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COUP-TF gene: a structure unique for the steroid/thyroid receptor superfamily

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ABSTRACT

Two different genomic genes for the COUP-transcription factor, COUP-TF I and COUP-TF II, have been isolated from a human cosmid genomic library using a [^{32}P]-labeled cDNA probe. Data obtained from Southern blot analysis of these cosmid clones indicated that two closely related genes exist in the human genome and have a similar genomic organization. The genes are similar in the hormone and DNA binding domains but diverge from one another in the N-terminal region. Using DNA sequencing and polymerase chain reaction (PCR) techniques we have determined that the structure of COUP-TF I consists only of three exons and two introns. Surprisingly, both zinc fingers (i.e., F1 and F2) are located in the first exon. Therefore, COUP-TF I is unique among the members of the steroid/thyroid hormone receptor superfamily which have been described to date.

INTRODUCTION

The COUP-TF (Chicken Ovalbumin Upstream Promoter Transcription Factor) promotes transcription of the chicken ovalbumin gene (16,19). COUP-TF has been purified from human HeLa cells (23) and chicken oviduct (1), and subsequently cloned from a human cDNA library (24). Sequence analysis indicates that it is a member of the steroid/thyroid hormone receptor superfamily.

COUP-TF was found to be expressed in A431 squamous carcinoma cells (14), hamster insulinoma cells (8) and human embryo fibroblasts, lung, brain, kidney, liver and human placenta at term (14). COUP-TF binding sites have been identified in the promoter regions of the chicken ovalbumin gene, rat insulin, apolipoprotein VLDLII, apolipoprotein A1 and POMC genes (4,8,9,10,16,26). Deletion or mutation of this sequence drastically alters the corresponding promoter activity. All these results suggest that COUP-TF plays a central role in the regulation of several important genes.

Interestingly, a Drosopohila protein (encoded by the seven-up gene, svp) shared a 75% homology with human COUP-TF. The homologies between these two proteins in the functional domains are strikingly high, 94% in the DNA binding domain and 93% in the hormone binding domain. Rubin and coworkers (15) demonstrated that the svp gene was expressed in the photoreceptor cell precursors R1, R3, R4 and R6 during Drosophila eye development. In the absence of this gene, these four photoreceptor precursors turned into another cell type, photoreceptor R7. Therefore, the svp gene controlled photoreceptor differentiation during neuronal development (15).

Given the diversity of species and tissue types in which COUP-TF or COUP-TF-like proteins exist, we were interested in determining the genomic organization of the COUP-TF gene. We report here on our findings that two COUP-TF genes are present in the human genome and that they have a unique genomic organization with respect to their exon/intron and zinc finger arrangements when compared to other members of the steroid/thyroid hormone receptor superfamily.

MATERIALS AND METHODS

Plasmid Library Screening

A human genomic cosmid library (3) was screened by standard methods (11). Briefly, nitrocellulose filters with immobilized colonies were prehybridized at 68°C, for 6 hr in 6×SSC containing 2 mM EDTA and 0.25% (wt/vol) nonfat dry milk, then hybridized with a ^{32}P-labeled 700 bp DNA fragment (containing the COUP-TF N-terminal region, DNA binding domain and a portion of region 2) under the same condition for 16 hr. The filters were washed twice with 2×SSC, 0.5% SDS for 1 hr at room temperature. The filters were washed sequentially with 1×SSC and 0.5% SDS for 1 hr at room temperature and 0.2×SSC, 0.5% SDS at 68°C for 1 hr before autoradiography.

Southern Blot Analysis of the Cosmid Clones

Six clones were isolated from the human cosmid library and digested with restriction enzyme EcoRI. Southern blot analysis (22) was employed to identify the DNA fragments containing the COUP-TF sequence using probes representing different parts of the COUP-TF cDNA. DNA fragment probes were ^{32}P-labelled by the nick-translated method or the random priming method (12). Oligoprimers were ^{32}P-labeled by T4 polynucleotide kinase (12).

Southern Blot Analysis of Human Genomic DNA

Ten μg of human genomic DNA was digested with EcoRI enzyme and subjected to electrophoresis on a 0.6% agarose gel. The separated DNA fragments were then transferred to nitrocellulose

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filters. The [^{32}P]-700 bp probe was then used for genomic blotting analysis (12).

DNA Sequencing

Restriction fragments (12 kb, 2.4 kb, 9 kb; 7.2 kb, 2.2 kb Eco RI DNA fragments) were subcloned into pGEM-7Z(+) for DNA sequencing by the chain termination method (20). A 2 kb PstI DNA fragment derived from the 12 kb DNA fragment was also subcloned into pGEM-5Z(+) for sequencing. Thirteen oligoprimers (1a, 1b; 2a, 2b; 3a, 3b; 4a, 4b; 5a, 5b; 6a, 6b; 7a, 7b; 8; 9) complementary to COUP-TF cDNA sequence were designed to cover the entire gene and to obtain the sequence information across predicted intron-exon boundaries.

Polymerase Chain Reaction (PCR)

Cosmid clones were linearized and analyzed by PCR with seven oligoprimer sets (1a, 1b; 2a, 2b; 3a, 3b; 4a, 4b; 5a, 5b; 6a, 6b; 7a, 7b) according to manufacturer's instructions (Perkin-Elmer/Cetus). 0.1 μg of linearized cosmid clone was amplified by PCR with a 4 min extension at 70°C, a 1-min denaturation at 95°C, and a 2-min primer annealing at 48°C for 25 cycles.

RESULTS

Human Cosmid Genomic Library Screening

Six cosmid clones (HC-1, HC-2, HC-3, HC-5, HC-6 and HC-8) having the strongest signals were isolated from a human cosmid genomic library with the (^{32}P)-700 bp probe (Fig. 1A). Secondary and tertiary screenings were performed to confirm the identity of these clones. Other colonies, which reacted only weakly with the probe, may represent more distantly related genes.

Southern Blot Analysis of the Cosmid Clones

Probes covering different portions of human COUP-TF cDNA are displayed in Fig. 1A. EcoRI restriction enzyme analysis showed that clones HC-1, 3 and 5 had overlapping DNA fragments (Fig. 2A). By Southern blot analysis, 12 kb, 2.4 kb and 9 kb DNA fragments were recognized by the COUP-TF cDNA probes in the cosmid clones HC-1, 3 and 5. The 12 kb EcoRI DNA fragment was recognized by oligoprobes # 171 and # 169 (Figs. 2G, 2H), as well as by 176 bp and 700 bp DNA probes (Figs. 2B and 2C). These results indicated that the 12 kb DNA fragment consisted of the COUP-TF 5′ untranslated region, N-terminal region and DNA binding domain. The 2.4 kb DNA fragment was detected by 700 bp and 303 bp DNA fragments (Figs. 2C and 2D). This fragment contained the hinge region, region 2 and region 3. Regions 2 and 3 share significant similarity among the members of the steroid/thyroid hormone receptor superfamily (24). The 9 kb DNA fragment was recognized by 303 bp and 160 bp DNA fragments (Figs. 2D, 2E and 2F). Therefore, the 9 kb DNA fragment had the 3′ coding and the 3′ untranslated regions of the COUP-TF.

Cosmid clones HC-2, 6 and 8 also showed overlapping DNA fragments (Fig. 2A) after EcoRI enzyme digestion. However, only 7.2 kb and 2.2 kb DNA fragments were detected by the COUP-TF cDNA probes. The 7.2 kb EcoRI DNA fragment from cosmid clones HC-2, 6 and 8 was recognized by 700 bp and 303 bp DNA probes (Figs. 2C and 2D). The 2.2 kb EcoRI DNA fragment was detected by 278 bp and 160 bp DNA probes (Figs. 2E and 2F). The above results suggest that cosmid clones HC-1, 3, 5 represent one gene for COUP-TF (termed COUP-TF I) and

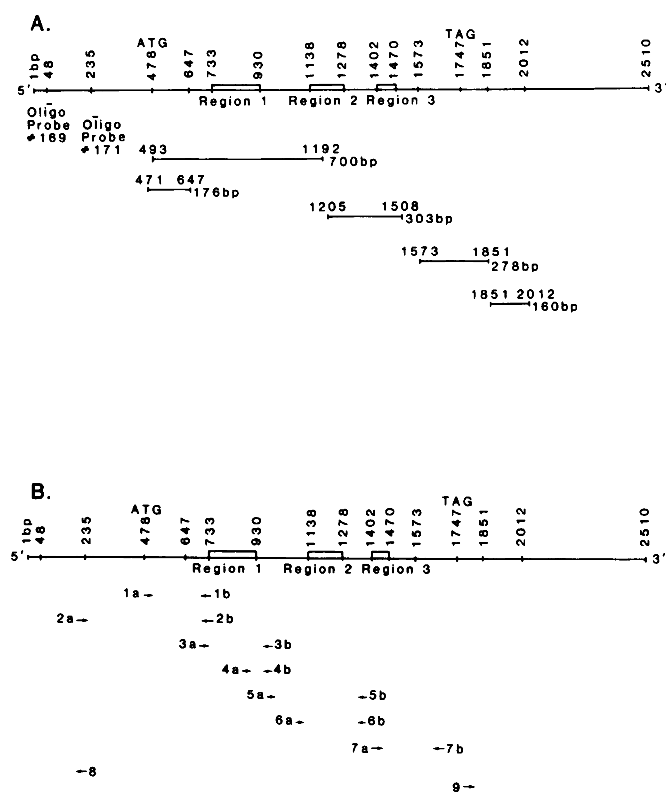

Figure 1. The organization of the COUP-TF cDNA. Panel A displays the DNA probes used for library screening and Southern blot analysis. The 5′-untranslated region starts from position 1 to position 477. The N-terminal region starts from position 478 to position 732. Region 1 represents the DNA binding domain. Regions 2 and 3 are the highly conserved regions in the hormone binding domain. ATG is the translation start site. TAG is the termination codon. Panel B illustrates the thirteen oligoprimers (1a/1b, 2a/2b, 3a/3b, 4a/4b, 5a/5b, 6a/6b, 7a/7b, 8, 9) used for DNA sequencing and polymerase chain reaction. Arrows indicate the directions of the oligoprimers for DNA sequencing and PCR amplification. Primer sets 1a/1b (1a is located at position 481 while 1b is located at position 748), 2a/2b (2a is located at position 205 while 2b is located at position 748), 3a/3b (3a is located at position 704 while 3b is located at position 998), 4a/4b (4a is located at position 881 while 4b is located at position 998), 5a/5b is located at position 983 while 5b is located at position 1380), 6a/6b (6a is located at position 1088 while 6b is located at position 1380, 7a/7b (7a is located at position 1417 while 7b is located at position 1690). Primer 8 is located at position 235. Primer 9 is located at position 1783.

cosmid clones HC-2, 6, 8 represent a second COUP-TF gene (termed COUP-TF II). It is worth noting that probes (oligoprimers # 171 and # 169) directed against the 5′ untranslated region and the probe (176 bp DNA) directed against the N-terminal region of COUP-TF cDNA hybridized only weakly to DNA fragments in clones HC-2, 6 and 8. This suggests that COUP-TF II diverged from COUP-TF I at some point 5′ to the DNA binding domain.

Genomic Blot Confirms Two COUP-TF Genes

By Southern blot analysis of the six cosmid clones, we demonstrated that COUP-TF has at least two closely related genomic genes. To confirm this finding, we carried out Southern analysis of the human genomic DNA. When the separated EcoRI DNA fragments were probed with the 700 bp probe, we detected 12 kb, 2.4 kb and 7.2 kb DNA fragments (Fig. 2I). These DNA fragments corresponded to the 12 kb and 2.4 kb DNA fragments in cosmid clones HC-1, 3, 5 and to the 7.2 kb DNA fragment

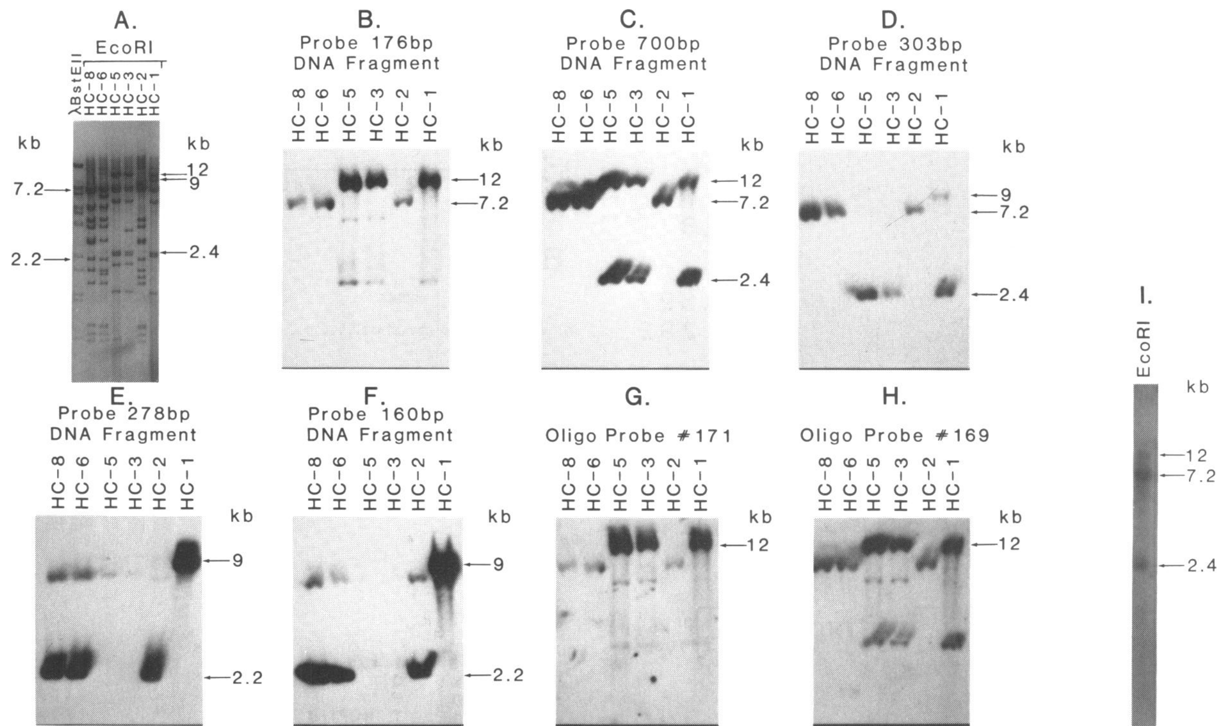

Figure 2. Southern Blot Analysis of the Human Cosmid Clones and the Human Genomic DNA. Cosmid clones HC-1, 2, 3, 5, 6 and 8 were subjected to EcoRI digestion, electrophoreses on a 0.8% agarose gel and then transferred to nitrocellulose filters. The resulting digestion patterns are shown in Figure 2A. The Southern blot analysis of these clones using specific oligoprobes (see Figure 1A) are shown in Figures 2B–2H. Human genomic DNA (see Methods) was also digested with EcoRI and subjected to Southern blot analysis (Figure 2I).

in cosmid clones HC-2, 6, 8 which were all recognized by the same probe (Fig. 2C). Thus the genomic blot data confirm that two very similar COUP-TF genes exist in the human genome.

COUP-TF I and II Have Similar Genomic Organization

Seven sets of oligoprimers (1a/1b; 2a/2b; 3a/3b; 4a/4b; 5a/5b; 6a/6b; 7a/7b) were used to analyze the genomic organization of COUP-TF I and COUP-TF II genes (Fig. 1B). When the primer set 1a/1b was used, a 267 bp PCR DNA fragment was generated from COUP-TF I (Fig. 3A, lane 1). Primer 1b is located at position 748 (i.e., within the DNA binding domain) and primer 1a is located at position 481 (i.e., 4 nucleotides away from the ATG translation start site). The appearance of this 267 bp PCR DNA fragment, which is identical to the distance between these two primers on the cDNA, indicated that no intron existed between the N-terminal region and the DNA binding domain of COUP-TF I. Since a similar size DNA fragment was amplified by the 1a/1b primer set from COUP-TF II (Fig. 3A, lane 2), the two genes must have similar genomic structures between positions 481 and 748.

When primer set 2a/2b was used for PCR amplification, a major 543 bp DNA fragment was obtained from COUP-TF I (Fig. 3A, lane 3); however, several DNA fragments were generated from COUP-TF II after PCR amplification with this primer set (Fig. 3A, lane 4).

Primer set 3a/3b generated a 2.45 kb PCR DNA fragment from the COUP-TF I template (Fig. 3A, lane 5). Since the distance between primers 3a and 3b in COUP-TF cDNA is only 294 bp, an intron must be present within this area. Primer set 4a/4b also generated a large (approximately 2.4 kb) DNA fragment (Fig. 3A, lane 6). Therefore both of these primer sets demonstrated that a 2.4 kb intron was located between the DNA binding domain and hinge region in COUP-TF I. Primer set 3a/3b did however generate several small DNA fragments from COUP-TF II (Fig. 3A, lane 7) and a 0.8 kb DNA fragment was detected by a [^{32}P]-oligoprobe (data not shown) directed against the DNA binding domain. Thus the intron between the DNA binding domain and the hinge region of COUP-TF II (approximately 0.5 kb) is smaller than the one ($\sim$2.4 kb) in COUP-TF I.

Primer sets 5a/5b and 6a/6b amplified DNA fragments with the sizes of 397 bp and 292 bp respectively from both COUP-TF I and COUP-TF II DNA templates (Fig. 3A, lanes 8, 9, 10 and 11). Since the sizes of these DNA fragments were identical to the distances between each individual primer set, we conclude that no intron is present between positions 983 and 1380 (Fig. 1B). These data also indicate that these two genes are very similar or identical in this region.

We next amplified the COUP-TF II template using the primer set 7a/7b (see Fig. 1B). This primer set generated a 3.3 kb PCR DNA fragment (Fig. 3A, lane 12) which most likely represents an intron between positions 1417 and 1690. Summary of these PCR results is present in Figure 3B.

COUP-TF Genomic Organization and Exon-Intron Boundaries

The relative locations of thirteen oligoprimers (1a, 1b; 2a, 2b; 3a, 3b; 4a, 4b; 5a, 5b; 6a, 6b; 7a, 7b; 8; 9) complementary to the COUP-TF cDNA sequence are shown on Fig. 1B. The oligoprimers were used to directly sequence the genomic COUP-TF subclones. A counterpart to the entire cDNA sequence was

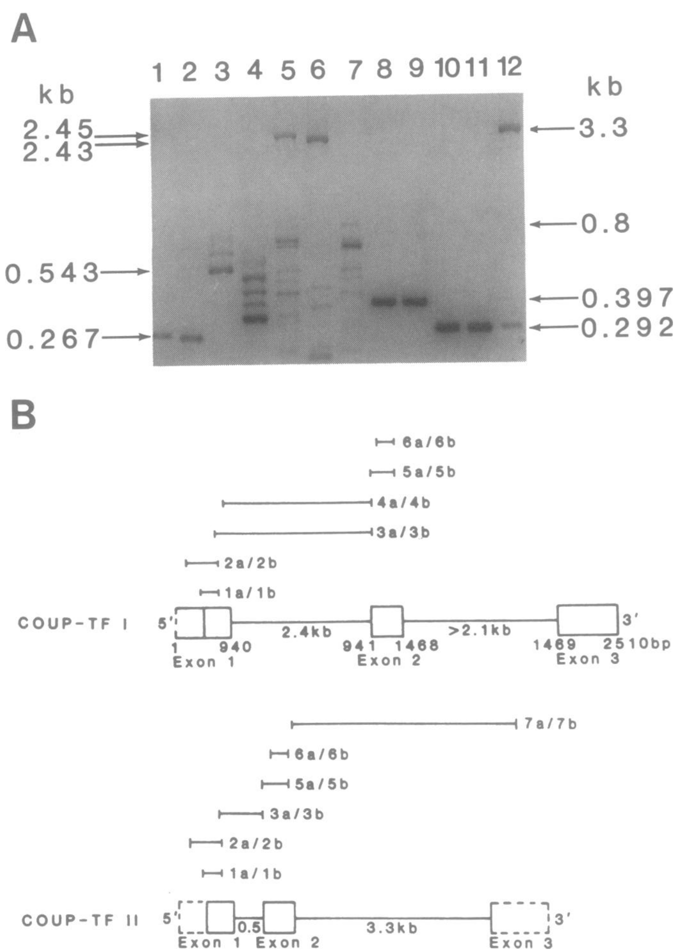

Figure 3. Genomic Organization of COUP-TF I and COUP-TF II Genes. Using seven sets of oligoprimers, COUP-TF I and COUP-TF II cosmid clones were amplified by polymerase chain reaction. The resulting DNA fragments are shown in Figure 3A. Lane 1: 267 bp DNA fragment generated by primer set 1a/1b from COUP-TF I (267 bp, 1a/1b, I); Lane 2: ~267 bp, 1a/1b, II; Lane 3: 543 bp, 2a/2b, I; Lane 4: several DNA fragments, 2a/2b, II; Lane 5: 2.45 kb, 3a/3b, I; Lane 6: 2.43 kb, 4a/4b, I; Lane 7: several fragments, 3a/3b, II: Lane 8: 397 bp, 5a/5b, I; Lane 9: 397 bp, 5a/5b, II; Lane 10: 292 bp, 6a/6b, I; Lane 11: 292 bp, 6a/6b, II; Lane 12: 3.3 kb, 7a/7b, II. Figure 3B illustrates the orientation of these PCR generated DNA fragments in relation to the individual genomic organization.

identified in the genomic clones. From the DNA sequence data, we concluded that COUP-TF I is the genomic counterpart of COUP-TF cDNA. The entire coding sequence for COUP-TF I is divided into only three exons (Fig. 4A). The DNA sequence located between the ATG translation start site (position 478) and position 940 resides in exon 1. This 463 bp coding sequence contains the COUP-TF N-terminal region and the DNA binding domain. The DNA binding domain encodes two zinc finger structures (abbreviated F1, F2). Furthermore, a 477 bp DNA sequence located 5′ to the ATG start site of COUP-TF cDNA was found in exon 1. Region 2 (from position 1138 to 1278) and region 3 (from position 1402 to 1470) in the hormone binding domain are located in exon 2. The 3′ end coding sequence adjacent to region 3 and the 3′ untranslated portion of the mRNA sequence are located in exon 3.

The DNA sequences in the exon-intron boundaries which follow the AG...GT splicing rule (21) are shown in Figure 4B. At position 940, an intron interrupts the amino acid codon for

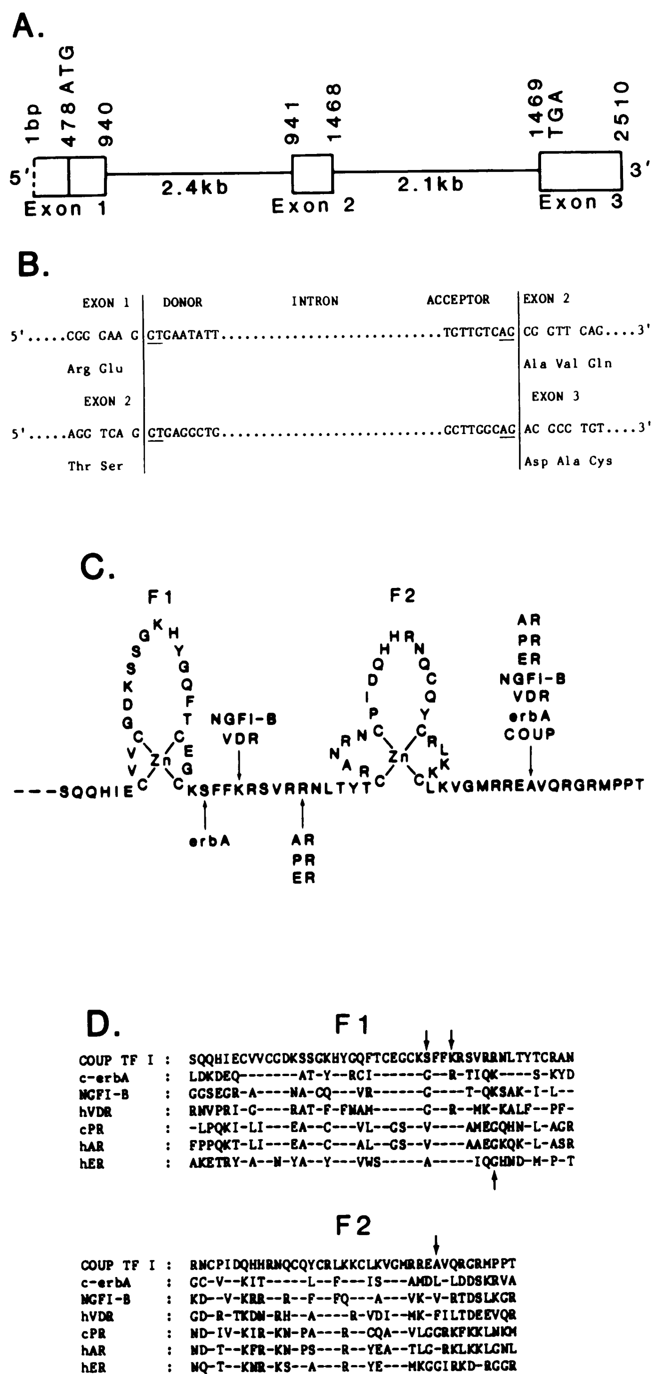

Figure 4. COUP-TF I Exon-Intron Boundaries. From the PCR results and the DNA sequencing data, the coding sequence for COUP-TF was determined to have three exons and two introns (see Panel A). Exon 1 (from position 478 to position 940) includes the translation start site and the whole DNA binding domain sequence. The 477 bp 5′ untranslated region is also located at exon 1. Exon 2 (from position 941 to position 1368) contains the conserved regions 2 and 3 in the ligand binding domain. From position 1469 to position 2510 represents exon 3 covering the sequence after region 3 and the 3′ end untranslated region. Intron 1 is 2.4 kb long and intron 2 is larger than 2.1 kb. The DNA sequences in the exon-intron boundaries of COUP-TF I are shown in Figure 4B. The sequence of COUP-TF I DNA binding domain and its zinc finger structure are shown in Figure 4C. The splice sites (indicated by arrows) in the DNA binding domain for the available members in the steroid/thyroid hormone receptor superfamily are presented in Figure 4C. The amino acid sequences near and at the splice sites (indicated by arrows) are shown in Figure 4D.

alanine (GCG) into G...intron...CG. CG represent position 941 and 942 which lie at the beginning of exon 2. The splice site at the end of exon 2 disrupts the aspartic acid codon (GAC) into G...intron...AC. Again, AC represent positions 1469 and 1470 which lie at the beginning of exon 3. Exon 3 then extends to position 2510.

Figure 4C summarizes the splice sites in the zinc finger region (i.e. DNA binding domain) for all the available steroid/thyroid hormone receptor family members. The receptors for human androgen (hAR), chicken progesterone (cPR), human estrogen (hER), chicken c-erbA (c-erbA), human vitamin D3 (hVDR), as well as rat nerve growth factor induced gene B (NGFI-B) and COUP-TF I all have the same splice site located 3′ to the F2 coding exon. This highly conserved splice site is located 9 amino acids away from the last cysteine residue of the second zinc finger. The individual splice sites for each of the aforementioned genes (5,6,7,18,25,27) are identified in Fig. 4D.

The splice sites between the F1 and F2 regions vary among the members of this superfamily. hAR, cPR, and hER, from the steroid hormone receptor group (18), share the same splice site which is 10 amino acids 3′ to the last cysteine of first zinc finger. hTR, cTR and hRAR from the thyroid receptor group, share another splice site at position 2 amino acids from the same cysteine. The splice site for rat NGFI-B and hVDR is 3 amino acids further downstream. Interestingly, COUP-TF I is the only member within this superfamily with no splice site between F1 and F2 (Fig. 4C).

Comparison of Genomic Organization Among Members of the Steroid/Thyroid Hormone Receptor Superfamily

The genomic organization of several members in this superfamily is shown in Figure 5. With the exception of COUP-TF I and II, similar patterns of genomic organization are readily apparent. Examination reveals that: (1) the DNA binding domains are located in two exons separated by introns of various sizes, (2) the hormone binding domains are located in 4–6 exons and (3) the N-terminal regions are located in one exon (i.e. steroid hormone receptors, cPR, hAR, hER and rat NGFI-B). In contrast, COUP-TF I reveals a single exon containing both zinc finger regions, F1 and F2, as well as the N-terminal region. In addition, the hormone binding region is located in only two exons. Therefore, the organization of the COUP-TF gene is unique among the members of this superfamily.

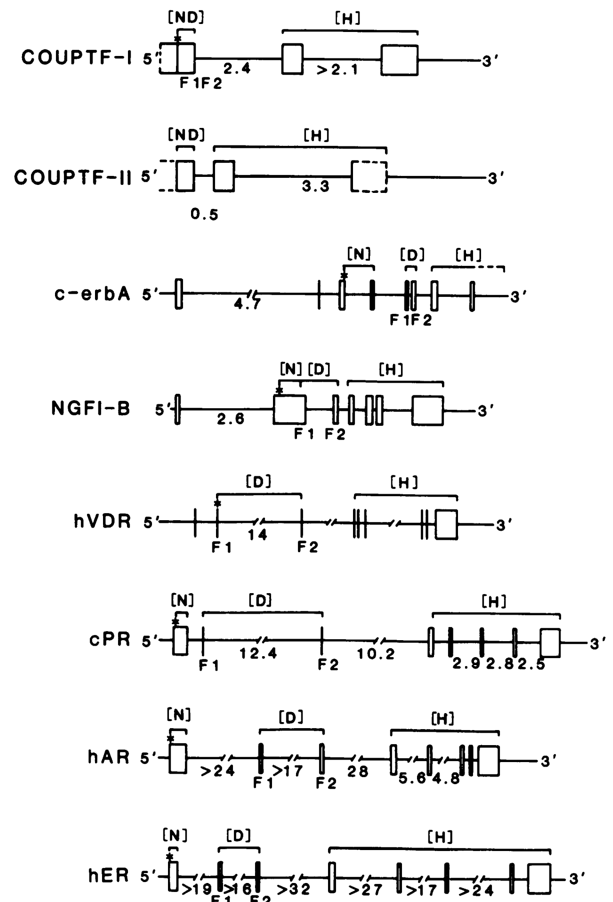

Figure 5. Comparison of the Genomic Organization among the Members in the Steroid/Thyroid Hormone Receptor Superfamily. The members for comparison include human estrogen receptor (hER), human androgen receptor (hAR), chicken progesterone receptor (cPR), human vitamin D3 receptor (hVDR), rat NGFI-B, chicken c-erbA (c-erbA), COUP-TF I and COUP-TF II. * represents ATG-the translation start site. Numbers indicate introns lengths in kilobases. [N] stands for N-terminal region, [D] for DNA binding domain and [H] for hormone binding domain.

DISCUSSION

Using a [^{32}P]-labeled 700 bp human cDNA probe, we have isolated two genes, COUP-TF I and COUP-TF II, from a human cosmid genomic library. A genomic blot analysis also confirms the existence of COUP-TF I and COUP-TF II in the human genome. We cannot rule out the possibility, however, that COUP-TF could be a small family containing additional more distantly related members. APR-1, a recently identified human regulatory protein factor for apolipoprotein A1 gene, shares extremely high sequence homology in the DNA binding and hormone binding domains with those of COUP-TF (9,10). Another gene, ear-2, also shows sequence homology (14). Thus, the COUP-TF subfamily consists of at least two and possibly four or more members.

Our PCR results suggest that COUP-TF I and COUP-TF II are very similar in their genomic organization. Most of PCR primer sets complementary to COUP-TF cDNA were able to amplify both genes and generate similar size DNA fragments. However, primers complementary to the 5′ untranslated region and the N-terminal region of the protein generated nonspecific DNA fragments in COUP-TF II. Thus, the two genes diverged from a region located 5′ to the COUP-TF DNA binding domain. The Southern blot analysis also agreed with this interpretation since the 176 bp probe (directed to the N-terminal region) and the oligoprobes (directed to the 5′ untranslated region) hybridized poorly with COUP-TF II cosmid clones HC-2, 6, 8.

The existence of two genes raises an interesting question as to their relative functions. We know that COUP-TF I is expressed in different human embryo tissues and different tumor cell lines (see Introduction). Given the differences in the N-terminal sequences between these two genes (see Figs. 2 and 3), along with the fact that N-terminal regions may be involved in specific transcriptional activation of some target genes during development (28), COUP-TF II may be expressed differently than COUP-TF I. Examples of differential expression among multigene members of the steroid/thyroid hormone receptor family include the retinoic acid receptor (RAR) genes which are expressed either constitutively in all tissues (i.e. alpha form), or have differential tissue specific expression (i.e. beta form), or are expressed predominately in skin (i.e. gamma form); another COUP-TF

related gene ear-2 (14), has been shown to be expressed differently than COUP-TF I.

Our DNA sequencing data and PCR results indicate that the COUP-TF I (the genomic counterpart of COUP-TF cDNA) consists of three exons and two introns. The absence of an intron between the two zinc finger regions (F1 and F2) in the DNA binding domain is notable since intron positions in many genes (i.e., globin, alphafetoprotein/albumin, ovalbumin as well as steroid/thyroid hormone (SH/TH) receptor members) are precisely conserved (2,18). One view on the origin of introns was that they have been present always in the genome and were therefore incorporated in the assembly of the first gene. Since COUP-TF shares a high sequence homology with other members of the SH/TH family (which again because of sequence homologies are presumed to be derived from a common ancestral gene), the COUP-TF F1/F2 intron was probably present early in its evolution but was lost at a later time. Examples of such intron loss include the rat pre-proinsulin gene and the chicken trisphosphate isomerase gene (13,17).

It remains an open question as to whether the COUP-TF gene may have diverged from the primordial SH/TH gene at an earlier time than the other members of this superfamily. Mlodzik *et al.* (15) have now found a COUP-TF counterpart (svp) in Drosophila which shares a 94% amino acid sequence homology in the DNA binding domain and a 93% amino acid sequence homology in the hormone binding domain. At this time, no other counterparts with high sequence homology to human SH/TH members have been identified in the Drosophila genome. It would be of interest to examine the genomic organization of the Drosophila COUP-TF counterpart to determine whether the F1/F2 intron is present.

All members of steroid/thyroid hormone receptor superfamily, including COUP-TF I, contain a highly conserved splice site at the 3′ end of the F2 exon. The splice sites at the 5′ end of F2 exon vary among the different members (see Figs. 4B and 4C). Since the position of the splice site between two fingers in the steroid hormone receptors for hER and cPR is different from that in the thyroid/retinoic acid receptors, Ponglikitmongkol *et al.* (18) suggested that the steroid hormone receptors belonged to a specific group which diverged from the thyroid hormone receptor group early in the evolutionary time frame. Based on the splice sites between F1/F2 (Figs. 4C and 4D), members of this superfamily can now be divided into four receptor groups: steroid hormone receptors, thyroid hormone receptors, COUP-TF, and NGFI-B/hVDR which diverged from each other very early during the evolutionary process.

Another unique aspect of COUP-TF I is that region 2 and the majority of region 3 (both within the hormone binding domain) are located in one exon (exon 2). Regions 2 and 3 have been found to be highly conserved areas in the hormone binding domain indicating that they serve a requisite function (24). In addition, a small portion of region 3, plus the 3′ coding sequence, and the 3′ untranslated region are located in one exon (exon 3). In other members of this superfamily (i.e. hER, cPR, hAR, chicken c-erbA, hVDR), both regions 2 and 3 are divided by introns. The significance of this structural arrangement remains to be determined. In summary, we have demonstrated that two closely related genes, COUP-TF I and COUP-TF II, exist in the human genome. We have shown also that the genomic structure of these two genes is unique in that the DNA binding domain was localized to a single exon (containing both zinc finger regions), and the hormone binding domain was distributed within two exons. Future studies designed to characterize those factors responsible for the constitutive as well as tissue-specific expression of COUP-TF genes should provide a more complete picture of this topical superfamily.

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