

Glucocorticoid-suppressible Hyperaldosteronism and Adrenal Tumors Occurring in a Single French Pedigree

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Abstract

Glucocorticoid-suppressible hyperaldosteronism is a dominantly inherited form of hypertension believed to be caused by the presence of a hybrid *CYP11B1/CYP11B2* gene which has arisen from an unequal crossing over between the two *CYP11B* genes in a previous meiosis. We have studied a French pedigree with seven affected individuals in which two affected individuals also have adrenal tumors and two others have micronodular adrenal hyperplasia. One of the adrenal tumors and the surrounding adrenal tissue has been removed, giving a rare opportunity to study the regulation and action of the hybrid gene causing the disease. The hybrid *CYP11B* gene was demonstrated to be expressed at higher levels than either *CYP11B1* or *CYP11B2* in the cortex of the adrenal by RT-PCR and Northern blot analysis. In situ hybridization showed that both *CYP11B1* and the hybrid gene were expressed in all three zones of the cortex. In cell culture experiments hybrid gene expression was stimulated by ACTH leading to increased production of aldosterone and the hybrid steroids characteristic of glucocorticoid-suppressible hyperaldosteronism. The genetic basis of the adrenal pathologies in this family is not known but may be related to the duplication causing the hyperaldosteronism. (*J. Clin. Invest.* 1995. 96:2236–2246.) Key words: hypertension • adenomas • hybrid genes • *CYP11B* gene expression • glucocorticoid-remediable aldosteronism

Introduction

In the normal human adrenal cortex the terminal steps in cortisol and aldosterone biosynthesis are mediated by the cytochrome P450 enzymes *CYP11B1* (11 β -hydroxylase) and *CYP11B2* (aldosterone synthase), respectively (1, 2), which are encoded by two highly similar genes located on chromosome 8q22 (3). In the zona fasciculata the 17-hydroxylated steroid precursor, 11-deoxycortisol, is hydroxylated at the 11 β position by *CYP11B1* to produce cortisol. The expression of both the *CYP11B1* and *CYP11B2* genes, and hence the 11 β -hydroxylase and 17 α -hydroxy-

lase activities in this zone, is controlled by secretion of ACTH from the anterior pituitary (4). In the zona glomerulosa, where 17 α -hydroxylase activity is suppressed, *CYP11B2* mediates 11 β -hydroxylation, 18-hydroxylation, and finally 18-oxidation of 11-deoxycorticosterone to yield aldosterone. Expression of the *CYP11B2* gene is principally influenced by serum levels of angiotensin II (AII)¹ and potassium (5). Limitation of the expression of *CYP17* to the zona fasciculata and *CYP11B2* to the zona glomerulosa results in the functional zonation of cortisol and aldosterone synthesis to those respective zones.

Glucocorticoid-suppressible hyperaldosteronism (GSH) is a disease in which the normal pattern of synthesis of the mineralocorticoid hormone, aldosterone, is disturbed. It is characterized by hypersecretion of aldosterone, in the face of a low plasma renin activity (6, 7), and the production of two normally rare steroids, 18-hydroxycortisol and 18-oxocortisol (8–10), which require the simultaneous presence of a 17 α -hydroxylase activity and the two C18 activities typical of the *CYP11B2* enzyme. All symptoms of the disease are normalized by the administration of glucocorticoid analogues and are exacerbated by administration of ACTH (6, 7). Thus, the disease can be viewed as one in which the activities characteristic of the *CYP11B2* gene product are inappropriately expressed in the zona fasciculata under the control of ACTH. The genetic basis of this disease remained a mystery until publication of a seminal paper (11) which demonstrated the presence of a hybrid *CYP11B1/CYP11B2* gene segregating with the disease in a large pedigree. The hybrid gene is assumed to be transcribed in the zona fasciculata of the adrenal cortex under the control of ACTH, as a result of containing 5' sequences from *CYP11B1*, and to encode a protein with 18-hydroxylase and 18-oxidase activities, due to the presence of 3' coding sequence from *CYP11B2*. The expression of these activities in the zona fasciculata under the influence of ACTH would be sufficient to explain all of the characteristics of the disease. Subsequent studies have confirmed that the presumed transcript of the hybrid gene has the required aldosterone synthetic activities, providing that the breakpoint of the hybrid gene is in or 5' of intron 4 (12, 13), as is the case for all genes studied so far (12–14). However, it has not been possible previously to demonstrate that the hybrid gene is in fact expressed in the adrenal cortex and more specifically in the zona fasciculata under the control of ACTH. In this paper, we report studies on a French pedigree with seven individuals affected with GSH of whom two also have adrenal tumors and two others have bilateral adrenal hyperplasia with micronodules. One of the adrenal tumors has been surgically

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1. Abbreviations used in this paper: AII, angiotensin II; AT1, type 1 AII receptor; GSH, glucocorticoid-suppressible hyperaldosteronism; UTR, untranslated region.

removed, providing a unique opportunity to study the hybrid gene believed to cause the inherited disorder in the tissue which is directly affected. Adrenal tissue from the tumor and the surrounding nontumorous GSH tissue has been studied by RT-PCR, in situ hybridization, and by analysis of steroids and RNA produced by the adrenal cells cultured in the presence of ACTH and AII. We also demonstrate the zonal pattern of expression of the *CYP11B* genes in a normal human adrenal, showing that it has characteristics of both the rodent and bovine adrenals.

Methods

Genomic DNA analysis. Genomic DNA was prepared from peripheral blood leukocytes and analyzed by Southern blot as described previously (12). DNA was amplified by PCR using oligonucleotides TCTCGA-AGGCAAGGCACCAG immediately 5' of exon 1 and TGAGGCTGG-ATCTTCCCATGT from intron 3 of *CYP11B1* and GACCTTCGCTCT-GAGAGTCT and TGGGGCTGGACCTTCCCGCAT from the corresponding regions of *CYP11B2*. The hybrid gene segment was amplified using the 5' oligonucleotide from *CYP11B1* and the 3' oligonucleotide from *CYP11B2* (oligonucleotides A and B in Fig. 1). All reactions were subjected to 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min increasing by 5 s each cycle. PCR products were sequenced directly as described previously (15) to determine the crossover point in the hybrid gene.

Removal and dissection of adrenal glands. The right adrenal was surgically removed and the 2.5-cm diameter tumor was dissected away from the surrounding tissue. The tumor and the remaining nontumorous GSH tissue were sectioned into smaller pieces and allocated to three different experimental protocols. Some of each type of tissue was used to make RNA for analysis of the *CYP11B* messages. Other pieces of tissue were fixed in paraformaldehyde for subsequent in situ hybridization studies and the remaining cells were used in cell culture experiments to determine ACTH and AII responsiveness and to measure the steroids produced.

Preparation and analysis of RNA. RNA was made from tumor and healthy adrenal tissue using RNazolB (Biotech Laboratories, Houston, TX). Total RNA (4 µg) was electrophoresed in 0.74 M formaldehyde 1% agarose gels at 100 V for 2.5 h, transferred to nylon membranes (Hybond N; Amersham, Paris, France), fixed by ultraviolet cross-linking, and hybridized with oligonucleotides which were radioactively labeled with [α -³²P]dATP (Amersham) by terminal deoxynucleotide transferase (Pharmacia, Uppsala, Sweden or GIBCO-BRL Life Technologies, Paris, France) and cDNA probes which were radioactively labeled by random priming. The sequences of the oligonucleotide probes used were CACTGTCCTGGGGACCCGGGC and CACCGTCCTAGG-GGCCCGAGC from the region 5' of the breakpoint and TGCCAC-GATGCTGGTGTACTG and TGCCACGATGCCTGTGTAGTG 3' of the breakpoint, for *CYP11B1* and *CYP11B2*, respectively. Hybridizations were performed in 6 × SSPE/1% SDS solution containing 50% formamide, 5 × Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA at 37°C. Final stringent washes were in 0.5 × SSPE/0.3% SDS at 50°C (5' end probes) or 2 × SSC/0.3% SDS at 42°C (3' end probes). Specificity of the oligonucleotide hybridizations was confirmed by simultaneous hybridization to RNA prepared from COS cells that had been transfected with a *CYP11B1*, *CYP11B2*, or a *CYP11B1/CYP11B2* hybrid cDNA construct.

RT-PCR. Total RNA was reverse transcribed using RNaseH⁻ reverse transcriptase (SuperScript; GIBCO-BRL) and random hexamers. Aliquots were then used to perform PCR amplification of specific *CYP11B* mRNA sequences. Amplification was performed with oligonucleotides GCCCGGGTCCCCAGGACA and TGCCACGATGCTGGT-GTACTG from exon 2 and exon 3 of *CYP11B1* and GCTCGGGCC-CCTAGGACG and TGCCACGATGCCTGTGTAGTG from the corresponding regions of *CYP11B2*. Hybrid cDNA was amplified using the 5' oligonucleotide corresponding to the *CYP11B1* sequence and the 3' oligonucleotide corresponding to the *CYP11B2* sequence. All reactions

were subject to 38 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min increasing by 5 s each cycle. Magnesium concentration was optimized at 0.75 mM to give specific amplification of all products. The specificity of the PCR amplification was confirmed by the inclusion of reactions using cloned templates corresponding to *CYP11B1*, *CYP11B2*, and the hybrid *CYP11B1/CYP11B2* cDNAs.

In situ hybridization. Tissue from both the tumor and the surrounding adrenal tissue was dissected into ~5-mm slices and fixed in 4% paraformaldehyde for 24 h. After routine dehydration and paraffin embedding, 7-µm sections were mounted on silanized slides and used for in situ hybridization as described previously (16). Identification of the three different types of *CYP11* mRNAs in situ required the use of gene-specific probes from the 3' and 5' ends of *CYP11B1* and *CYP11B2* mRNAs, as the three types of transcripts all share common sequences. *CYP11B1* and *CYP11B2* are 93% similar in coding sequence and the hybrid transcript is identical to *CYP11B1* for the first two exons and identical to *CYP11B2* for the remainder of the transcript. In the 3' untranslated region (UTR) there are regions where the sequence is quite different between the two genes, so that the use of short sense and antisense cRNA probes for that region was possible. Radiolabeled cRNA probes were prepared by transcription reactions incorporating α -³⁵S-UTP using linearized recombinant plasmids containing the oligonucleotide sequences CCTGGCATTGCTGCTTAGCCTGGCAAACCTGG-TCCTAG from *CYP11B1* and GCCTTGCTATTTGACAAGCCTGGC-AAGCCCCAGTCCTGG from *CYP11B2* as templates. T3 and T7 RNA polymerases were used as appropriate to generate sense and antisense RNA strands. In the 5' region, where the sequences were similar, specificity of oligonucleotide probe hybridizations was demonstrated by a novel competitive hybridization technique. A labeled oligonucleotide was incubated with 20 times the amount of the same unlabeled oligonucleotide (homologous competition) or 20 times the amount of the corresponding unlabeled oligonucleotide from the other gene (heterologous competition). Empirical melting profiles and T_m s for the oligonucleotide sequences were generated by separate experiments with Northern blots (data not shown) (17) and hybridization conditions were then chosen so that the hybridization step was competitive for the heterologous oligonucleotides. The hybridization mixture contained 6 × SSC, 50% formamide, 1% sarcosyl, 1 µg/ml denatured salmon sperm DNA, 4.5% dextran sulfate, and 1 × Denhardt's solution, and sections were incubated overnight with 30 µl of hybridization buffer containing 5 × 10⁵ cpm of the radiolabeled probe in a humid chamber at 50°C for cRNA probes or 40°C for oligonucleotide probes. Subsequent most stringent washes were in 0.2 × SSC, 50% formamide at 50°C for the cRNA probes, and in 2 × SSC at 40°C for oligonucleotide probes. Under these conditions the homologous competition lead to a quenching of the hybridization signal, whereas the heterologous competition gave the same result as no competition, demonstrating the specificity of the hybridization. Oligonucleotide probes were radioactively labeled with [α -³³P]dATP as described above. Positive and negative serial sections were mounted on the same slide and developed at the same time. The sections were counterstained with toluidine blue for microscopic examination.

Culture of adrenal cells. After removal of surrounding adipose tissue, the capsule, and the medulla, the remaining adrenal cells were dispersed and cultured in the presence of various concentrations of ACTH and A II as described previously (18, 19). The cells were stimulated for a period of 48 h with replacement of medium at 24 and 48 h. At the end of 48 h the cells were washed twice in 0.9% NaCl, followed by 4 min in 50 mM glycine and two more washes in 0.9% NaCl, and restimulated with a ACTH (10⁻⁸ M) for 2 h. At the conclusion of the experiments, total RNA was prepared from the cells (20) and the medium was stored at -20°C until determination of steroid concentrations.

Measurements of steroids. Aldosterone, cortisol, 18-hydroxycortisol, and 18-oxocortisol were measured by ELISA as described previously (21, 22).

Results

PCR amplification of the hybrid gene and its message. Genomic DNA from all family members was amplified by PCR using

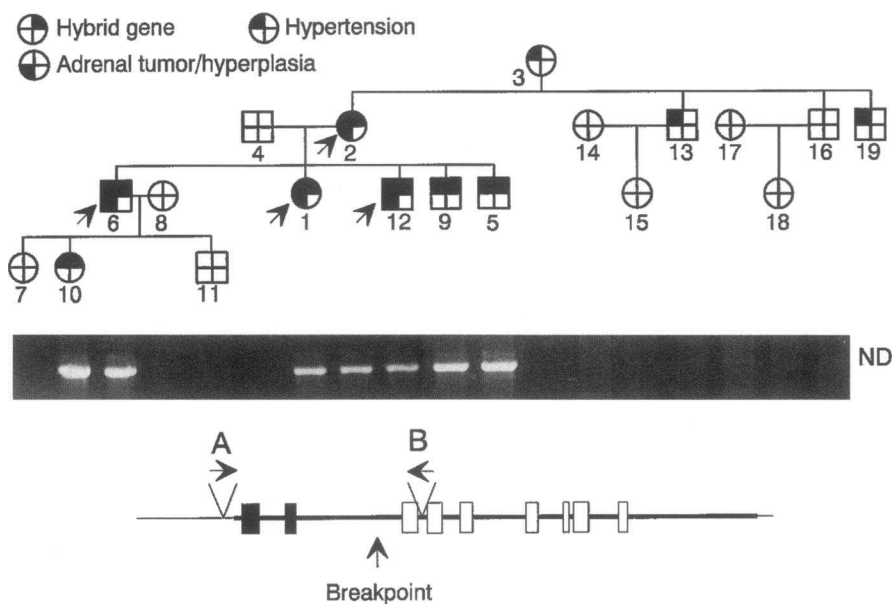


Figure 1. PCR amplification of the hybrid gene segment from members of a pedigree with GSH. Genomic DNA samples from all family members were used as templates in PCR reactions using oligonucleotides A and B, as indicated. Successful amplification, as shown by ethidium bromide staining of amplified DNA, was only seen in reactions using DNA prepared from individuals who had GSH by clinical criteria and Southern blot analysis. Individual 2 had the adrenal tumor which was removed for this study and individual 6 had an adrenal tumor which was removed before this study. Individuals 1 and 12 were diagnosed as having micronodular adrenal hyperplasia.

CYP11B1 or *CYP11B2* oligonucleotides to amplify the corresponding *CYP11B1* or *CYP11B2* genes and a 5' *CYP11B1* oligonucleotide with a 3' *CYP11B2* oligonucleotide to amplify the hybrid *CYP11B1/CYP11B2* gene. The amplification of a hybrid product occurred only in samples from individuals diagnosed to have GSH by clinical characteristics and by Southern blot analysis (Fig. 1). The use of PCR to amplify hybrid sequences is thus a useful screening test to detect affected members within a pedigree, in whom the presence of the hybrid gene can then be confirmed by Southern blot analysis. A full clinical description of these patients will be published elsewhere (23). PCR products from the hybrid gene were then sequenced to reveal the crossover point of the hybrid gene, which lay in the same position (near the 3' end of intron 2) for all affected members.

The hybrid mRNA was detected by a similar amplification of cDNA derived from both the adrenal tumor and the nontumorous adrenal tissue removed from our patient. No corresponding product was amplified from control normal adrenal cDNA, whereas *CYP11B1* and *CYP11B2* messages were amplified from all samples studied (Fig. 2). The hybrid mRNA was found to be composed of the first two exons from *CYP11B1* and the remaining sequence from *CYP11B2*, as predicted from the genomic analysis.

Northern analysis of RNA from tumor and normal adrenal tissue. Northern blots of RNA from the tumor, the surrounding nontumorous tissue, and tissue from a normal adrenal were hybridized with oligonucleotide probes from the 5' and 3' ends of both *CYP11B1* and *CYP11B2* (Fig. 3). Three mRNAs, 4.2, 3.6, and 2.2 kb in length, were detected with an oligonucleotide probe corresponding to sequence at the 3' end of *CYP11B1* (which will not detect the hybrid mRNA). The different mRNA lengths are assumed to result from alternate usage of different polyadenylation signals and are similar to the sizes observed in a previous analysis of mRNA from cultured adrenal cells (24) and an aldosterone-secreting tumor (1). The 2.2-kb mRNA was difficult to detect with an oligonucleotide probe and evidently represents a minor transcript in these tissues. No transcripts were detected by a 5' *CYP11B2* probe, apart from those in the control RNA from COS cells transfected with *CYP11B2* cDNA

constructs, a result which is consistent with previous studies showing low expression of this gene (2, 3). Furthermore, the failure to detect *CYP11B2* transcripts with oligonucleotide probes is to be expected in a patient with GSH, where the renin-angiotensin system is normally suppressed.

When either a 5' *CYP11B1* or a 3' *CYP11B2* probe was used, two mRNAs of 4.2 and 3.2 kb were detected and these evidently correspond to the mRNA transcripts from the hybrid gene. The sizes of these hybrid transcripts correspond to sizes observed for *CYP11B2* mRNA in a previously reported North-

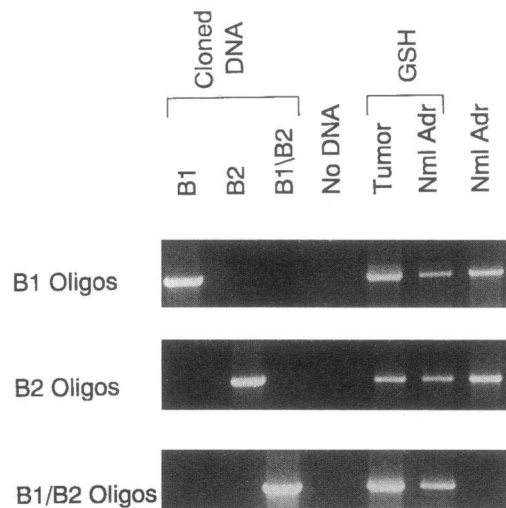


Figure 2. RT-PCR amplification of GSH and normal adrenal RNA. RNA isolated from adrenal tissue from a GSH patient with an adrenal tumor, from the adrenal tumor itself, and from a normal adrenal was amplified by RT-PCR using oligonucleotides specific for *CYP11B1*, *CYP11B2*, or a 5' oligonucleotide from *CYP11B1* and a 3' oligonucleotide from *CYP11B2*, as indicated. Amplification of *CYP11B1* and *CYP11B2* mRNAs is seen in all tissue samples, but amplification of the hybrid mRNA is only seen in samples from the GSH adrenal. PCR reactions using cloned cDNA templates demonstrate the specificity of the amplifications.

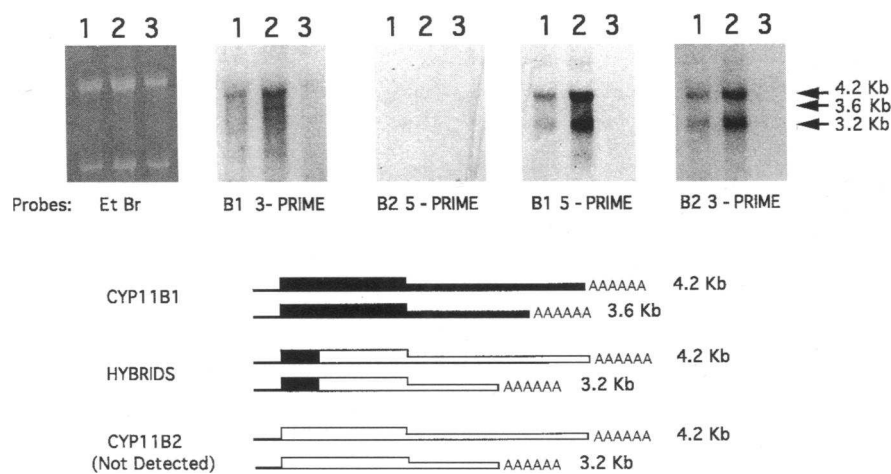


Figure 3. Northern blots of RNA prepared from the adrenal of a patient with GSH and an adrenal tumor and from a normal adrenal hybridized with gene-specific oligonucleotide probes. Lanes 1, 2, and 3 in each photograph contain RNA from the tumor, the surrounding nontumorous tissue, and the normal adrenal, respectively. Ethidium bromide-stained RNA and autoradiographs of hybridizations with gene-specific oligonucleotide probes from regions 5' and 3' of the hybrid gene breakpoint for both *CYP11B1* and *CYP11B2* are shown. An interpretation of the principal *CYP11B* transcripts detected, which are assumed to arise from the use of alternative polyadenylation addition signals, is diagrammed below the photographs.

ern analysis of RNA from an aldosterone-producing tumor (1) and presumably also arise from the use of two different polyadenylation recognition sites.

It is evident that mRNAs containing the 3' UTR from *CYP11B1* are less abundant than the hybrid mRNA and that these transcripts are mildly degraded in the RNA blots compared with those from the hybrid gene. The apparent mRNA degradation was seen using several different *CYP11B1* oligonucleotide probes but was never observed with *CYP11B2* probes. The sizes of the *CYP11B* mRNA transcripts were similar in RNA prepared from the tumor and RNA prepared from the surrounding GSH adrenal tissue. No *CYP11B* mRNAs were detected by the oligonucleotide probes in RNA from the control normal adrenal sample used in this analysis, despite the apparent integrity of the RNA as judged from ethidium bromide-stained RNA gels. This result could be due to downregulation of these mRNAs as a result of perfusion of the organ with anticoagulants and isotonic fluids before transplantation of the accompanying kidney or prior undetermined medical treatment. Prolonged exposure of the same blot hybridized with a random-primed *CYP11B1* cDNA probe showed that *CYP11B* mRNA was present in the normal adrenal, with sizes consistent with those observed in the GSH samples and in RNA from the cultured adrenal cells (discussed below).

In situ hybridization. The results of in situ hybridization experiments performed on fixed tissue from the tumor and surrounding GSH adrenal tissue are shown in Fig. 4. At low magnification the GSH adrenal had a normal appearance, but the zona fasciculata lacked its usual columnar organization. Nuclei in this zone were organized in clumps and there were intervening areas devoid of nuclei, suggesting that the cells are arranged in distinct islets or alveoli. In this respect the adrenal resembled previously studied adrenals from GSH patients (25–27, and our unpublished observations). The zona reticularis was also irregular and intercalated into the zona fasciculata, in some areas almost invading it. The tumor itself was black when cut and was well circumscribed but not encapsulated. Microscopically it was composed of large polygonal cells with regular central nuclei and finely granulated cytoplasm. The cellular morphology was similar to that of the zona reticularis and could be argued to have arisen from cells within it. Serial sections from the GSH tissues, as well as control normal adrenals, were hybridized with sense and antisense cRNA probes from the 3' UTRs of *CYP11B1* and *CYP11B2* and oligonucleotide probes

corresponding to sequence from *CYP11B1* or *CYP11B2* 5' of the breakpoint of the hybrid gene to detect the *CYP11B* transcripts, as discussed below.

Expression of *CYP11B* genes in normal adrenal tissue. In tissue sections of normal adrenals, expression of *CYP11B1* was detected most strongly in the zona fasciculata and zona reticularis but could also be easily detected in the zona glomerulosa. The *CYP11B2* mRNA was found to be strongly expressed in the zona glomerulosa (although not in all samples studied), but was barely distinguishable above background signals in the zona fasciculata and zona reticularis, suggesting at most a low level of expression in these zones. This pattern of expression differs from the strict zonation of expression seen in rodent adrenal cortex (28, 29) and the ubiquitous expression seen in bovine adrenal cortex (30, 31).

Expression of *CYP11B* genes in a GSH adrenal. In the GSH adrenal both *CYP11B1* and hybrid *CYP11B1/CYP11B2*, but not *CYP11B2*, transcripts were found to be strongly expressed in the tumor tissue. Expression of the former transcripts in the tumor would be expected to result in production of both cortisol and aldosterone and subsequent downregulation of *CYP11B* mRNAs in the nontumorous tissue. Nevertheless, it was possible to detect expression of *CYP11B1* and hybrid gene transcripts in various zones of the surrounding adrenal cortex. *CYP11B1* transcripts were detected in the zona glomerulosa and at significantly lower levels in the zona fasciculata and zona reticularis, suggesting that the mechanism for downregulation of this gene may be limited to the latter two zones. Consistent with this idea, expression of the hybrid gene was also observed in the zona glomerulosa and at lower levels in the other two zones, irrespective of whether a 5' *CYP11B1* (detecting both *CYP11B1* and the hybrid mRNAs) or a 3' *CYP11B2* probe (detecting only the hybrid gene) was used. In spite of the apparent downregulation of these genes in the nontumorous tissue, detection of the expression of the hybrid gene in the zona fasciculata and zona reticularis supports the notion that it is this gene which is responsible for the production of aldosterone, 18-hydroxycortisol, and 18-oxocortisol in these zones in GSH patients. Hybridizations performed with a *CYP17* probe (data not shown) confirmed that expression of *CYP17* mRNA was limited to the tumor, the zona fasciculata, and the zona reticularis.

Cell culture experiments. A principal feature of the explanatory model for GSH is that the hybrid gene should be responsive to stimulation by ACTH and that this increased expression

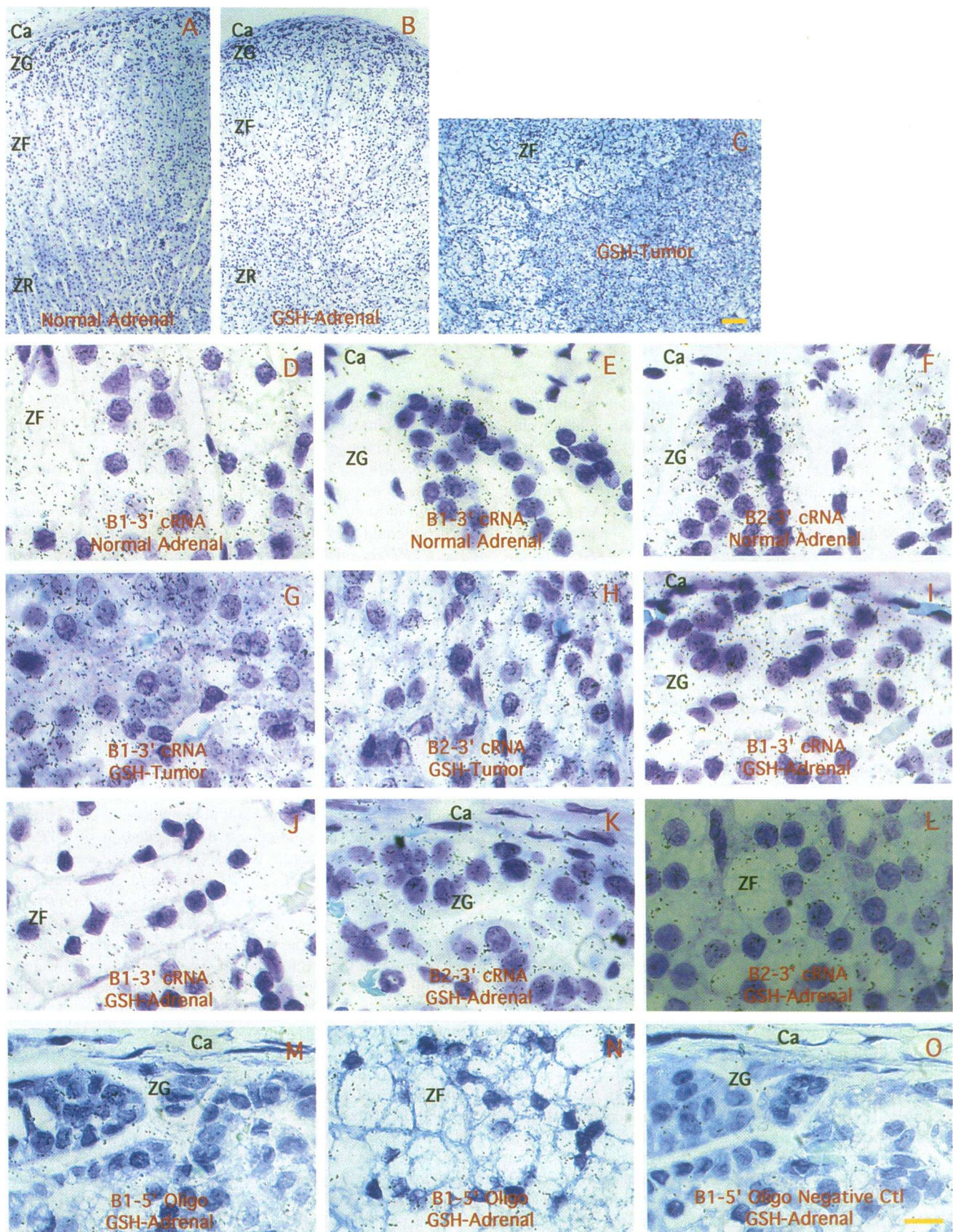


Figure 4. Histological structure and in situ hybridization analysis of tissue sections of the adrenal tumor removed from the patient with GSH, of the surrounding nontumorous GSH tissue, and of a normal adrenal. Low power photomicrographs of the normal adrenal (A), nontumor GSH tissue (B), and tumor tissue (C) are shown for comparison. In situ hybridization of 3' cRNA probes to normal adrenal tissue shows expression of *CYP11B1* in the zona fasciculata (D) and *CYP11B1* and *CYP11B2* in the zona glomerulosa (E and F). In the tumor tissue, as well as in the

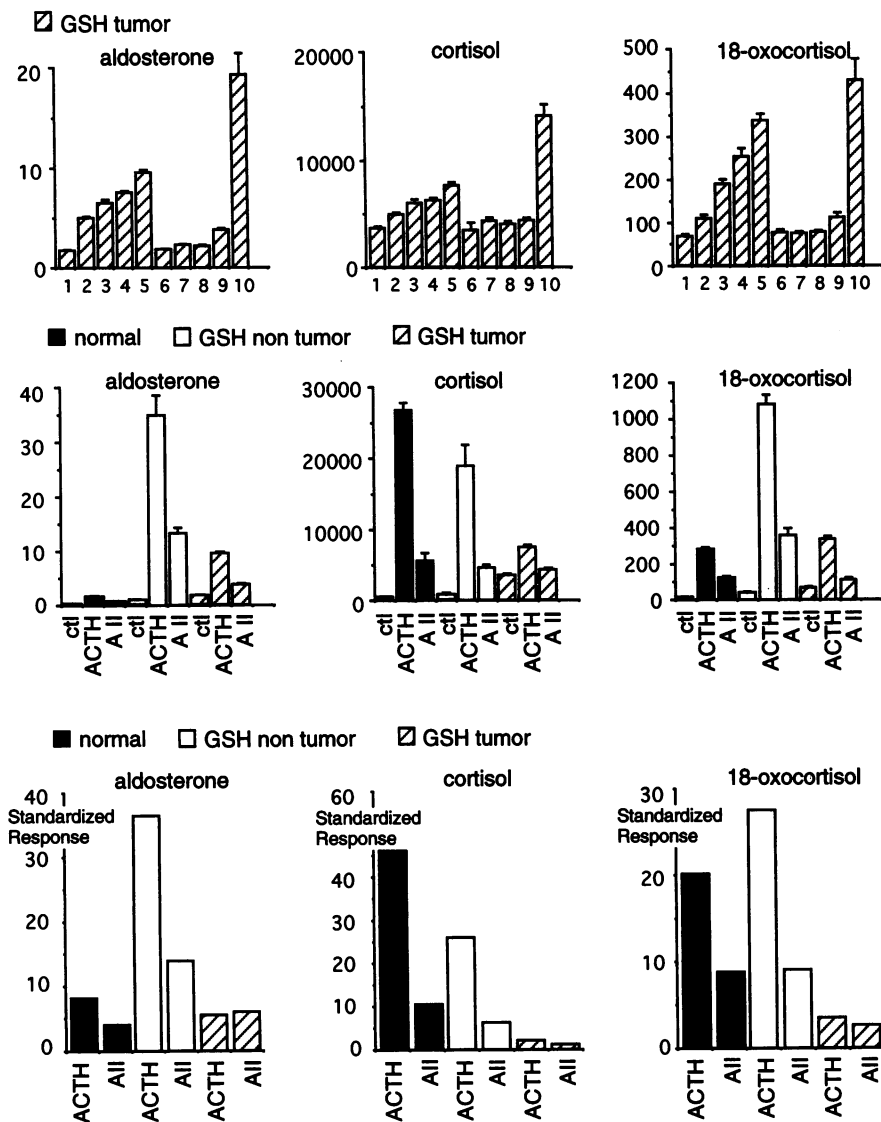


Figure 5. Steroid production in cultured adrenal cells. Results determined from media incubated with cells 24–48 h after initiation of the experiments are shown; media collected for the 0–24-h period and after the final 2 h of stimulation gave comparable results. The results of 18-hydroxycortisol (not shown) and 18-oxocortisol determinations were highly correlated. In the upper panel, amounts (nanograms per milliliter) of aldosterone, cortisol, and 18-oxocortisol produced by GSH adrenal tumor cells cultured in the presence of various concentrations of ACTH and AII are shown. Lanes 1–5 depict results from cultures containing no ACTH, 10⁻¹¹ M, 10⁻¹⁰ M, 10⁻⁹ M, and 10⁻⁸ M ACTH, respectively; lanes 6–9 depict the results from cultures containing 10⁻¹⁰ M, 10⁻⁹ M, 10⁻⁸ M, and 10⁻⁷ M AII, respectively; and lane 10 depicts results from cultures containing both 10⁻⁸ M ACTH and 10⁻⁷ M AII. In the center panel, steroid determinations are shown from media incubated with normal adrenal cells, nontumorous GSH cells, and GSH tumor cells cultured under three different conditions: with no addition of ACTH or AII (*ctl*), with 10⁻⁸ M ACTH (*ACTH*), or with 10⁻⁷ M AII (*AII*). In the lower panel, the amounts of steroids produced by the three cell types on incubation with 10⁻⁸ M ACTH (*ACTH*) or 10⁻⁷ M AII (*AII*) are shown as the fold induction over the basal levels observed in the corresponding unstimulated cells.

should result in the production of aldosterone, 18-hydroxycortisol, and 18-oxocortisol by the hybrid gene product. Consequently, we cultured adrenal cells from the tumor, from the surrounding nontumorous tissue from the GSH patient, and from normal adrenal tissue, in the presence of various concentrations of ACTH and AII, and measured the steroids produced (Fig. 5). Northern blots of RNA prepared from the cultured cells at the conclusion of the experiments were analyzed to determine relative levels of expression of the *CYP11B* transcripts as well as those for the steroidogenic genes *CYP11A1* (encoding

P450_{scc}), *3βHSD*, and *CYP17* and the ACTH and type 1 angiotensin II receptors (AT1) (Fig. 6).

Production of steroids by cultured cells. The effects on the cells of incubation with ACTH and AII at 0–24 h (data not shown) and 24–48 h (Fig. 5) were similar but more pronounced after the longer time period. Unstimulated tumor cells produced between five and nine times the amount of steroids produced by similarly cultured control normal adrenal cells (nine, seven, eight, and five times for aldosterone, cortisol, 18-hydroxycortisol, and 18-oxocortisol, respectively). In the cells from the

surrounding GSH adrenal tissue, no signal was detected with a 5' *CYP11B2* probe (not shown). However, strong signals are seen in the tumor with a 3' *CYP11B2* probe (*H*), which thus detects the hybrid *CYP11B* transcripts, and a 3' *CYP11B1* probe (*G*). In the nontumorous GSH tissue *CYP11B1* mRNA is detected by the 3' cRNA probe in the zona glomerulosa (*I*) and at a lower level in the zona fasciculata (*J*). The hybrid gene mRNA, detected by the 3' *CYP11B2* probe, is also seen in both zones (*K* and *L*, respectively). Hybridization with a 5' *CYP11B1* oligonucleotide probe in the presence of an unlabeled heterologous (*CYP11B2*) competitor allows the simultaneous detection of the expression of the *CYP11B1* and hybrid mRNAs in the zona glomerulosa and zona fasciculata (*M* and *N*, respectively). A section hybridized with the same 5' *CYP11B1* probe, but in the presence of its unlabeled homologous (*CYP11B1*) competitor, was negative (*O*). Indicated in the photographs are the capsule (*Ca*), zona glomerulosa (*ZG*), zona fasciculata (*ZF*), and zona reticularis (*ZR*). The yellow bar is 500 μm in the upper panel and 50 μm in the other panels.

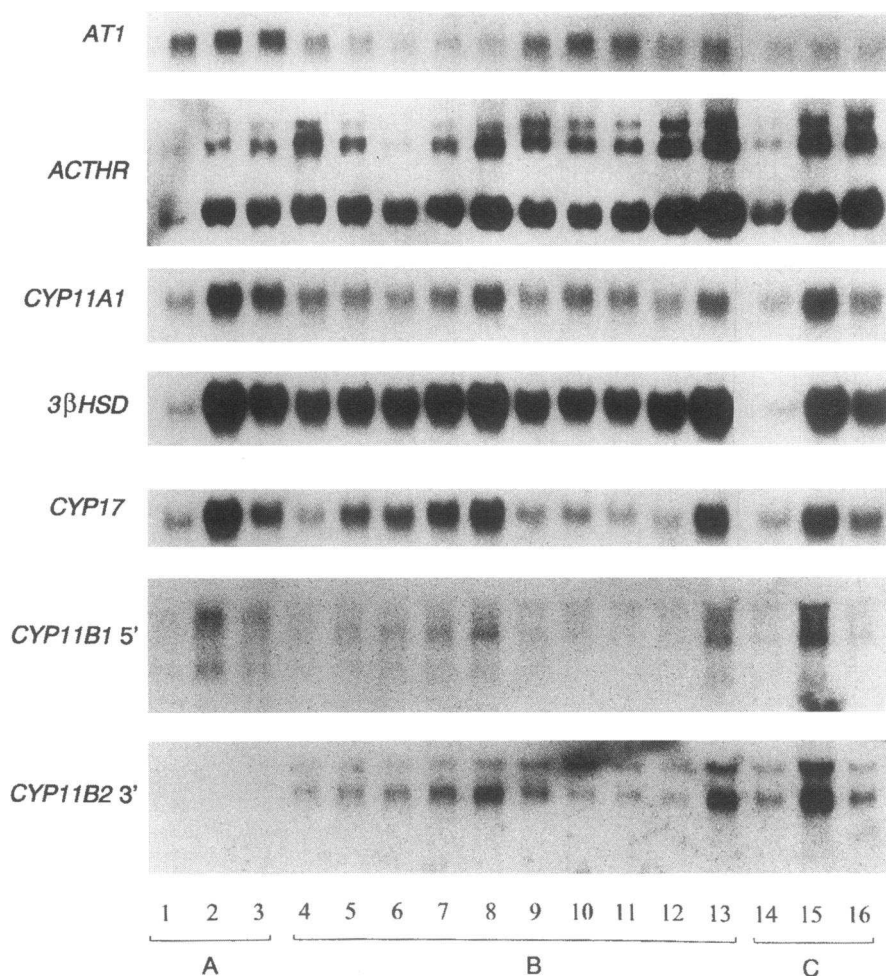


Figure 6. Northern blots of total RNA from cultured adrenal cortical cells hybridized with the radioactively labeled probes indicated at the left of the figure (see text for details). (A) Normal adrenal cells incubated with no addition of ACTH or AII (lane 1), 10^{-8} M ACTH (lane 2), or 10^{-7} M AII (lane 3); (B) adrenal tumor cells from a patient with GSH incubated with no addition of ACTH or AII (lane 4), 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M ACTH (lanes 5–8), 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M AII (lanes 9–12), or both 10^{-8} M ACTH and 10^{-7} M AII (lane 13); (C) nontumorous adrenal cells from a patient with GSH incubated with no addition of ACTH or AII (lane 14), 10^{-8} M ACTH (lane 15), or 10^{-7} M AII (lane 16).

adrenal tumor, we observed a dose-dependent response to increasing levels of ACTH for aldosterone, cortisol, 18-hydroxycortisol, and 18-oxocortisol production. The maximal responses compared with basal levels were, respectively, five-, two-, six-, and fivefold increases for these four steroids. These maximal responses are considerably lower than those observed in experiments with cells from the surrounding nontumorous GSH tissue or normal adrenal cells (as can be seen from the standardized responses shown in Fig. 5), suggesting a defect in the signaling or regulatory pathways or defective steroid synthesis in the tumor.

Tumor cells cultured in the presence of increasing levels of AII did not produce more steroids than unstimulated cells except at the supraphysiological concentration of 10^{-7} M AII, where there was a significantly increased production of all C18-modified steroids, which was lower than that induced by ACTH. Coincubation with both 10^{-8} M ACTH and 10^{-7} M AII led to a synergistic increase in production of all steroids as expected (18).

Less tissue was available for cell culture experiments from the surrounding nontumorous cells from the GSH patient than from the tumor itself. Consequently, the nontumorous cells were incubated with a single level of ACTH (10^{-8} M) or AII (10^{-7} M) that had been shown previously to give a maximal response of steroidogenic mRNA and steroid production in human adrenal cell culture experiments (19). The basal production of C18-modified steroids in the unstimulated nontumorous GSH cells

was three to five times higher than that of unstimulated normal adrenal cells, whereas cortisol production was similar. Incubation with ACTH strongly stimulated aldosterone (36-fold), cortisol (26-fold), 18-hydroxycortisol (12-fold), and 18-oxocortisol (27-fold) production in the nontumorous GSH cells. Similarly cultured normal adrenal cells showed a comparable increase in cortisol production (50-fold) with ACTH stimulation, but the increase in aldosterone production (8-fold) induced by ACTH was diminished compared with that observed in GSH cells. The much stronger aldosterone response to ACTH in the nontumorous GSH cells, despite an already fourfold higher production in the unstimulated cells compared with the normal control cells, starkly demonstrates the effect of the presence of the hybrid gene in the GSH adrenal cells. Similar differences were observed for 18-oxocortisol production. The nontumorous GSH cells and the normal adrenal cells also respond to incubation with 10^{-7} M AII, though not as strongly as to ACTH. The responses for the nontumorous cells were 14-, 6-, 4-, and 9-fold induction for aldosterone, cortisol, 18-hydroxycortisol, and 18-oxocortisol, respectively, while for normal cells the induction was 4-, 10-, 6-, and 8-fold, respectively.

Northern blot analysis of mRNA from cell culture experiments. Induction of the expression of the hybrid *CYP11B* mRNAs by increasing levels of ACTH is clearly evident in the Northern blot which was hybridized with an oligonucleotide probe containing sequence specific to the 3' end of the *CYP11B2* gene (Fig. 6). No *CYP11B2* mRNAs are detected on

this blot when a 5' end *CYP11B2*-specific probe was used (even in AII-stimulated GSH tumor cells or similarly stimulated cells from a normal adrenal), consistent with all our experimental data to date which indicates that the level of expression of *CYP11B2* mRNA is below the level of detection possible when gene-specific oligonucleotides are used as probes in Northern blot analyses. An induction of these mRNAs in the nontumorous cells from the GSH adrenal, which is greater than that observed in the tumor cells, is also observed on incubation with 10^{-8} M ACTH, consistent with the steroid results. Incubation with AII clearly has no effect on the level of expression of the hybrid gene.

It was not possible to obtain a clear hybridization signal when the blot shown in Fig. 6 was probed with the 3' *CYP11B1*-specific oligonucleotide, so the effect of ACTH on *CYP11B1* expression in these cultured cells could not be determined directly. Hybridization of the blot with an oligonucleotide specific to the 5' end of *CYP11B1* also produced a diffuse hybridization signal, but the simultaneous detection of the hybrid mRNA species aids in its interpretation. Induction of expression of the hybrid gene by ACTH is again most apparent in the cells cultured from the nontumorous portion of the GSH adrenal. The 4.2-, 3.6-, and 2.2-kb *CYP11B1* transcripts are seen in the control normal adrenal RNA and are induced in the presence 10^{-8} M ACTH and more weakly by 10^{-7} M AII. *CYP11B1* mRNAs are also weakly induced by 10^{-8} M ACTH in both the tumor and nontumorous GSH cells, but the effect of AII is not apparent. There is the suggestion in this and the previous hybridization that induction of the expression of the hybrid mRNA and the *CYP11B1* gene is more marked by incubation with both ACTH and AII.

The level of *CYP11A1* mRNA expression was similar in unstimulated cultures of normal adrenal cells, nontumorous GSH cells, and GSH tumor cells. Expression of this mRNA was increased by both ACTH and AII in the normal adrenal cells and the nontumorous GSH cells, however there was virtually no increase in expression in GSH tumor cells incubated with either ACTH or AII at any concentration, suggesting a dysregulation of the expression of this mRNA in the tumor cells. The expression of *CYP17* mRNA was similar in all three cell types and was induced by both ACTH and AII in the normal adrenal cells, consistent with previous experiments (19), and in the nontumorous GSH cells. In the GSH tumor cells, the *CYP17* mRNA was induced in a dose-dependent fashion by increasing concentrations of ACTH, but its expression was not stimulated by incubation with AII. Transcripts of the *3 β HSD* gene were detected at similar levels in the unstimulated control normal adrenal cells and the nontumorous GSH cells, and in both cases their expression was strongly increased by ACTH (50-fold) and AII (10-fold). Basal expression of *3 β HSD* was much higher in the unstimulated tumor cells, and mRNA levels were further increased by both ACTH (up to 15-fold) and AII (up to 4-fold) stimulation in a dose-dependent fashion.

Consistent with previous studies, the expression of the ACTH receptor mRNA levels in the normal and nontumorous GSH cells was strongly induced by both ACTH and AII (18). In the tumor cells, the basal expression of the mRNA was much higher and was only induced by the highest concentrations of 10^{-8} M ACTH and 10^{-7} M AII. Incubation with both hormones resulted in a synergistic increase in mRNA production. In view of the strong expression of the ACTH receptor in the tumor cells, the lack of induction of *CYP11A1* mRNA and the weak

increase in steroid production on stimulation by ACTH were surprising. AT1 mRNA was readily detected in unstimulated control normal adrenal cells and was mildly induced by both ACTH and AII treatment. In the nontumorous GSH cells, expression of AT1 mRNA was less easily detectable in the unstimulated cells and there was minimal response to ACTH or AII. The tumor cells showed no response to incubation with increasing levels of ACTH and a complex response to incubation with AII; incubation with concentrations of AII up to 10^{-8} M increased AT1 mRNA but 10^{-7} M AII was a less effective stimulant.

Discussion

Expression of CYP11B genes in the normal adrenal cortex. In rodent adrenal cortex, expression of the *CYP11B1* and *CYP11B2* genes is strictly delimited to the zona fasciculata and the zona glomerulosa, respectively (28, 29). Thus, synthesis of aldosterone is carried out by the product of the *CYP11B2* gene in the zona glomerulosa, whereas glucocorticoid hormone (corticosterone in rodents) is synthesized in the zona fasciculata by the product of the *CYP11B1* gene. In bovine species all *CYP11B* genes, which are almost identical in sequence, have C18 activities when expressed in COS cells (32), but the C18 activity is suppressed in the zona fasciculata by a poorly understood factor associated with the mitochondrial membrane (30, 33, 34). In principle the human adrenal cortex could follow the rodent, the bovine, or a completely different mechanism of zonation. Studies showing that *CYP11B2* but not *CYP11B1* has the enzymatic activities required for aldosterone synthesis (1, 2) seemed to suggest that humans more closely resembled the rodent than the bovine model, but it remained possible that C18 activity in the zona fasciculata was still suppressed by a bovine-like mechanism, as no definitive *in situ* hybridization study has been previously reported. Our *in situ* hybridization results demonstrate that *CYP11B1* is expressed in both the zona fasciculata (as expected) and the zona glomerulosa, a result which is consistent with previously reported RT-PCR experiments showing expression of *CYP11B1* in cultured human zona glomerulosa cells (2).

In contrast, *CYP11B2* expression seems to be largely limited to the zona glomerulosa, although it is not possible to rule out a low level of expression in the zona fasciculata. That expression of *CYP11B2* is essentially limited to the zona glomerulosa is supported by the observation that the 17-hydroxylated steroids 18-hydroxycortisol and 18-oxocortisol are normally produced in relatively small quantities (urinary excretion of 10–150 μ g/24 h and < 20 μ g/24 h, respectively). A significant expression of *CYP11B2* in the zona fasciculata, but with a suppression of 18-hydroxylase activity, is contradicted by the phenotypes of patients with 11 β -hydroxylase deficiency. Patients with mutations in the *CYP11B1* gene which completely abrogate 11 β -hydroxylase activity fail to make cortisol efficiently (35). In the 11 β -hydroxylase-deficient patient, ACTH levels are elevated, the adrenal is hyperplastic, and a low level of cortisol is produced at the expense of high levels of 11-deoxysteroid precursors. This would not be the case if *CYP11B2* were expressed in the zona fasciculata, as it encodes an enzyme with an 11 β -hydroxylase activity equivalent to that of *CYP11B1* (1, 2). However, the fact that cortisol is made at all suggests that some expression of *CYP11B2* in the inner adrenal cortex may be possible.

In vitro studies show that CYP11B1 has ~ 10% of the 18-hydroxylase activity of CYP11B2 (36). This result, combined with the fact that cortisol is normally produced at up to 1,000 times the level of aldosterone, leads to the expectation that the amount of 18-hydroxycortisol produced should be ~ 100 times the amount of 18-hydroxycorticosterone. In fact, the ratio is usually < 10 times, suggesting that 18-hydroxylase activity is suppressed in the zona fasciculata, as it is in the bovine adrenal. However, suppression of 18-hydroxylase activity must be either incomplete or specific to the CYP11B1 enzyme or the hybrid steroids typical of GSH could not be made. The human adrenal would then seem to be intermediate in character between the rodent and bovine adrenals. It expresses two CYP11B enzymes with specialized activities and expression of CYP11B2 is largely limited to the zona glomerulosa, as in rodents, but CYP11B1 is expressed in all zones of the adrenal cortex and a separate mechanism by which its 18-hydroxylase activity is inhibited persists in the zona fasciculata, as in cattle.

Action of the hybrid gene in GSH cells. The occurrence of adrenal tumors in the pedigree studied here has enabled us to study the hybrid CYP11B1/CYP11B2 gene which causes GSH in the tissue in which it is expressed. The hybrid genes in this pedigree have been detected by both Southern blot analysis (23) and PCR of the hybrid gene, and the crossover point was determined by sequencing of the amplified DNA. Expression of the hybrid gene has been detected by RT-PCR of RNA from adrenal tissue and by Northern blot analysis of that RNA. By in situ hybridization, the expression of the hybrid gene has been demonstrated to occur in the zona fasciculata of the adrenal cortex, a key feature of the explanatory model for this disease. The hybrid gene is also expressed in the zona glomerulosa, where it appears not to be downregulated to the same extent as CYP11B2 by the reduced plasma renin activity. The expression of the hybrid gene has further been shown to respond to stimulation by ACTH in cell culture experiments and in fact to be present at higher levels than the normal CYP11B1 mRNAs, a result which could not have been predicted in advance. The higher expression of the hybrid gene was observed in both the cultured cells and the RNA prepared directly from the adrenal tissue. Expression of the hybrid mRNA is expected to result in increased production of aldosterone and the hybrid steroids 18-hydroxycortisol and 18-oxocortisol, all of which are hypersecreted in GSH. In summary, we have been able to confirm all of the principal features of the expression and regulation of the hybrid gene that are postulated to occur in GSH.

The strong expression of the hybrid gene relative to the CYP11B1 gene was an unexpected result in this study. Since the hybrid gene contains 5' sequences from CYP11B1, it has been assumed that these would result in a similar level of expression of hybrid gene and CYP11B1 mRNAs. Given that there are two copies of the CYP11B1 gene and only one copy of the hybrid gene, the hybrid gene mRNA would therefore be expected to comprise about one-third of the CYP11B mRNA in the zona fasciculata. In fact, the hybrid mRNA is slightly more abundant than CYP11B1 mRNA. The 5' sequences from CYP11B1 in the hybrid gene are sufficient to ensure expression of the hybrid mRNA in the zona fasciculata, but its greater abundance must be explained by sequences in the 3' end of the gene. For example the hybrid gene may lack CYP11B1 genomic sequences, which lead to downregulation in response to cortisol production or lower ACTH levels, or sequences which lead to a more unstable mRNA under these conditions. These sequences

would have to lie downstream of the breakpoint, perhaps in intron 5 or the 3' untranslated region which differ in size between the two genes. Destabilizing sequences are frequently found in the 3' UTRs of inducible mRNAs. Similarly, although CYP11B2 mRNA was downregulated in the GSH adrenal, both CYP11B1 and hybrid gene transcripts were readily detected in the zona glomerulosa. This is consistent with there being a regulatory element in the 5' region of CYP11B2 which is responsible for controlling its expression but which is absent in the hybrid and CYP11B1 genes. The sequences involved in the regulation of CYP11B genes have not been studied extensively but will presumably be of great significance in disorders where the genes are rearranged (37).

Comparison of tissue from the nontumorous GSH adrenal and normal adrenals. The levels of expression of the ACTH receptor, CYP11A1, 3βHSD, and CYP17 mRNAs in the cells cultured from the nontumorous part of the GSH adrenal were similar to those detected in cells cultured from a normal adrenal (Fig. 6). Furthermore, the regulation of expression of these mRNAs by ACTH and AII was equivalent. Thus, the ACTH receptor mRNAs were strongly induced by both AII (10^{-7} M) and ACTH (10^{-8} M), while the mRNAs encoding CYP11A1, 3βHSD, and CYP17 were more strongly induced by ACTH than by AII. The cells cultured from the nontumorous portion of the GSH adrenal differed from those of a normal adrenal in that the level of expression of the AT1 receptor was lower, a result which is not unexpected in an adrenal in which the renin-angiotensin system has been downregulated throughout life (38). The CYP11B1-specific transcripts were difficult to detect, but the unique 2.2- and 3.6-kb bands seen in normal adrenals are also evident in cells of the GSH adrenal. While in normal adrenals these are induced by both ACTH and AII (with the effect of ACTH being much stronger), induction by AII is not evident in the GSH adrenal. Finally, the most important difference between these two cell types is that hybrid gene transcripts are expressed only in the GSH adrenal. As with the CYP11B1 transcripts, these are clearly induced by 10^{-8} M ACTH but appear to be unresponsive to 10^{-7} M AII.

The principal difference in steroid production between the nontumorous GSH cells and the normal adrenal cells was the greater production of the C18-modified steroids aldosterone, 18-hydroxycortisol, and 18-oxocortisol and the greater response of all three steroids to stimulation by ACTH in the GSH cells. These differences in steroid production can be explained by the expression of the hybrid gene in the nontumorous GSH cells. Expression of the hybrid gene results in production of aldosterone, 18-hydroxycortisol, and 18-oxocortisol in the zona fasciculata cells of the GSH adrenal, and the higher production of these steroids on incubation with ACTH is consistent with the increased expression of the hybrid gene. ACTH stimulation results in both an enhanced transport of cholesterol substrate to the mitochondria, which is a rate-limiting step in all steroid synthesis, and increased transcription of steroidogenic and ACTH receptor mRNAs (39). However, the chronic response to ACTH and our experimental results can be best explained in terms of differences in mRNA production between the two cell types. With respect to cortisol production, the nontumorous GSH cells and the normal adrenal cells showed similar responses to incubation with ACTH and AII as expected.

Steroid and mRNA production in the tumor cells. The cells cultured from the GSH tumor differed from the nontumorous tissue in several respects. Most striking were the high basal

levels of expression of the ACTH receptor and 3β HSD mRNAs in the tumor. While induction of the ACTH receptor by ACTH was no longer possible, its level of expression was still augmented by AII (indicating that the AT1 receptors were functional). ACTH inducibility of *CYP17* was maintained and induction of 3β HSD was still possible (indicating that the ACTH receptors were also functional), but the ability of ACTH to induce *CYP11A1* gene expression was lost. While ACTH receptor and 3β HSD mRNA were induced by AII, mRNA responses to AII were generally poor in the tumor cells, consistent with the low level of expression of AT1 mRNA.

Basal production of cortisol and the C18-modified steroids was greater in the tumor cells than in the other two cell types but did not respond as strongly to incubation with ACTH. This relative failure of the tumor cells to augment steroid production could be explained by the failure of induction of *CYP11A1* mRNA which encodes the enzyme mediating the first step in steroid synthesis. The product of the *CYP11A1* gene is functional as is evidenced by the production of the steroid hormones, but levels of this mRNA in the stimulated tumor cells were lower than in the corresponding nontumor GSH or normal adrenal cells. As expected from the mRNA results, steroid production in the tumor cells was relatively unresponsive to incubation with AII.

The underlying reason for the adrenal hyperplasia and tumors in this pedigree is not known. The fact that both of the micronodular hyperplasias as well as the two tumors occurred in individuals affected with GSH suggests that the chromosomal region containing the hybrid gene may be involved but is not conclusive. Involvement of that region could be mediated directly by a defect in *CYP11B* gene expression, by other genes in the duplicated intergenic region or by another gene linked to the *CYP11B* genes. Alternatively, the genetic disposition to adrenal pathologies could be due to an unlinked gene which segregates by chance with the hybrid gene in this pedigree. The adrenals removed from the patients with tumors also displayed the presence of micronodules, suggesting that these nodules may predispose to tumor formation. If this is the case, other family members are at risk to develop adrenal micronodular hyperplasia and tumors which may not become evident until an advanced age. While it is possible to speculate on scenarios in which the defect in *CYP11A1* regulation could have led to a local hyperplastic response or conversion of a nodule to a tumor, it should be recognized that adrenal tumors are often defective in steroid synthesis and have aberrant regulation of steroidogenic mRNAs. In fact, the tumor studied was unusual in that it produced all of the normal steroid hormones in a regulatable fashion.

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