Construction and Expression of Human/Bovine $P450_{17\alpha}$ Chimeric Proteins: Evidence for Distinct Tertiary Structures in the Same P450 from Two Different Species†

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ABSTRACT: In the human and bovine adrenal cortex, 17α-hydroxylase ($P450_{17\alpha}$) catalyzes reactions involved in the production of C21-glucocorticoids (17α-hydroxylation) and C19-androgens (17,20-lyase). The bovine and human forms of $P450_{17\alpha}$ share 71% primary sequence identity. Using naturally occurring restriction sites common to cDNAs encoding both human and bovine $P450_{17\alpha}$, we have constructed bovine/human (bovine amino terminus and human carboxy terminus) and human/bovine (human amino terminus and bovine carboxy terminus) cDNAs that have been expressed in COS 1 cells, and the enzymatic properties of the resultant chimeric proteins have been examined. The three bovine/human chimeras studied have 17α-hydroxylase activities intermediate between those of the wild-type bovine and wild-type human enzymes, although the 17,20-lyase activity of these chimeras is significantly lower than that of either of the wild-type enzymes. Surprisingly, the opposite chimeras (those containing a human amino-terminal sequence and a bovine carboxy-terminal sequence) are all virtually inactive, even though they appear to be expressed at normal levels. These results indicate that the folding of $P450_{17\alpha}$ initiated by the bovine amino terminus can accommodate human $P450_{17\alpha}$ sequences of various lengths to produce a relatively normal 17α-hydroxylase having decreased 17,20-lyase activity. On the other hand, folding initiated by the human $P450_{17\alpha}$ amino terminus does not easily accommodate bovine carboxy-terminal sequences to produce a functional enzyme. Presumably this difference arises from the fact that the tertiary structures of the bovine and human forms of $P450_{17\alpha}$ are sufficiently different so that interchanging sequences will not lead to functional enzymes in a predictable fashion.

The compositions of the different gene families within the cytochrome P450 superfamily are quite varied; some gene families such as CYP1, CYP11, CYP17, and CYP21 have very few or only a single member, while others such as CYP2, CYP3, and CYP4 have several to many members and are divided into subfamilies (Nebert et al., 1989). Recent advances in genetic engineering and development of heterologous expression systems have made it possible for investigators to begin to locate specific amino acid segments in P450 primary structures that are essential for particular enzymatic activities. The identification and characterization of cDNAs encoding closely related members of specific P450 subfamilies and the construction of chimeric cDNAs between such subfamily members within a given animal species (Uno & Imai, 1989; Kronbach et al., 1989; Aoyama et al., 1989) or the use of site-directed mutagenesis (Lindberg & Negishi, 1989) based on sequence relatedness between subfamily members has led to important new understanding concerning the functional role of specific regions within the P450 primary structure.

The steroid 17α-hydroxylase ($P450_{17\alpha}$[1]) located in adrenal cortex, testis, ovary, and, in certain species, placenta, is a unique form of P450 in that it catalyzes distinct reactions which can be separated temporally. In the adrenal this enzyme catalyzes the 17α-hydroxylation of pregnenolone and progesterone in the pathway leading from cholesterol to cortisol. In addition, this same enzyme catalyzes the conversion of the C21-steroid, 17α-hydroxypregnenolone, to the C19 steroid, dehydroepiandrosterone, via the 17,20-lyase reaction, thereby producing a precursor for sex hormones. Accordingly, in the adrenal cortex of humans and cows, for example, a certain fraction of the 17α-hydroxypregnenolone is used in glucocorticoid production, the remainder being used for adrenal androgen production. Of course, in other steroidogenic tissues, the enzymes needed for cortisol production ($P450_{C21}$ and $P450_{11\beta}$) are not expressed, and the 17α-hydroxypregnenolone is used only for sex hormone production. In addition, human $P450_{17\alpha}$ catalyzes 16α-hydroxylation of progesterone, while bovine $P450_{17\alpha}$, for all practical purposes, does not. The amino acid sequence identity between human $P450_{17\alpha}$ and bovine $P450_{17\alpha}$ is 71%. We have wondered whether regions of this relatively specific form of P450 catalyzing the 17α-hydroxylation and 17,20-lyase reactions in two different species could be interchanged between species without significantly altering the enzymatic properties of either reaction and whether we might localize specific sequences in human $P450_{17\alpha}$ that are essential for 16α-hydroxylation by construction of bovine/human $P450_{17\alpha}$ chimeric proteins.

To this goal, we have constructed chimeric $P450_{17\alpha}$ cDNAs utilizing naturally occurring common, unique restriction sites

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[1] Abbreviations: $P450_{17\alpha}$, P450XVII, the gene product of the CYP17 gene (Nebert et al., 1989); DMEM, Dulbecco's modified Eagle's medium; GBSS, Gey's balanced salt solution; TE, 10 mM Tris, 1 mM EDTA buffer, pH 7.4; 1×SCC, 0.15 M NaCl, 15 mM sodium citrate; SDS, sodium dodecyl sulfate.

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                .         .         .         .         .         .         .
human   MWELVALLLLTLAYLFWPKRRCPGAKYPKSLLSLPLVGSLPFLPRHGHMHNNFFKLQKKYGPIYSVRMGT   70
        || | |  ||||||||||||    ||||| || ||||||||||||| |  | |||||| ||||||| | |
bovine  MWLLLAVFLLTLAYLFWPKTKHSGAKYPRSLPSLPLVGSLPFLPRRGQQHKNFFKLQEKYGPIYSFRLGS
                .         .      .XbaI     .         .         .         .
human   KTTVIVGHHQLAKEVLIKKGKDFSGRPQMATLDIASNNRKGIAFADSGAHWQLHRRLAMATFALFKDGDQ   140
        ||||  ||||||  ||| |||| |||||  ||||| | | |||||||  || ||||| ||   ||| |||
bovine  KTTVMIGHHQLAREVLLKKGKEFSGRPKVATLDILSDNQKGIAFADHGAQWQLHRKLALNAFALSKDGNL
                .         .         . BstEII  .         .         .         .
human   KLEKIICQEISTLCDMLATHNGQSIDISFPVFVAVTNVISLICFNTSYKNGDPELNVIQNYNEGIIDNLS   210
        ||||||  ||   ||| |||  |  ||  | |   |||| || |||| | ||  ||  |  ||| | ||   ||
bovine  KLEKIINQEANVLCDFLATQHGEAIDLSEPLSLAVTNIISFICFNFSFKNEDPALKAIQNVNDGILEVLS
                .         .         .         .         .         .         .
human   KDSLVDLVPWLKIFPNKTLEKLKSHVKIRNDLLNKILENYKEKFRSDSITNMLDTLMQAKMNSDNGNAGP   280
        |  | |  | |||||  |  ||  |  || ||| |||   | | ||||||  |  | ||| | || ||||
bovine  KEVLLDIFPVLKIFPSKAMEKMKGCVQTRNELLNEILEKCQENFSSDSITNLLHILIQAKVNADNNNAGP
                .         .         .         .         .         .         .
human   DQDSELLSDNHILTTIGDIFGAGVETTTSVVKWTLAFLLHNPQVKKKLYEEIDQNVGFSRTPTISDRNRL   350
        ||||  |||  | | ||||||||||||||||  ||  | |||||  ||     |||  || ||||||||||
bovine  DQDSKLLSNRHMLATIGDIFGAGVETTTSVIKWIVAYLLHHPSLKKRIQDDIDQIIGFNRTPTISDRNRL
                .         .         .         .         .         .         .
human   LLLEATIREVLRLRPVAPMLIPHKANVDSSIGEFAVDKGTEVIINLWALHHNEKEWHQPDQFMPERFLNP   420
         |||||||||||  |||||  ||||||  |||||    ||||  |  |||||||  ||||  ||  |||||||  |
bovine  VLLEATIREVLRIRPVAPTLIPHKAVIDSSIGDLTIDKGTDVVVNLWALHHSEKEWQHPDLFMPERFLDP
                .         .      .SacI     .         .         .         .
human   AGTQLISPSVSYLPFGAGPRSCIGEILARQELFLIMAWLLQRFDLEVPDDGQLPSLEGIPKVVFLIDSFK   490
         |||||||  ||||||||||||  ||  ||||||||  |  |||||  ||  ||||  ||||||    |  |  ||
bovine  TGTQLISPSLSYLPFGAGPRSCVGEMLARQELFLFMSRLLQRFNLEIPDDGKLPSLEGHASLVLQIKPFK
                .
human   VKIKVRQAWREAQAEGST*  509
        |||  |||||  ||||||||
bovine  VKIEVRQAWKEAQAEGSTP
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FIGURE 1: Alignment of the amino acid sequences of the human and bovine forms of $P450_{17\alpha}$ (Fevold et al., 1989; Nelson & Stobel, 1988). The amino acid residues spanning the unique restriction enzyme sites used in the construction of the chimeric molecules are underlined, with the restriction site indicated above the sequence. Identical residues between the two enzymes are indicated with a vertical match line. Amino acid residue numbers are indicated at the right.

within these two cDNAs and have expressed them in COS 1 cells. The enzymatic properties of these chimeric enzymes have been analyzed in situ by using HPLC and resultant protein levels determined by immunoblot analysis. In some cases we find that regions can be interchanged without greatly altering certain activities, but in other cases this is not true. For example, when human $P450_{17\alpha}$ sequences are coupled to the bovine $P450_{17\alpha}$ amino terminus, the resultant 17α-hydroxylase activities are relatively normal, while the opposite, bovine $P450_{17\alpha}$ sequences coupled to the human $P450_{17\alpha}$ amino terminus, tended to produce inactive proteins. The results described herein demonstrate that differences exist in the tertiary structures of bovine and human $P450_{17\alpha}$ such that sequence swapping will not produce a functional protein in a predictable way.

Experimental Procedures

Materials. All bacterial culture media were from Difco; agarose, ultrapure phenol, T4 DNA ligase and ligase buffer, and all restriction enzymes were from Bethesda Research Laboratories; DEAE-dextran [(diethylaminoethyl)dextran], and all ribonucleotide triphosphates, obtained as 100 mM, pH 7.0 solutions, were from Pharmacia. Nitrocellulose filter paper (BA85, 0.45 μm) was from Schleicher and Schuell; Sequenase DNA sequencing kits were from United States Biochemicals; and [α-^{32}P]UTP (3200 Ci/mmol) was from ICN. DMEM (Dulbecco's modified Eagle's medium), GBSS (Gey's balanced salt solution), and all other tissue culture media were from GIBCO; methanol and methylene chloride were of HPLC grade (Burdick and Jackson); [1,2,6,7-^{3}H]progesterone (96 Ci/mmol), [1,2,6,7-^{3}H]-17α-hydroxyprogesterone (63 Ci/mmol), [4,7-^{3}H]pregnenolone, and [7(n)-17α-hydroxypregnenolone were purchased from Amersham and used without further purification.

Plasmid Constructions. Plasmids were cleaved with the appropriate restriction enzyme and fractionated on a 1.5% low melting point agarose (SeaPlaque; FMC Marine Colloids) gel. The desired fragment was excised from the gel, melted at 65 °C in a final volume of 0.4 mL of TE (Maniatis et al., 1982) plus 100 mM NaCl, and extracted two times with 1 volume of TE-saturated phenol (which had been preheated to 65 °C). The extracted fragment was precipitated twice with ethanol, resuspended in TE, and analyzed by agarose gel electrophoresis. Purified vector and insert fragments were ligated together overnight, transformed into HB101 competent cells (Bethesda Research Laboratories), and isolated for restriction enzyme analysis by an alkaline lysis miniprep procedure (Birnboim & Doly, 1979). Plasmids prepared for transfection were purified by cesium chloride–ethidium bromide density gradient centrifugation (Davis et al., 1980) using a Beckman TL-100 ultracentrifuge.

Construction of Chimeric 17α-Hydroxylase cDNA. The nucleotide sequences of the bovine (Zuber et al., 1986a) and human (Bradshaw et al., 1987) forms of adrenal 17α-hydroxylase, and their derived amino acid sequences were aligned (Fevold et al., 1989; Nelson & Stobel, 1988) on the basis of sequence identity and similarity and determined to be 78% and 71% identical at the nucleotide and amino acids levels, respectively. As illustrated in Figure 1, these two protein sequences align very closely, with the bovine form being one amino acid longer due to the presence of a proline residue at the carboxy terminus (although the nucleotide sequence determined for the bovine $P450_{17\alpha}$ gene identified this carboxy-terminal residue as serine; Bhasker et al., 1989).

Chimeric cDNA constructs were generated by using pcD17α-2 (Zuber et al., 1986a), which contains the wild-type bovine $P450_{17\alpha}$ coding sequences, and pcD17αH (Bradshaw et al., 1987), which contains the wild-type human $P450_{17\alpha}$ coding sequences, both in an Okayama–Berg (Okayama & Berg, 1983) expression vector. Sequences were exchanged between the two wild-type cDNAs by using three unique, naturally occurring restriction sites already present in the two

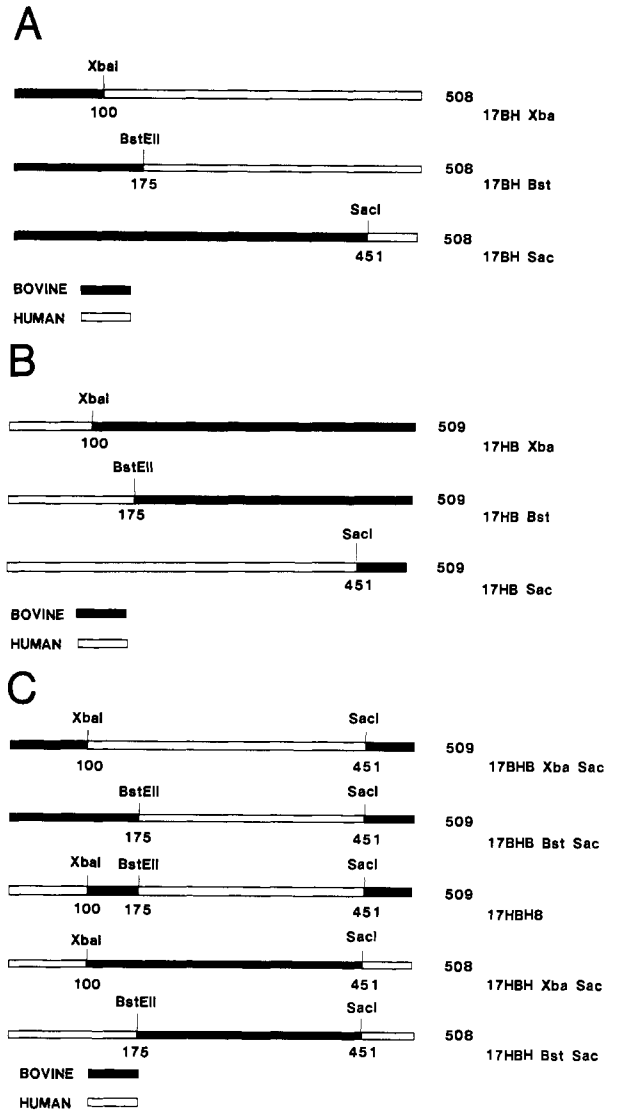

FIGURE 2: Schematic representation of the various chimeric constructs. (A) Group 1: N-Terminal bovine and C-terminal human. (B) Group 2: N-Terminal human and C-terminal bovine. (C) Group 3: N-Terminal and C-terminal of one form with a central segment from the other. The length (in amino acids) and the construct name are indicated at the right of the figure. The restriction site and its central residue number are also indicated.

cDNA sequences (Figure 1) but not present in the vector sequences. An *Xba*I restriction site (TCTAGA) is located at the sequences encoding amino acid residues 100–102 (Thr-Leu-Asp), a *Bst*EII site (GGTNACC) is located at the sequences encoding residues 174–176 (Ala-Val-Thr), and a *Sac*I site (GAGCTC) is located at the sequences encoding residues 450–451 (Glu-Leu).

To generate the two-fragment chimeric cDNA constructs illustrated in Figure 2A,B, pcD17α-2 and pcD17αH were cleaved at the desired restriction site in the cDNA sequences and with either *Acc*I (a unique restriction site located in the prokaryotic vector portion of the plasmid) to exchange a carboxy-terminal segment or *Hin*dIII (a unique site located at the junction of the SV40 early promoter and procaryotic vector sequences) to exchange an amino-terminal segment. The resulting fragments were purified separately from low melting point agarose and ligated together, as described above. These two fragment chimeras were then utilized in the construction of the multiple-fragment chimeras shown in Figure 2C. For example, to construct pcD17BHB *Xba*/*Sac*, the *Hin*dIII–*Xba*I fragment from pcD17α-2 was substituted for the corresponding fragment in pcD17HB *Sac*. The restriction sites used in construction of the chimeric cDNAs (*Xba*I, *Bst*II, or *Sac*I) were found in all cases to be cleaved in the final expression vectors, establishing in all cases that religation had occurred correctly. In addition, in three cases where little or no activity or P450$_{17\alpha}$ protein was detectable (HB *Bst*, HB *Sac*, and HBH *Bst*), the junctions were sequenced approximately 100 nucleotides in each direction to further establish the fidelity of religation.

COS 1 Cell Transfections and Enzymatic Assays. COS 1 cells were grown in DMEM plus 10% iron-supplemented bovine serum and transfected with 5.0 μg of plasmid DNA/mL of transfection medium in 60-mm dishes by using DEAE-dextran as described previously (Zuber et al., 1986b). Radiolabeled steroid substrate (100000 cpm/mL of medium) was added, along with radioinert substrate, to a final concentration of 2.5 μM, 72 h after transfection. Aliquots of media (0.5 mL) were removed at various time intervals and extracted with 5.0 mL of methylene chloride, and the organic phase was evaporated. The residue was dissolved in methanol and subjected to reverse-phase HPLC using a Waters 30-cm μBondaPak C_{18} column with a gradient solvent delivery system of methanol and water. Radioactive material was detected by an in-line liquid scintillation spectrophotometer (Radiometric Flo-One).

Northern Hybridization Analysis. Following enzymatic assays, cells were washed once with GBSS to remove any residual medium and lysed in the presence of 7.5 M guanidine hydrochloride (MacDonald et al., 1987). Total RNA (10 μg/lane) was resolved on a 1.5% agarose/5 mM methylmercury hydroxide gel (Bailey & Davidson, 1976) and transferred overnight to a nitrocellulose filter. Following prehybridization, the filter was probed overnight with an [α-^{32}P]UTP (3000 Ci/mmol; ICN) labeled RNA probe synthesized from either bovine P450$_{17\alpha}$ cDNA sequences (encoding residues 1–451) or human P450$_{17\alpha}$ cDNA sequences (encoding residues 3–451). The filters were washed at 65 °C in 0.1 × SSC and 0.1% SDS and subjected to autoradiography.

Western Immunoblot Analysis. Following enzymatic assays, cells were washed once with GBSS, collected by scraping in a buffer containing 1% sodium cholate and 0.1% SDS, and lysed by three freeze–thaw cycles. The cell debris was pelleted, and the total protein concentration of the supernatant was determined by BCA protein assay (Pierce Chemicals). Total protein (100 or 200 μg) was resolved on a 10% SDS–PAGE gel and electroblotted to Immobilon P membrane (Millipore). The blot was probed with anti-P450$_{17\alpha}$ (Zuber et al., 1985) primary antibody, followed by ^{125}I-labeled protein A (9.66 μCi/μg, Du Pont-NEN) as the second antibody.

Results

The P450$_{17\alpha}$ chimeras have been divided into three groups for the sake of convenience in presentation. Group 1 (Figure 2A) are those having a bovine amino terminus and a human carboxy terminus (bovine/human chimeras), and the results of enzymatic assays for this group of chimeras are summarized in Figure 3A. With respect to 17α-hydroxylation, it can be seen that wild-type human P450$_{17\alpha}$ is 2–3 times less active than wild-type bovine P450$_{17\alpha}$ when either pregnenolone or progesterone was used as substrate. Each of the bovine/human chimeras had about the same 17α-hydroxylase activity with either pregnenolone or progesterone, and these activities were intermediate between the bovine and human wild-type activities. However, when the human/bovine chimeras (Figure 2B) (group 2) were examined (amino-terminal human and carboxy-terminal bovine), the results were surprisingly different (Figure 3B). Note that only 17HB Xba had detectable ac-

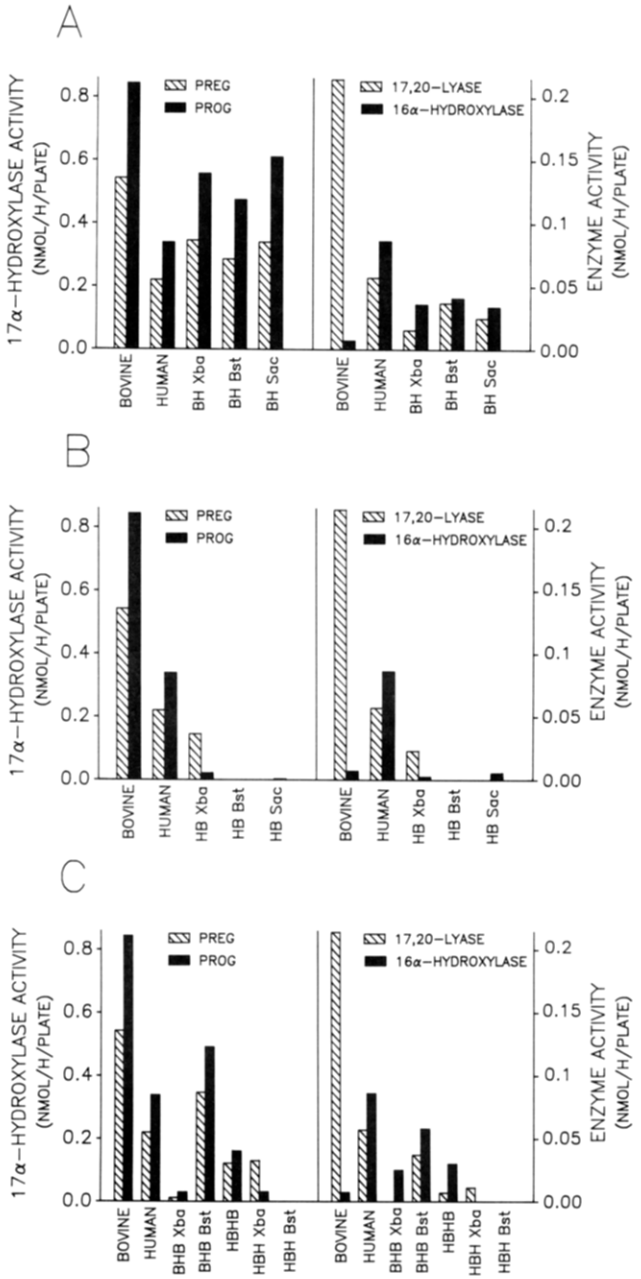

FIGURE 3: Relative rates of enzymatic activities of the various chimeras as compared to the wild-type forms of $P450_{17\alpha}$. Rates were determined from the linear portion of velocity plots of enzymatic activity over the course of three independent experiments. 17α-Hydroxylase activity was measured by using either pregnenolone (Preg) or progesterone (Prog) as substrate. 17,20-Lyase activity was measured by using 17α-hydroxypregnenolone as substrate, while 16α-hydroxylase activity was measured by using progesterone as substrate. (A) Group 1 chimeras. (B) Group 2 chimeras. (C) Group 3 chimeras. Data shown are the average from three separate experiments.

tivity. Furthermore, 17HB *Xba* was more efficient at 17α-hydroxylating pregnenolone than progesterone, just the opposite of the wild-type bovine or human enzymes an the bovine/human chimeras. The very low or absent activity of human/bovine chimeras raised the question as to whether their expression might be dramatically reduced. Figure 4 shows that Northern analysis does not indicate reduced expression of any of the chimeric mRNAs. However, mRNA concentration need not parallel protein levels, and consequently we examined whether the $P450_{17\alpha}$ levels might be different, thereby suggesting differences in translation of the chimeras or, more likely, differences in their stability in COS 1 cells. When examining the immunoblot in Figure 5B, it is important

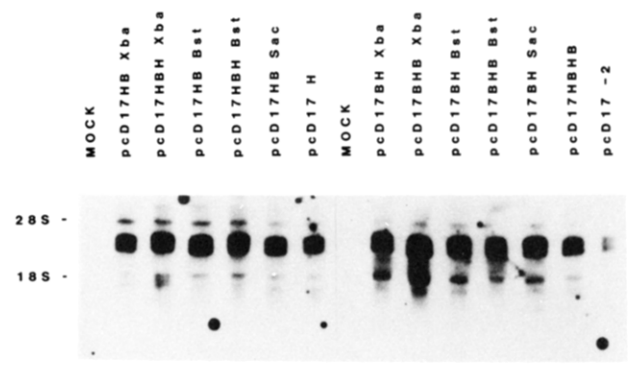

FIGURE 4: Northern hybridization analysis of wild-type and chimeric transcript abundancies in transfected COS 1 cells. Total RNA (10 μg/lane) was resolved on a 1.5% agarose/5 mM methylmercury hydroxide gel, transferred to nitrocellulose, and screened with a riboprobe synthesized from either human or bovine cDNA coding sequences. The positions of the 18S and 28S ribosomal RNAs, to which there is weak cross-hybridization, are indicated. The $P450_{17\alpha}$ cDNA is encoded by two messages of different size in COS 1 cells, presumably due to alternative use of polyadenylation signals, one encoded by the $P450_{17\alpha}$ cDNA and one encoded by the SV40 sequences in the vector. pcD17 H is wild-type human $P450_{17\alpha}$ cDNA (Bradshaw et al., 1987), pcD17-2 is wild-type bovine $P450_{17\alpha}$ cDNA (Zuber et al., 1986a), and MOCK is RNA from untransfected COS 1 cells.

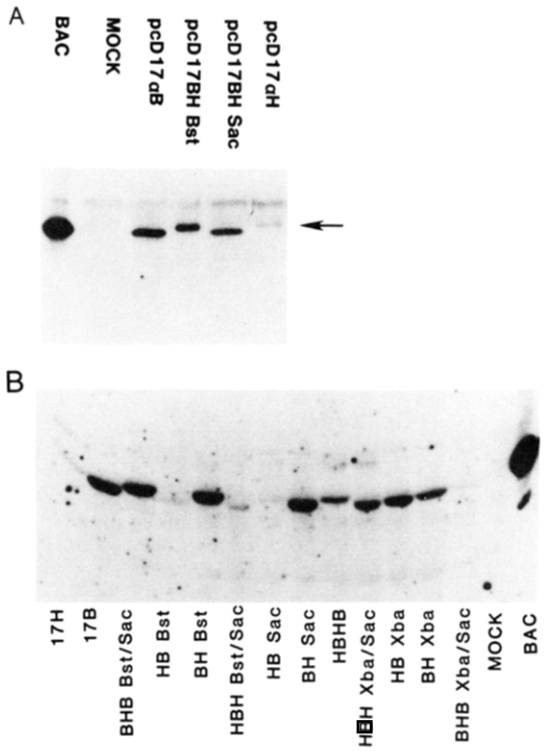

FIGURE 5: Western immunoblot analysis of wild-type and chimeric proteins in transfected COS 1 cells. Total protein from cell homogenates was resolved by 10% SDS–PAGE and electroblotted to Immobilon P (Millipore). The blot was probed with anti-$P450_{17\alpha}$ antibody (Zuber et al., 1985), followed by ^{125}I-labeled protein A as the second antibody. (A) Immunoblot of 100 μg of cell homogenate showing difference in intensity of human and bovine $P450_{17\alpha}$ when probed with the antibody used in this laboratory. The arrow shows the position of the human $P450_{17\alpha}$ in COS 1 cells having activities similar to those shown in Figure 3. MOCK is 100 μg of cell homogenate from untransfected cells, and BAC is 2 μg of microsomes isolated from bovine adrenal cortex (BAC). (B) Immunoblot of chimeric 17α-hydroxylases in 200 μg of cell homogenates from cells having activities as shown in Figure 3. 17H is pcD17αH; 17B is pcD17αB; MOCK and BAC are as in (A).

to note that the antibody which is used in this laboratory is about 10 times more efficient at detecting bovine $P450_{17\alpha}$ epitopes than human $P450_{17\alpha}$ epitopes (compare pcD17αH and

pcD17αB in Figure 5A). From a comparison of the immunoblots of the group 1 chimeras, it is apparent that 17BH *Xba*, 17BH *Bst*, and 17BH *Sac* are all readily detectable, with the detectable level of 17BH *Xba* being less than that of either 17BH *Bst* or 17BH *Sac* (Figure 5B). We believe that a major epitope for our antibody lies between the *Xba* and *Bst* sites in bovine $P450_{17\alpha}$ because the activities of these three chimeras are equivalent (Figure 3A), even though by immunoblot analysis the 17BH *Xba* chimera appears to be less abundant. Also the immunodetectable level of 17HB *Xba* is higher than that of 17BH *Xba*, further supporting the contention of a strong epitope between the bovine *Xba* and *Bst* sites, yet 17HB *Xba* is a very poor 17α-hydroxylase, particularly for progesterone (Figure 3B). 17HB *Bst* and 17HB *Sac* are inactive with either substrate. However, in this case, the immunoblot analysis shows low levels of both proteins, levels similar to that of 17H (wild-type human $P450_{17\alpha}$). Thus, we cannot distinguish from the immunoblot whether the reduced activity of 17HB *Bst* and 17HB *Sac* results from lowered stability of these chimeric proteins or from proteins expressed at the same level as 17H but which are simply inactive, although we favor the latter possibility because the immunodetectable levels of 17HB *Bst* and 17HB *Sac* are similar to that of 17H. As noted under Experimental Procedures, the nucleotide sequences surrounding the ligation positions in both 17HB *Bst* and 17HB *Sac* were found to be correct.

A third, more complicated, series of chimeras (group 3) were prepared by using either bovine amino- and carboxy-terminal sequences and a central human sequence or vice versa (Figure 2C). Of these chimeras, 17BHB *Bst/Sac* is the most active (Figure 3C), having levels of activity very similar to those of 17BH *Bst*. Also, this protein is expressed at approximately the same level as 17BH *Bst* (Figure 5B). Chimeric 17BHB *Xba/Sac* is observed to be present at a very low level by immunoblot analysis and shows very low activity. The 17HBH *Xba/Sac* chimeric is produced at normal levels and shows low levels of 17α-hydroxylase activities similar to 17HB *Xba*. These are the only two chimeras (17HB *Xba* and 17HBH *Xba/Sac*) that show readily detectable levels of 17α-hydroxylation of pregnenolone with little or no activity toward progesterone. The fact that the activities of both 17BHB *Bst/Sac* and 17HBH *Xba/Sac* resemble their respective two-fragment chimeras (17BH *Bst* and 17HB *Xba*) suggests that the addition of the carboxy-terminal *Sac* fragment from either species has no effect on resultant activities. However, the insertion of the bovine *Sac* fragment into 17BH *Xba*, producing 17BHB *Xba/Sac*, dramatically reduces both the enzymatic activities (Figure 3C) and the level of immunodetectable protein (Figure 5B). Presumably in this particular case, addition of the bovine *Sac* fragment to 17BH *Xba* renders the resultant protein unstable in COS 1 cells. 17HBH *Bst/Sac* shows similar levels of enzyme to 17HB *Bst* by immunoblot analysis and no enzymatic activities, similar to 17HB *Bst*. Finally, the single four-fragment chimera prepared, 17HBHB, shows detectable levels of all activities and the protein is clearly present by immunoblot analysis. However, we would expect more protein to be detectable on the basis of our contention of a major epitope located between the bovine *Xba* and *Bst* sites, and we suggest that the stability of this four-fragment chimeric protein in COS 1 cells may be somewhat lowered.

Discussion

The use of genetic engineering and heterologous expression systems makes it possible to evaluate the functional role of specific sequences in enzymes. Even when taking into account the caveat that mutagenesis can lead to altered structure and consequently to changes in kinetic properties not directly related to changes of a specific amino acid, these technologies have provided new insights into the role of specific amino acids in certain forms of P450. In the present study we have taken the approach of constructing chimeric proteins of the same enzyme ($P450_{17\alpha}$) derived from two different species (human and bovine) to evaluate sequence relatedness. As seen in Figure 3A, following expression in COS 1 cells, $P450_{17\alpha}$ from both species catalyzes the 17α-hydroxylation of progesterone more efficiently than it does the 17α-hyroxylation of pregnenolone. However, the bovine enzyme is 2–3 times more active at catalyzing both activities. Also, the bovine enzyme is about 4 times more efficient in catalyzing the 17,20-lyase conversion of 17α-hydroxypregnenolone to dehydroepiandrosterone. In addition to these differences, human $P450_{17\alpha}$ catalyzes 16α-hydroxylation of progesterone greater than 10 times more efficiently than does bovine $P450_{17\alpha}$. Therefore, we reasoned that by utilizing common, unique restriction sites within the cDNAs encoding these two 17α-hydroxylases we might be able to focus on regions of the sequence which are important for these various differences in activities.

The first group of chimeras studied, those having a bovine amino terminus and a human carboxy terminus, all catalyzed 17α-hydroxylase activities that were intermediate between the wild-type bovine and human levels and were about the same for each chimeric. In addition, they demonstrated that the 17α-hydroxylase activity was much less affected by the mixing of bovine and human sequences than was the 17,20-lyase activity because the 17,20-lyase activity of the three chimeras was decreased by 80% or more when compared to the wild-type bovine activity and lowered by about 50% when compared to the wild-type human activity. Furthermore, the 16α-hydroxylase activity that arises from the human $P450_{17\alpha}$ was also affected by the presence of bovine amino-terminal sequences because it was found to be decreased from the human wild-type activity by about 50% in each of the three bovine human chimeras (Figure 3A). The second group of chimeras studied, those having a human amino terminus and a bovine carboxy terminus, were quite surprising. These chimeras tended to be inactive. This was clearly not the result of altered expression of the chimeric mRNAs, as seen in Figure 4, although from the immunoblot shown in Figure 5B we cannot rule out the possibility that certain of the human/bovine chimeras have lowered stability in COS 1 cells. Clearly the 17HB *Xba* protein is found to be present in COS 1 cells at the same level as the first group of chimeras, and yet all activities of this chimera are very low. This suggests that the folding of the 17BH *Xba* chimera is more normal than that of the 17HB *Xba* chimera. Since the two proteins are exactly the same length except for an additional proline at the C terminus of the bovine $P450_{17\alpha}$, the most obvious explanation of the striking difference between BH chimeras and HB chimeras is that bovine and human 17α-hydroxylases have slightly different tertiary structures. The folding pattern initiated by the bovine amino terminus upon anchoring in the membrane of the endoplasmic reticulum can accommodate human carboxy-terminal sequences without severe steric hindrance, leading to stable, functional enzymes having relatively normal 17α-hydroxylase activity and lowered but readily detectable 16α-hydroxylase and 17,20-lyase activities. On the other hand, the human amino terminus initiates a folding pathway that does not readily accommodate bovine carboxy-terminal sequences of any size, and consequently proteins are produced that have little or no enzymatic activity and in certain cases may be unstable in COS 1 cells.

Studies of chimeras of other forms of P450 have not shown decreased activity upon interchanging amino-terminal sequences. When cDNAs encoding rat P450c and -d were used for chimeric constructions, the first 146 amino acids of rat P450c (P450IA1) could be used to replace the same sequence of rat P450d (P450IA2) without affecting the enzymatic properties (Sakaki et al., 1987). Likewise, the amino-terminal 143 amino acids of rabbit P450LM4 (P450IA2) could be used in place of those of rabbit P450LM6 (P450IA1) without altering activity (Pompon et al., 1988). Furthermore, the amino-terminal 144 amino acids of rabbit P450LM4 could be used to replace those of mouse P_1450 (P450IA1) without affecting activity (Pompon & Nicolas, 1989), and the 40 amino-terminal amino acids of P_1450 could be replaced by the 36 amino-terminal amino acids of P_3450 (P450IA2) with no change in activity (Cullin & Pompon, 1988). Only when the middle one-third of P450c is replaced with a similar sequence from P450d, producing a cdc three-fragment chimera, has an inactive chimeric enzyme been produce (Sakaki et al., 1987), resulting from instability of the chimeric protein in the microsomes of the heterologous yeast expression system.

In most cases the three-fragment chimeras in this study resemble the two-fragment chimeras from which they are derived. The addition of the human *Sac* fragment in place of the bovine fragment in both 17HB *Xba* and 17HB *Bst* has no effect on the activities or the levels of chimeric protein. Both 17HB *Xba* and 17HBH *Xba/Sac* are stable proteins having little activity. Likewise, the addition of the bovine carboxy-terminal *Sac* fragment to the 17BH *Bst* has no effect on the activity. However, the addition of this same fragment to 17BH *Xba* renders this protein unstable (Figure 5B).

The results described herein show that sequence swapping between P450s which are closely related by amino acid sequence and enzymatic activities cannot always be relied upon to produce functional and/or stable chimeric proteins that may lead to new insights on the P450 structure–function relationship. Furthermore, they suggest that differences in folding initiated by the amino-terminal sequences and the resultant tertiary structures are the bases of the differences observed between bovine/human and human/bovine $P450_{17\alpha}$ chimeras. The tertiary structures of the human/bovine $P450_{17\alpha}$ chimeras are inappropriate for 17α-hydroxylase and 17,20-lyase activities. These studies also indicate that the 17,20-lyase activity is much more sensitive to structural alterations than is either 17α-hydroxylase activity, a result that may not be surprising since the former activity is thought to require three hydroxylation steps of which the 17α-hydroxylation is first. Consequently, the hydroxylation at C20 or the cleavage of the carbon–carbon bond between C17 and C20 is dramatically reduced in all three bovine/human chimeras relative to 17α-hydroxylation. Finally, we conclude that small differences in the tertiary structures of bovine and human $P450_{17\alpha}$ are responsible for the observation that the bovine enzyme is more active with respect to both the 17α-hydroxylase and 17,20-lyase activities and that the human enzyme is a much better 16α-hydroxylase.

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