated proteins.

Based on the significant sequence conservation, it is reasonable to speculate that COUP-TFs play a vital role for cellular function in vertebrates. This hypothesis is supported by the finding that null mutants of dsvp are lethal (24, 29). In addition, null mutants of mCOUP-TF1 and II, generated in our laboratory by homologous recombination, lead to perinatal and embryonic lethality, respectively, in mice, which will be discussed in more detail in a later section.

III. Biochemical Characteristics

A. DNA-binding sites

COUP-TFs were first identified by binding to the COUP element which is an imperfect GTGTCA AAGGTCA direct repeat separated by one nucleotide in the ovalbumin promoter (8, 9). Methylation interference assays indicate that two guanine residues in both half-sites are important for binding (11, 30). Mutation of either one of the G residues in

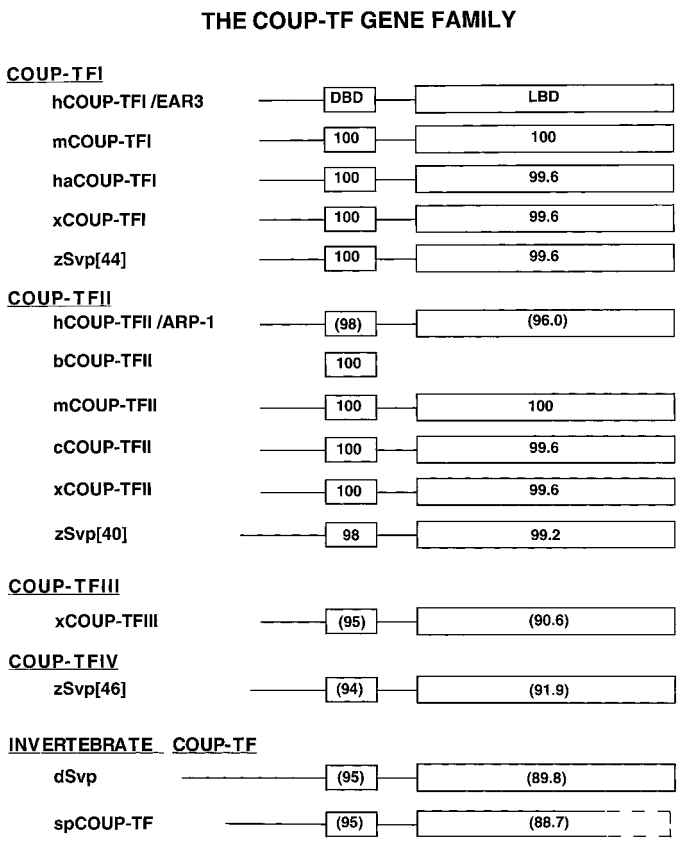

FIG. 4. Homologies in the COUP-TF gene family. The *numbers in the boxes* represent the percentage of homology compared with the hCOUP-TFI. The *dashed box* indicates the putative sequence that has yet to be cloned.

the downstream half-site completely abolished binding, indicating that these two G residues are critical contact sites (30). Subsequently, COUP-TFs have been shown to bind to a GGGTCA imperfect direct repeat separated by six nucleotides in the rat insulin II promoter (30). Again, all three guanine residues are important contact sites. COUP-TFs bind to the ovalbumin promoter with much higher affinity than to the COUP element in the insulin promoter, possibly a consequence of variation in the sequence of the downstream half-site from the consensus element and the longer spacing between the two half-sites (30).

To systematically analyze the effect of spacing and orientation of half-sites on the relative binding affinity of COUP-TFs, a series of oligonucleotides containing a direct repeat of the core half-site, GGTCA separated by a 0, 2, 5, 7, 9, or 12 nucleotide spacing, were synthesized and used as competitors in a gel mobility shift binding assay (31). The relative binding affinity for the direct repeats with different spacing is as follows: DR1, DR6, DR4, DR8, DR0, and DR11. COUP-TFs also bind to inverted and everted repeats of the consensus sequence (31). In general, COUP-TFs display higher binding affinity for direct repeats in comparison to the inverted palindromic sequence. Among the palindromic sequences, COUP-TFs bind to the consensus element with 0 spacing with the highest affinity, yet their binding is about 3-fold lower than the binding affinity of the DR1 element. As expected, homo- and heterodimers of COUP-TFI and II bind to the different repeats with similar relative affinity, consistent with the function of a nearly identical DBD of the two factors. Taken together, COUP-TFs display highest preference to bind DR1 elements (31).

In fact, the most common COUP-TF-binding site found in natural promoters is the DR1 consensus sequence, which is an AGGTCA direct repeat with one nucleotide spacer. COUP-TFs have been shown to bind and repress DR1 consensus-regulatory elements in the promoter of many genes, including rat and human apolipoprotein CIII (32–34), human Apolipoprotein AI (32, 33), chicken apolipoprotein VLDLII (35, 36), mouse lactoferrin (37, 38), mouse mammary tumor virus promoter (39), and mouse OCT4 promoter (40). In addition to the DR1 element, COUP-TFs can also bind to the DR0 sequence in oxytocin (41) and hemopexin promoters (42), the DR2 element in the sea urchin actin III B gene (27), the DR6 RIPE-1 element of the rat insulin 2 promoter (30), the DR7 of arrestin gene (43), the DR9 of HIV-LTR (44), and the everted repeats of eight- and 14-nucleotide spacings of the acyl-coA dehydrogenase gene promoter (45).

Biochemical studies indicate that COUP-TFs exist in solution as dimers (9, 12, 46) and have been shown to bind to the consensus response elements as dimers in gel mobility shift assays (9, 46). The ability of the dimeric COUP-TFs to bind to diverse structural motifs, such as direct repeats, inverted repeats, and everted repeats with a variety of spacings, suggests that COUP-TFs must be able to assume different conformations to accommodate the structural and spatial changes in the recognition sequences (31, 47). Indeed, the COUP-TF dimer bound to DR1 or DR6 elements possesses distinct conformations, as shown by its different susceptibilities to protease digestion (31). The binding versatility of COUP-TFs for direct repeats has tremendous biological implications because many of these binding sites are also response elements for the retinoid (DR2 and 5), thyroid hormone (DR4), and vitamin D (DR3) receptors (46, 48). Retinoids, thyroid hormones, and vitamin D are well known hormones and morphogens for vertebrate development and differentiation. Therefore, it is of great interest to delineate the molecular mechanism by which COUP-TFs modulate the processes of cellular development and differentiation.

B. Molecular mechanism of COUP-TFs action

Vitamin D receptor (VDR), TR, and RAR have been demonstrated to activate target genes containing DR3, DR4, and DR5 response elements, respectively. By virtue of their promiscuous DNA binding, COUP-TFs are expected to downregulate the hormonal induction of target genes by VDR, TR, and RAR. Cotransfection of COUP-TFI or II expression vectors inhibited the hormonal induction of VDR-, TR-, and RAR-dependent activation of reporter activity (31, 46, 47, 49). The inhibition of transcriptional activity by COUP-TFs is dose-dependent, as the reporter activity is progressively inhibited by increasing concentrations of the transfected COUP-TF expression vector. COUP-TFs not only inhibit the hormone response of reporters containing synthetic AGGTCA repeats with various spacings, they also inhibit the expression of reporters containing natural vitamin D re-

sponse element (VDRE), thyroid response element (TRE), and retinoic acid response element (RARE) sequences as in the respective cases of the osteocalcin, myosin heavy chain, and βRAR promoters (46). In addition, COUP-TFs have been shown to antagonize the HNF4-dependent transcriptional activation of many liver-specific genes (33, 50, 51) and to suppress OCT3/4 expression during retinoid-induced differentiation of P19 embryonic carcinoma cells (52). How do COUP-TFs inhibit the transactivation of other members of the steroid receptor superfamily? Four mechanisms are proposed, which are the subject of discussion here (Fig. 5).

1. Competition for occupancy of the binding sites. COUP-TFs have been shown to bind promiscuously to a variety of direct repeats, including DR3, DR4, and DR5 of the AGGTCA motif, and hormone response elements of VDR, TR, and RAR, respectively (46, 47, 49). Through direct competition with VDR, TR, and RAR for the available binding sites, COUP-TFs have been shown to repress the hormonal induction of target genes of VDR, TR, and RAR in transient transfection assays (46, 47). The repression is released by increasing the expression of RAR, which suggests that COUP-TFs negatively regulate retinoid responses by competing for binding to the retinoid response elements of the target reporter. COUP-TFs have also been demonstrated to interfere with the transactivation of TR, RXR, and peroxisome proliferator-activated receptor (PPAR) through a similar mechanism (46, 47, 49). In addition, COUP-TFs have been shown to inhibit the transactivation of SF1 and HNF4 due to mutually exclusive binding to the promoter of many genes (53–55). Finally, COUP-TF has been shown to antagonize ER activation of the lactoferrin and oxytocin promoters by binding to a binding site that overlaps the estrogen response element (38, 56).

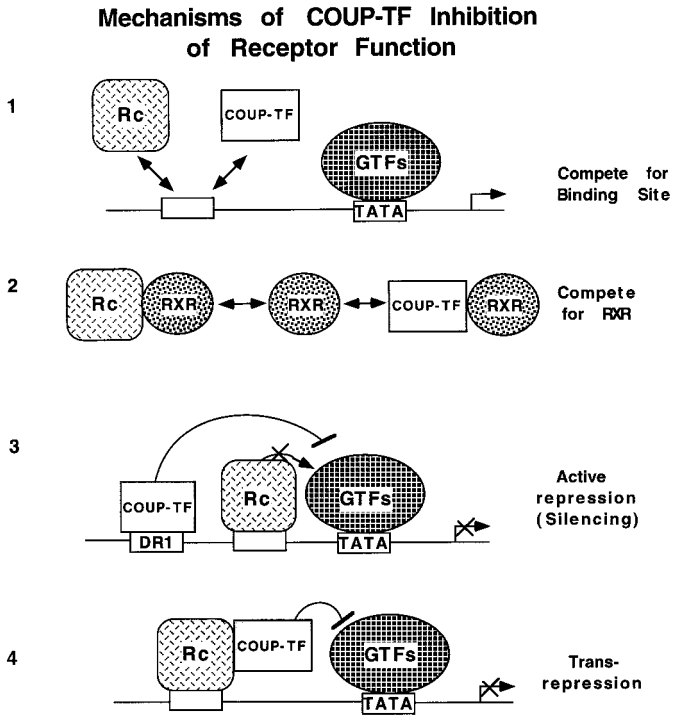

FIG. 5. Mechanism of COUP-TF inhibition of receptor function. 1, Competition for the DNA-binding site; 2, competition for RXR; 3, active repression; 4, transrepression. Rc, Activated receptor; GTFs, general transcription factors.

2. Competition for RXR. It is well documented that RXR is a universal heterodimeric partner of RAR, TR, VDR, PPAR, and other orphan receptors (57–61). Homodimers of RAR, TR, VDR, and PPAR either bind poorly or not at all to their cognate response elements (3). Through association with RXR, the heterodimeric receptors can then bind to the cognate response elements with high affinity and, thus, enhance the transactivation potential of this group of receptors (3). Because the direct repeat recognition sequence is asymmetric, it has been shown that RXR occupies the 5′ half-site while the other partner occupies the downstream 3′ half-site, which confers the hormone responsiveness (62, 63). RXR can also bind to DR1 elements as a homodimer and as a heterodimer with RAR and PPAR (60, 61). The RXR homodimer is an activator that responds to 9-*cis*-retinoic acid. RXR/PPAR heterodimers respond to both 9-*cis*-retinoic acid- and PPAR-specific ligands (61). However, RAR/RXR heterodimers, in which RAR binds to the 5′ half-site, are transactivationally inactive. It has been shown that RAR and TR bind to a corepressor [either silencing mediator for retinoid and thyroid hormone receptors (SMRT) or nuclear receptor corepressor (N-CoR)] in the absence of hormone (64, 65). Binding of these corepressors is necessary for receptors to silence the promoter activity. Binding of hormone then releases the corepressor and, thus, abolishes silencing activity of receptors. However, when RAR/RXR binds to DR1, the retinoic acid ligand is not able to release the corepressor from RAR; therefore, RAR/RXR heterodimer is not able to activate the DR1 reporter (63).

Although COUP-TFs exist in solution as homodimers and fail to form stable heterodimers with RXR in coimmunoprecipitation assays (12, 31), they do readily form DNA-binding heterodimers with RXR (31, 47, 49). Therefore, COUP-TFs are able to sequester the universal partner RXR in a functionally inactive complex and reduce the available concentrations of RXR (31, 46, 47, 49, 66). The loss of RXR indirectly decreases the DNA-binding affinity of TR, VDR, RAR, and PPAR and thereby interferes with the potential of this subgroup of receptors to transactivate their target genes (46, 47, 61). This notion is further verified by the relief of COUP-TF inhibition when RXR is overexpressed (46). In addition, it has been demonstrated that COUP-TFs form heterodimers with TR and RAR and disrupt their functions (67–69). Thus, the ability of COUP-TFs to form heterodimers with RXR, TR, and RAR may contribute significantly to the negative regulatory role of COUP-TFs in modulating hormone responsiveness of a large number of receptors of the TR and RAR subfamily (3).

Svp, the *Drosophila* homolog of COUP-TF, has also been shown to modulate the *in vivo* and *in vitro* function of Ultraspiracle (Usp), the *Drosophila* homolog of RXR (70, 71). Usp is the heterodimeric partner of ecdysone receptor. Like COUP-TFs, Svp binds to the consensus response element of ecdysone receptor and competes with the ecdysone/Usp heterodimer for the same binding site as well as forms heterodimers with Usp; thereby, it interferes with the signaling pathway of the ecdysone receptor in *Drosophila* (72). The induction of lethality during early metamorphosis by ectopic

expression of Svp and the reversal of lethality by concomitant overexpression of Usp are consistent with the hypothesis that Svp negatively modulates the ecdysone signaling pathway in *Drosophila* in a manner similar to COUP-TFs' modulation of thyroid hormone and retinoid function in mammalian systems.

3. Active repression. Like unliganded TR, COUP-TFs have been shown to repress basal transcriptional activity of a number of thymidine kinase reporters containing DR3, DR4, or DR5 hormone response elements (31, 46). This silencing of basal transcriptional activity is response element specific and is unlikely due to squelching of TFIIB, which interacts with COUP-TFs or other general transcription factors, since reporter genes lacking COUP-TF binding sites show little COUP-TF-mediated repression (69). Subsequently, it has been demonstrated that COUP-TFs, similar to TR and RAR, possess an active silencing domain within the C terminus of the putative LBD (69). This repressor domain can be transferred to a heterologous GAL4 DBD and can be shown to retain its ability to repress basal transcriptional activity (69).

In addition, we have recently shown that COUP-TFs can function as an active repressor to inhibit transactivation mediated by acidic (Gal4-RII), glutamine-rich (Gal4-ftzQ), proline-rich (Gal4-CTF1P), and Ser/Thr-rich (Gal4-ZenST) transactivators (69). The active repressor function of COUP-TFs is position independent, *i.e.* the binding sites of COUP-TFs can either be localized upstream of the activator binding site or downstream of the reporter gene without significantly affecting the active repression. The fact that COUP-TFs can repress such diverse groups of transactivators suggests that it is unlikely due to COUP-TFs directly quenching these transactivators or interfering with their interaction with their respective targets. Perhaps it is more likely that COUP-TFs interact with a common target, a putative corepressor that mediates the repression. Therefore, it is possible that COUP-TFs can interact with cellular repressors, such as SMRT and N-CoR, to silence basal and active transcription in a manner similar to RAR and TR (64, 65). Whether SMRT or N-CoR mediates the active silencing activity of COUP-TFs has yet to be defined.

4. Transrepression. As discussed earlier, COUP-TFs form heterodimers with RAR, TR, and RXR (46, 47, 49, 69). The dimerization is presumably mediated through interactions between the LBDs of COUP-TFs and these receptors. We have recently demonstrated that heterodimeric interactions can take place between DNA-bound wild type TR, RAR or RXR, and Gal4-COUP-TF (LBD). This dimeric interaction is sufficient for subsequent inhibition of the basal or activated promoter activity (69). Therefore, COUP-TFs can be tethered to DNA in the absence of their cognate response elements via LBD-LBD interactions with other receptors such as TR, RAR, and RXR to transrepress the ligand-dependent transactivation of the above nuclear receptors.

5. The role of COUP-TFs in transactivation. Although accumulated evidences indicate that COUP-TFs function as negative regulators in transient transfection assays, COUP-TF was initially found as an activator of chicken ovalbumin gene expression. COUP-TF has been shown to stimulate the transcription of transferrin promoter in Hep3B cells, but not in Sertoli cells (55). In addition, it has been shown that COUP-TF can activate fatty acid-binding protein (73), mouse mammary tumor virus (74), vHNF1 (75), and ornithine transcarbamylase (76) promoter activities. Recently, Hall and Sladek (77) demonstrated that COUP-TF and HNF-4 bind to the AF-1 element in the PEPCK gene and serve as accessory factors to augment glucocorticoid response in activation of the PEPCK gene expression. However, the physiological importance of COUP-TFs as activators is unclear, inasmuch as some of the activity has been observed only when a response element was analyzed out of the context of its promoter. In addition, the inducibility is low in general, and it is only observed in a few particular cell types.

IV. Expression Patterns of COUP-TFs During Development

The expression patterns of various COUP-TFs have been described in mouse, chick, zebrafish, frog, and *Drosophila* (16). The general patterns of expression in different species are fairly similar. COUP-TFs are expressed in restricted regions of the central nervous system (CNS) during embryonic development. The segmental expression of COUP-TFs in the forebrain suggests that they might play a role in patterning of the rostral brain, whereas their specific expression within the hindbrain rhombomere primordia suggests that they might be important for hindbrain segmentation. COUP-TFs are also expressed in the mesenchymal tissue of many organs that require mesenchymal-epithelial interactions for their development. The expression levels decrease after organogenesis. It is also expressed in the developing eye. In *Drosophila*, svp gene is expressed in the embryonic CNS and the fat body as well as in the precursor of photoreceptor cells (24, 29) and has been shown to be essential for neurogenesis and for photoreceptor development (24). The conservation in expression patterns and in protein structure throughout evolutionarily diverged species suggests a functional conservation of COUP-TFs. Thus, COUP-TFs likely play a central role in embryonic development and differentiation.

Both COUP-TFI and II are widely expressed in the CNS and in many organs, with patterns that are overlapping yet distinct from each other (16, 17, 19–21). In general, COUP-TFI expression is higher in the CNS and lower in internal organs as compared with COUP-TFII. Because the lower vertebrates include many members of COUP-TFs that are not found in the higher vertebrates, we will describe the expression patterns of the two groups separately. To simplify the presentation, we will primarily describe results obtained from one species and discuss others in parallel so that the results can be presented in more meaningful and less complex terms.

A. Expression patterns in zebrafish and Xenopus

COUP-TFI/Svp[44] transcripts are first detected in 11- to 12-h embryos of zebrafish (25). In the rostral brain of 13-h embryos, it is expressed within the anterior half of the midbrain and the posterior part of the diencephalon (25). In the presumptive hindbrain, it appears in a segment-like stripe in the anterior region, resembling the presumptive rhom-

bomere units of the hindbrain (25). It is also detected in the intermediate mesoderm posterior to the first somite. As somitogenesis proceeds, its expression extends posteriorly and flanks the 10 most anterior somites (25). The expression changes extensively both in level and expansion of domains between 13 and 20 h. In the rostral brain, its expression extends to include a major part of the diencephalon and a caudal portion of the telencephalon. Within the hindbrain, it is strongly expressed in the two most anterior rhombomeres, and a lower but uniform expression is seen to extend throughout rhombomere 7. In 28-h embryos, higher and more uniform expression of COUP-TFI/Svp[44] is seen in both rostral and hindbrain areas. Also, COUP-TFI/svp[44] is expressed in the retina of the eye.

Similar to COUP-TFI/svp[44], COUP-TFII/svp[40] is expressed as early as the 10-h stage (26). By 12 h, it is expressed in two domains of the brain: one at the border between diencephalon and hindbrain and the other located just posterior to the optic vesicles (26). In contrast to COUP-TFI/svp[44], there is no expression in the presumptive telencephalon. It is also expressed in the anterior part of somites 3–6 (26). In 16-h embryos, the expression in the hindbrain is higher and more sharply demarcated, corresponding to the primordia of the six most rostral rhombomeres (26). In 20-h embryos, COUP-TFII/svp[40] is expressed as a step gradient with specific levels in each of the first six rhombomeres and the expression domains corresponding directly to rhombomeres (26). The expression of COUP-TFII/svp[40] in the rhombomeres resembles that of xCOUP-TFB, having higher expression in r1, r2, r5, and r6 than in r3 and r4 in the hindbrain and very little expression in the major part of the midbrain (26).

The third COUP-TF member in zebrafish, COUP-TFIV/svp[46], is expressed in the primordia of the diencephalon, midbrain, hindbrain, and the anterior part of the spinal cord at the 11-h stage (25). In the hindbrain, it is expressed in the presumptive rhombomeres 1, 2, 4, and 5, but the expression is transient and is not detected by the 17-h stage (25). COUP-TFIV/svp[46] is also expressed in paraxial mesoderm in different developmental stages. In general, the expression is intense in the last somites being formed and in the anterior portion of each somite (25). Finally, COUP-TFIV/svp[46] is highly expressed in the optic stalk. The overall expression patterns of COUP-TFIV/svp[46] are very similar to xCOUP-TFIII/xCOUP-TFA, which is intensely expressed in rhombomeres 1, 2, 4, and 6 of the hindbrain and the eye anlagen (78). However, xCOUP-TFIII/xCOUP-TFA is also highly expressed in telencephalon (78).

B. Expression patterns in mouse and chick.

COUP-TFI and II from mouse and COUP-TFII from chick are expressed in the developing CNS (19, 21). The expression patterns of COUP-TFI and II are extremely similar to that observed with svp[44] and svp[40] of the zebrafish, respectively (25, 26). Expression of mCOUP-TFI and II was first detected at 7.5 days post coitus (p.c.), peaked at 14–15 days p.c., and declined sharply before birth (19). Whole-mount staining of mCOUP-TFI at 8.5 days p.c. indicates that it is expressed in specific regions of the rostral brain marked by sharp expression domains and two intensively expressed stripes in the presumptive hindbrain, correlating well with migratory neural crest cells (Y. Qiu, F. A. Pereira, F. J. DeMayo, J. P. Lydon, S. Y. Tsai, and M.-J. Tsai, unpublished results). It is also expressed in the anteriormost somites (X. Qiu *et al.*, unpublished results). In contrast, the expression domains of COUP-TFII are much less defined in both the rostral and hindbrain, and expression is seen in all somites (Y. Qiu, F. Pereira, M.-J. Tsai, and S. Y. Tsai, unpublished result).

As development proceeds, mCOUP-TFI and II establish overlapping yet distinct patterns of expression (19). At 13.5 days p.c., COUP-TFI expression is seen throughout the pallium, while COUP-TFII is restricted to the caudal region. Interestingly, mCOUP-TFs are expressed in a segmental fashion in the diencephalic neuromeres. According to Figdor and Stern (80), the neuromeres are designated as D1 (ventral and hypothalamus), D2 (dorsal thalamus), and D3 and D4 (pretectal region). Both mCOUP-TFI and II are expressed at equally high levels in D1 but at low levels in D3 and D4. However, high levels of mCOUP-TFI, but not mCOUP-TFII, are seen in D2 (19).

In the midbrain, mCOUP-TFI is distributed in the tectum with an anterioposterior gradient, with highest intensity at the rostral end, while the expression of mCOUP-TFII is limited to the rostral third of the tectum (19). mCOUP-TFII is also expressed at high levels in the ocular motor nucleus, whereas mCOUP-TFI transcripts are barely detectable. In the hindbrain, mCOUP-TFI is highly expressed in rhombomeres 1–5, and expression gradually declines within rhombomere 6 (Y. Qiu, F. A. Pereira, F. J. DeMayo, J. P. Lydon, S. Y. Tsai, and M.-J. Tsai, unpublished results). mCOUP-TFII is expressed highly in the anteriormost rhombomeres, and expression is significantly reduced in r4 and gradually increases to a higher level in r6 (Y. Qiu, F. Pereira, M.-J. Tsai, and S. Y. Tsai, unpublished observation). The hindbrain expression pattern is strikingly similar to its counterpart, svp[40] of zebrafish, suggesting the importance of COUP-TFs in hindbrain segmentation. In the spinal cord, mCOUP-TFI is expressed throughout the neural tube with slightly higher intensity in the motor neurons (19). In contrast, mCOUP-TFII expression is restricted to the motor neurons. In chick, it was also shown that cCOUP-TFII expression correlates with the differentiation, but not determination, of motor neurons (21).

In addition to the neuronal expression, mCOUP-TFs are also differentially expressed in a restricted manner during organogenesis (17, 20). mCOUP-TFI is expressed in the stroma of the nasal septum, in the tongue, in the follicles of vibrissae, and in the cochlea (17). mCOUP-TFII is expressed in the same regions, but at a considerably lower level (Fig. 6) (17). In contrast, mCOUP-TFII is highly expressed in the salivary gland, lung, esophagus, stomach, pancreas primordium, mesonephros, prostate, and kidney and at lower levels in testes, ovary, retina, limb bud, skin, and inner ear, while mCOUP-TFI, with the exception of salivary gland, is expressed at much lower levels (Fig. 6) (17). In general, in organs that require mesenchymal and epithelial interactions, COUP-TFs are only expressed in the mesenchymal cells of the developing organs, not in the terminally differentiated epithelium. Thus, we hypothesize that COUP-TFs may be

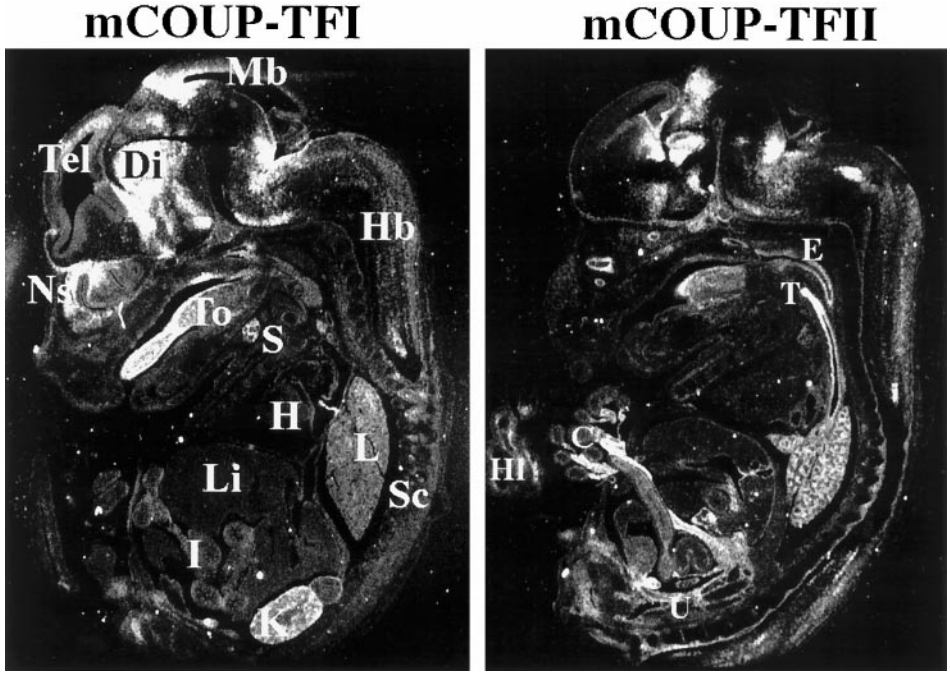

FIG. 6. Expression of mCOUP-TFI and mCOUP-TFII in 15.5-day embryos. The sections are not immediately adjacent sections, but are used to show a representative profile of mCOUP-TFI and II expression. Not shown is expression of mCOUP-TFII in the kidney, adrenal and salivary glands, prostate, tooth, mesonephros, and inner ear. K, Kidney; I, intestines; Li, liver; L, lung; Sc, spinal cord; H, heart; S, salivary gland; To, tongue; Ns, nasal septum; Tel, telencephalon; Di, diencephalon; Mb, midbrain; Hb, hindbrain; E, esophagus; T, trachea; Hi, hindlimb; C, umbilical cord; and U, urogenital sinus.

important signals required for induction of epithelial differentiation.

The expression of COUP-TFs is dramatically reduced in adult mice (81). Both COUP-TFI and II display restricted and distinct expression patterns in the adult brain (81). COUP-TFI is expressed in the rostral and caudal part of the mouse brain, while COUP-TFII is expressed in the middle part of the brain. Higher expression levels of COUP-TFI are detected in the olfactory nucleus, the neocortex, and the dentate gyrus and in various regions of the hippocampus and cerebellum. In contrast, COUP-TFII is detected in the reticular and thalamic nuclei as well as the amygdaloid and the arachnoid membrane. Both factors are also expressed at low levels in many other different regions (81).

V. Physiological Function of COUP-TFs

The expression patterns of COUP-TFs during embryonic development of vertebrates and invertebrates suggest that COUP-TFs may be important for diencephalic neuromere and hindbrain rhombomere segmentation and eye development. Also, the biochemical and *in vitro* studies implicate COUP-TFs as negative regulators of retinoid- and thyroid hormone-signaling pathways. Finally, regulation studies indicate that COUP-TFs are themselves targets of retinoic acid signaling. Therefore, it is important to understand the physiological function of COUP-TFs during development. For these reasons, studies involving ectopic expression and loss of function have been initiated in various laboratories to address these questions (24, 26, 82, 83).

A. Ectopic expression of svp in Drosophila

It is known that svp in *Drosophila* is required to specify photoreceptor subtype in the development of the compound eye, preventing photoreceptors, R1/R3/R4/R6, from adopting R7 cell fate (24, 83). Ectopic expression of svp in cone cells converts the cone cells to neuronal cells, and ectopic expression in other photoreceptor subtypes maintains the neuronal characteristics but loses the specific subtype identity (24). These results suggest distinct processes are required for achieving neural and subtype identities and that svp plays a role in the determination of both processes. Therefore, svp acts as a cell fate switch, and the specific phenotype depends on the developmental stage of the ommatidium at the time of svp expression (83). Molecular and genetic studies suggest that *ras* signaling is required for svp activity (84). However, the detailed mechanism has yet to be defined.

B. Ectopic expression of COUP-TFI in Xenopus embryos

Misexpression of COUP-TFI dramatically affected early *Xenopus* development (82). Overexpression of COUP-TFI in the two-cell *Xenopus* embryos resulted in anterior truncation at the tadpole stage. At four-cell embryos, overexpression of COUP-TFI in the dorsal half, but not the ventral half, of the embryos led to alterations in anterior development, including truncation of head structure, loss of eyes, deletion of the cement gland, and malformation of brain structure in a large percentage of tadpoles. The abnormal early development might result from perturbation of transcription of anterior early genes because expression of the anterior neural mark-

ers, EN-2 and Krox-20, and the anterior neural crest markers, XDLL-1, XAP-2, and Xtwi, were all greatly reduced when COUP-TFI was overexpressed (82). Whether ectopic COUP-TFI expression will cause similar anterior head developmental defects in higher vertebrates that mimic those observed in *Xenopus* has yet to be defined.

C. Loss of function of COUP-TFI in mice

We have recently generated null mutants of COUP-TFI using homologous recombination technology. The homozygous mutants died perinatally between 8 and 36 h post birth (Y. Qiu, F. A. Pereira, F. J. DeMayo, J. P. Lydon, S. Y. Tsai, and M.-J. Tsai, unpublished results). The newborn mutant neonates appear highly dehydrated, lack milk in their stomach, and contain air in the intestines. The apparent symptoms resemble the phenotype of the knockout mice of neurotrophins or their receptors, possibly resulting from an inability to feed (85, 86). Since COUP-TFI is highly expressed in the CNS, the nerve ganglia, and the pharyngeal region, we examined the nerve ganglia of the null mutants.

For this purpose, we used an antibody specific for the neurofilament heavy chain to probe for axonal pathways of cranial nerve ganglia. Among all the null mutants that have been examined, 95% of them have asymmetric fusion of the glossopharyngeal (IX) and vagus (X) cranial nerves. In addition, the arborization of axons, as seen by neurofilament staining, is severely affected in the cervical plexus regions (Y. Qiu *et al.*, unpublished results). Therefore, lack of appropriate neuronal function due to the defect in these neurons may impair sensory and motor functions, which affect feeding behavior and result in perinatal death.

Retinoids are known to regulate the expression of Hox genes, which play a major role in pattern formation and bone morphogenesis (87–90). Because COUP-TFI is hypothesized to antagonize retinoid function and its expression is known to be regulated by retinoids, it is of interest to assess whether loss of COUP-TFI function in the null mutants will affect bone formation. We noted that the left or the right exoccipital bone is fused to the basioccipital bone in 98% of the null mutants. This result suggests that COUP-TFI plays a major role in the development of these bones. Similar ossified fusions are also observed in the mutant mice of the double knockout of the RARα1 and RARβ genes (91). Since COUP-TFI has been shown to be regulated by retinoids during differentiation of embryonic carcinoma cells, it is considered as one of the downstream targets of the retinoid-signaling pathways (20, 92). Thus, it is not surprising that mutation of either COUP-TFI or RARs will give rise to some common phenotypes. On the other hand, many of the other defects seen in the RAR double knockouts, including hyoid bone abnormality, renal hypoplasia, etc., are not seen in the COUP-TFI null mutants (91). Whether this is due to functional redundancy of two COUP-TFs or limited convergence of the signaling pathways shared by COUP-TFI and retinoids has yet to be defined.

D. Loss of function of COUP-TFII in mice

Embryonic lethality is seen with COUP-TFII null mutants. Few homozygous COUP-TFII mutants survive past 10 days p.c. The cause of the death has yet to be defined. Since the mutants die very early, it is difficult to assess the role of COUP-TFII during organogenesis or during motor neuron differentiation. The function of COUP-TFII during organogenesis must await a conditional, inducible knockout in the future.

VI. Regulation of the Expression of COUP-TF Genes

A. Regulation of COUP-TF by retinoids

High levels of expression of COUP-TFI and II are shown to be preferentially induced by all-*trans*- and 9-*cis* retinoic acid during retinoid-induced differentiation of P19 EC cells (20). The levels of expression and the kinetics of induction by both ligands are similar, suggesting the induction might be directly modulated by members of the RAR or RXR subfamilies. We have recently demonstrated that induction of COUP-TFII expression is first seen 16 h subsequent to retinoid treatment and reaches its maximum by 30 h. Regulation of COUP-TF gene expression is also shown *in vivo*. Treatment of zebrafish embryos with retinoic acid alters the rhombomere expression of COUP-TFII/svp[40] in the hindbrain (26) and the expression domain in the retina, which is consistent with the *in vitro* results that retinoids modulate COUP-TF expression. At the same time, misexpression of COUP-TFs also disrupts the retinoid-induced differentiation process in cell cultures and changes the anterior neural gene expression and the head development in *Xenopus* embryos (82). Finally, overexpression of COUP-TFI in teratocarcinoma PCC7 cell lines results in blockade of RA-induced neuronal differentiation of these cells (92). Taken together, it is likely that COUP-TFs and retinoids affect one another's function during neurogenesis and eye development.

B. Regulation of COUP-TF by sonic hedgehog (Shh)

COUP-TFs have been shown to be expressed at high levels in motor neurons of the spinal cord (19, 21). We have previously demonstrated that the expression of cCOUP-TFII correlates with the differentiation pattern of motor neurons in the spinal cord (21). In addition, we have shown that signals from the notochord, which induce motor neuron differentiation, also induce cCOUP-TFII expression when the notochord was ectopically transplanted to the dorsal regions of the spinal cord (21). Since Shh is one of the best known morphogens produced by the notochord, which is important for induction of differentiation of the floor plate and motor neurons, we began to ask whether Shh will induce COUP-TFII gene expression. We showed that Shh induced COUP-TFII mRNA synthesis when P19 cells were cocultured with bacterially produced Shh or with conditioned medium from COS-1 cells expressing sonic hedgehog (G. Krishnan, S. Y. Tsai, and M.-J Tsai, unpublished observation). We further delineated the regions in the promoter of mCOUP-TFII that are responsible for conferring Shh induction. The Shh induction is completely blocked by agents that activate cAMP signaling pathways, consistent with the accumulated evidence that cAMP antagonizes Shh function during limb

development in vertebrates and eye development in *Drosophila* (93, 94).

VII. Perspectives

Lethalities have been observed with mutation of svp in *Drosophila* and with mutation in either COUP-TFI or COUP-TFII in mice (Ref. 24 and Y. Qiu *et al.,* unpublished results). These results strongly indicate that COUP-TFs play a vital functional role in the development of vertebrates and flies. Furthermore, ectopic expression of COUP-TFs in flies and in frogs suggests that COUP-TFs are important for neural and eye development. The defects observed in the null mutants of COUP-TFs in mice support the notion that COUP-TFs are evolutionarily and functionally conserved and are important for neurogenesis during development.

The perinatal lethality caused by the COUP-TFI knockout in mice was not entirely anticipated, inasmuch as the expression patterns of COUP-TFI and COUP-TFII overlap in many different regions, particularly in the CNS during early development. For the same reasons, it is possible that the subtle defective phenotypes observed in COUP-TFI null mutants are contributed by the functional redundancy of the two factors and their overlapping expression patterns. Whether the double knockout of COUP-TFI and II will produce much more severe phenotypes must await future investigation.

The cranial nerve fusion defects detected in the COUP-TFI knockout mice are consistent with the fact that the expression of COUP-TFI mimics the migration patterns of the neural crest cells during early embryonic development. Although COUP-TFI is expressed highest in rhombomeres 2 and 4 of the hindbrain, little or no defects are seen with cranial nerves derived from these two rhombomeres. Perhaps the high expression of COUP-TFII in these regions can compensate for the loss of the COUP-TFI. It will be interesting to use organ cultures from the null mutants to assess whether the mutant cells will differentiate to the proper lineage or whether they will assume a different cell fate. Also, it will be interesting to examine whether other marker genes, which are expressed in these rhombomeres, are affected by the loss of COUP-TFI in the null mutants. These results should permit the identification of putative genes that are downstream of COUP-TFs. Also it is interesting to define what is COUP-TF's role in the formation of ganglia.

The problems associated with arborization and varicosity in the axons of the COUP-TFI null mutants are particularly intriguing. It will be interesting to study whether the axon guidance cues are affected in the COUP-TFI knockout or whether the receptors for the guidance signals are missing in the cell body. It is possible that the loss of COUP-TFI interferes with the proper timing of the expression of the cues or the receptors, thereby leading to the inappropriate migration of neurons and/or projection of the nerve fibers.

The fusion of exoccipital and basioccipital bones in the COUP-TFI null mutants is also seen in the double knockout mutants of the RARα and RARβ receptors. In addition, some of the cervical bones are ossified inappropriately. These results reaffirm the concept that COUP-TFs are involved in the retinoid-signaling pathways. Whether the same phenotypes are consequences of COUP-TFI being regulated by retinoic acid or COUP-TFs' misexpression interfering with the retinoid-signaling pathways has yet to be defined. Since retinoic acids have been implicated in the regulation of many Hox genes, it will also be of interest to determine whether the expression of some Hox genes are altered, leading to the abnormal fusion and ossification in the COUP-TFI knockout mice.

The early lethality of COUP-TFII null mutants does not permit us to examine the functional role of COUP-TFII in modulation of motor neuron differentiation in the spinal cord, nor does it allow us to properly investigate the possible role of COUP-TFII in organogenesis of tissues that require epithelial and mesenchymal interactions. Therefore, it will be necessary to generate an inducible knockout of the COUP-TFII gene so that we may begin to assess its role during organogenesis and during motor neuron differentiation and maturation.

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