Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8516–8520, November 1990
Biochemistry

Cloning and sequence of the human adrenodoxin reductase gene

(steroid hormones/electron transfer/flavoprotein/RNA splicing/ferredoxin reductase)

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Communicated by John A. Clements, August 22, 1990 (received for review July 16, 1990)

ABSTRACT **Adrenodoxin reductase (ferrodoxin:NADP$^+$ oxidoreductase, EC 1.18.1.2) is a flavoprotein mediating electron transport to all mitochondrial forms of cytochrome P450. We cloned the human adrenodoxin reductase gene and characterized it by restriction endonuclease mapping and DNA sequencing. The entire gene is approximately 12 kilobases long and consists of 12 exons. The first exon encodes the first 26 of the 32 amino acids of the signal peptide, and the second exon encodes the remainder of signal peptide and the apparent FAD binding site. The remaining 10 exons are clustered in a region of only 4.3 kilobases, separated from the first two exons by a large intron of about 5.6 kilobases. Two forms of human adrenodoxin reductase mRNA, differing by the presence or absence of 18 bases in the middle of the sequence, arise from alternate splicing at the 5′ end of exon 7. This alternately spliced region is directly adjacent to the NADPH binding site, which is entirely contained in exon 6. The immediate 5′ flanking region lacks TATA and CAAT boxes; however, this region is rich in G+C and contains six copies of the sequence GGGCGGG, resembling promoter sequences of "housekeeping" genes. RNase protection experiments show that transcription is initiated from multiple sites in the 5′ flanking region, located about 21–91 base pairs upstream from the AUG translational initiation codon.**

The conversion of cholesterol to pregnenolone is the first and rate-limiting step in the synthesis of all steroid hormones. This reaction is mediated by the mitochondrial cholesterol side chain-cleavage enzyme, cytochrome P450scc (reviewed in ref. 1). Like all mitochondrial forms of cytochrome P450, this enzyme receives electrons from NADPH via two electron transport intermediates—a flavoprotein and an iron-sulfur protein. Adrenodoxin reductase (ferrodoxin:NADP$^+$ oxidoreductase, EC 1.18.1.2), which is loosely associated with the inner mitochondrial membrane, receives electrons from NADPH. These electrons are passed to adrenodoxin, which is freely in solution in the mitochondrial matrix, shuttling electrons to all mitochondrial forms of P450 (2, 3). Adrenodoxin may then pass the electrons to a mitochondrial form of P450 bound to the inner mitochondrial membrane (4). In the adrenal, P450scc and P450c11, which mediates 11-hydroxylation, 18-hydroxylation, and 18-methyl oxidation of steroids, compete for available electrons borne by adrenodoxin. Hepatic P450c26/25, which mediates both bile acid 26-hydroxylation and vitamin D 25-hydroxylation (5), and renal vitamin D 1-hydroxylase (6) are examples of other mitochondrial P450 enzymes using the same electron-transport system. These proteins may also be termed "ferredoxin" and "ferredoxin reductase," but because this laboratory's work centers on the adrenal, we generally use the term "adrenodoxin reductase."

The human cDNAs for all three components of the cholesterol side chain-cleavage system have been cloned. Human P450scc cDNA (7) is encoded by a single gene (8) located on chromosome 15 (7) that consists of nine exons spanning about 30 kilobases (kb) (9). Adrenodoxin cDNA is found in three different forms, differing only in their sites of polyadenylylation (10). Nevertheless there are two genes for adrenodoxin (11, 12) on chromosome 11q13→qter (13), while two adrenodoxin pseudogenes are found on chromosomes 20cen→q13.1 (13) and on chromosome 21 (12). Adrenodoxin reductase cDNA sequencing suggests that the mRNA undergoes alternate splicing into two different forms, with the less abundant form containing 18 nucleotides encoding six amino acids in the middle of the protein (14). Both of these mRNAs must be encoded by the same gene, as blotting studies of human genomic DNA and of DNA from mouse/human somatic cell hybrids indicate the human genome contains only one gene for adrenodoxin reductase located on chromosome 17cen→q25 (14).

We now complete the final step in the genetic characterization of the cholesterol side chain-cleavage system—the cloning and sequencing† of the gene for adrenodoxin reductase. This gene eluded cloning from several libraries in bacteriophage λ but was readily cloned from cosmid libraries. It consists of 12 exons; 10 exons are confined to about 4.3 kb of DNA, but two upstream exons bring the overall size of the gene to 12 kb. The 5′ flanking DNA of the gene lacks TATA and CAAT boxes, possibly accounting for its multiple transcriptional initiation sites.

MATERIALS AND METHODS

Cloning and Sequencing. A library of human placental genomic DNA partially digested with *Mbo* I was cloned in the *Bam*HI site of cosmid pWE15 and propagated in *Escherichia coli* NM554 (Stratagene). Host cells were plated at 50,000 colonies per 150-mm plate on LB agar containing 100 μg of ampicillin per ml. Screening of the libraries, purification of the positive colonies, and preparation of cosmid DNA were done according to the Stratagene instruction manual with isolated ^{32}P-labeled fragments of the human adrenodoxin reductase cDNA from λhtAR (14). Genomic fragments hybridizing to the cDNA were subcloned into pBluescript II. Single- or double-stranded DNA for sequencing was isolated from *E. coli* XL1-blue transformed with various adrenodoxin reductase subclones. Dideoxy sequencing was performed by using the Sequenase sequencing kit (United States Biochemical) and ^{35}S-labeled dATP.

RNase Protection Analysis. RNase protection experiments were done essentially as described (15). Total cellular RNA was isolated from JEG-3 choriocarcinoma cells or human fetal adrenal tissues as described (16). A 0.9-kb *Bam*HI–*Nco* I fragment of the human adrenodoxin reductase gene containing only the 5′ flanking region was cloned into pBluescript, prepared by CsCl banding, digested with *Dde* I, and

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M38255 and M38256).

transcribed with T7 polymerase and [^{32}P]UTP to generate probe A. A 0.7-kb *Sac* I–*Stu* I fragment containing 5′ flanking DNA, exon 1, and part of intron 1 was similarly cloned and banded. This 0.7-kb fragment contains an internal *Stu* I site that was partially methylated and resistant to *Stu* I digestion when the plasmid was propagated in *dcm*$^+$ *E. coli* XL-1 blue. The pBluescript clone of this fragment was then propagated in *dcm*$^-$ *E. coli* GM2163, permitting digestion of the internal *Stu* I site. Transcription with T7 polymerase generated probe B (see Fig. 3). A 15-μg sample of total RNA was mixed with 5×10^5 cpm of riboprobe in 30 μl of hybridization buffer (80% formamide/40 mM Pipes, pH 6.7/0.4 M NaCl/1 mM EDTA), denatured by boiling for 5 min, and incubated for 12 hr at 58°C. After hybridization, RNA digestion buffer [350 μl containing 0.3 M NaCl, 10 mM Tris chloride (pH 7.5), 5 mM EDTA, 1–4 μg of RNase A, and 50 units of RNase T1] was added to the mix, which was incubated at 37°C for 30 min; 20 μl of 10% $NaDodSO_4$ containing proteinase K at 2 mg/ml was added, and the incubation was continued at 37°C for 30 min. After 1:1 (vol/vol) phenol/chloroform extraction, the protected RNA was precipitated twice with carrier tRNA and ethanol. The products were analyzed by electrophoresis on 5% acrylamide/7 M urea gels.

RESULTS

Cloning of the Adrenodoxin Reductase Gene. We initially tried to clone the adrenodoxin reductase gene from a phage library. Using the 1.1-kb *Eco*RI–*Hin*dIII fragment from our human adrenodoxin reductase cDNA (14) as probe, we screened 1.5×10^6 plaques from a library in EMBL3 (Clontech), 1×10^6 plaques from a library in phage λ Dash (17), and 1×10^6 plaques from a library in Charon 4A (18). No positive clone was identified. The same probe was later used to screen the amplified human cosmid library in TCD108 (19). Screening of 0.5×10^6 cosmids yielded five identical clones. These contained the middle of the adrenodoxin reductase gene but contained rearranged DNA of undetermined origin. Approximately 1×10^6 cosmids from a commercial cosmid library (Stratagene) were then screened with the 1.1-kb *Eco*RI–*Hin*dIII cDNA probe. Two types of clones gave positive hybridization signals and contained the human adrenodoxin reductase gene. One termed cAR111 contained all but the first and second exons of the gene; the other, termed cAR112, contained all but exon 1. A cosmid termed cAR113 containing the complete adrenodoxin reductase gene was subsequently obtained by screening another 10^6 colonies with the 387-base-pair (bp) *Eco*RI–*Pst* I fragment corresponding to the 5′ end of the adrenodoxin reductase cDNA (Fig. 1). These cosmid clones, which encompass over 60 kb of DNA, were initially characterized by comparing their restriction patterns on Southern blots with those of genomic DNA. Regions of the cosmid clones containing part of the adrenodoxin reductase gene were identified by finding identical *Eco*RI, *Hin*dIII, and *Bam*HI fragments in both the cosmid and genomic DNA. About 30 kb was mapped in this fashion (Fig. 1). Fragments were then subcloned into pBluescript for detailed restriction mapping and sequencing.

Structure of the Adrenodoxin Reductase Gene. The entire gene consists of 12 exons divided by 11 introns (Fig. 1). The gene is about 12 kb long, consistent with our previous estimation based on probing Southern blots of human genomic DNA with the full-length cDNA (14). The structural analysis of the isolated gene confirms that the whole gene is contained on a single *Xba* I or *Kpn* I fragment but that digestion with *Eco*RI, *Hin*dIII, or *Bam*HI generates more than one fragment (Fig. 1). Fig. 2 shows the sequence of the adrenodoxin reductase gene. We sequenced 6803 bp, including 904 bp of the 5′ flanking region and all of the exons and introns except about 5 kb in the second intron. All sequences were confirmed on overlapping clones. The sequences of the exons agree perfectly with our published cDNA sequence (14). Three allelic variations distinguished our human adrenal cDNA clone from the clone obtained from a testis cDNA library: CGG, arginine, instead of CAG, glutamine, at codon 123; CGG, arginine instead of CGA, arginine, at codon 327; and GCAA instead of GCAC following the AAATAAAA sequence in the 3′ untranslated region (14). The adrenodoxin reductase gene we cloned contains all three of these allelic variants but no others. The introns of the adrenodoxin reductase gene only interrupt the protein-coding sequences, not the 5′ or 3′ untranslated regions, and all of the intron/exon boundaries strictly follow the GT/AG rule (20). The additional 18 nucleotides found in one of the two forms of adrenodoxin reductase mRNA (14) are located at the 5′ end of exon 7 following the sequence TCTGATGACTCCAAG. Both this sequence at the 3′ end of intron 6 and the 18 bases at the beginning of exon 7 (GCCCTCCTTTTGTGCCAG) constitute 3′ splice donor sites. Thus, the presence or absence of these 18 nucleotides in the mRNA results from alternative splicing in this region of the primary transcript, as initially suggested from the cDNA sequence (14). However, CAG constitutes a better splice acceptor site than CAAG (20); this may explain why the form of adrenodoxin reductase

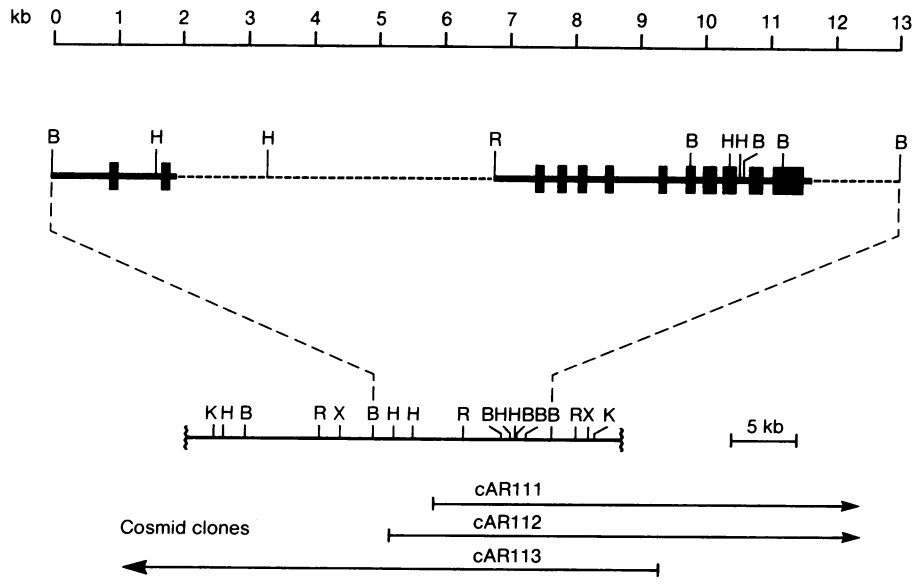

FIG. 1. Organization of the human adrenodoxin reductase gene. The upper line shows the scale in kb; the second line shows the gene. The 12 exons are indicated as boxes. The intronic and flanking DNA segments, shown as solid lines, have been sequenced; DNA segments shown as broken lines have not. The third line shows the restriction map of this region: B, *Bam*HI; K, *Kpn* I; H, *Hin*dIII; R, *Eco*RI; X, *Xba* I; note the scale to the right. Regions encompassed by the three cosmid clones obtained are shown at the bottom.

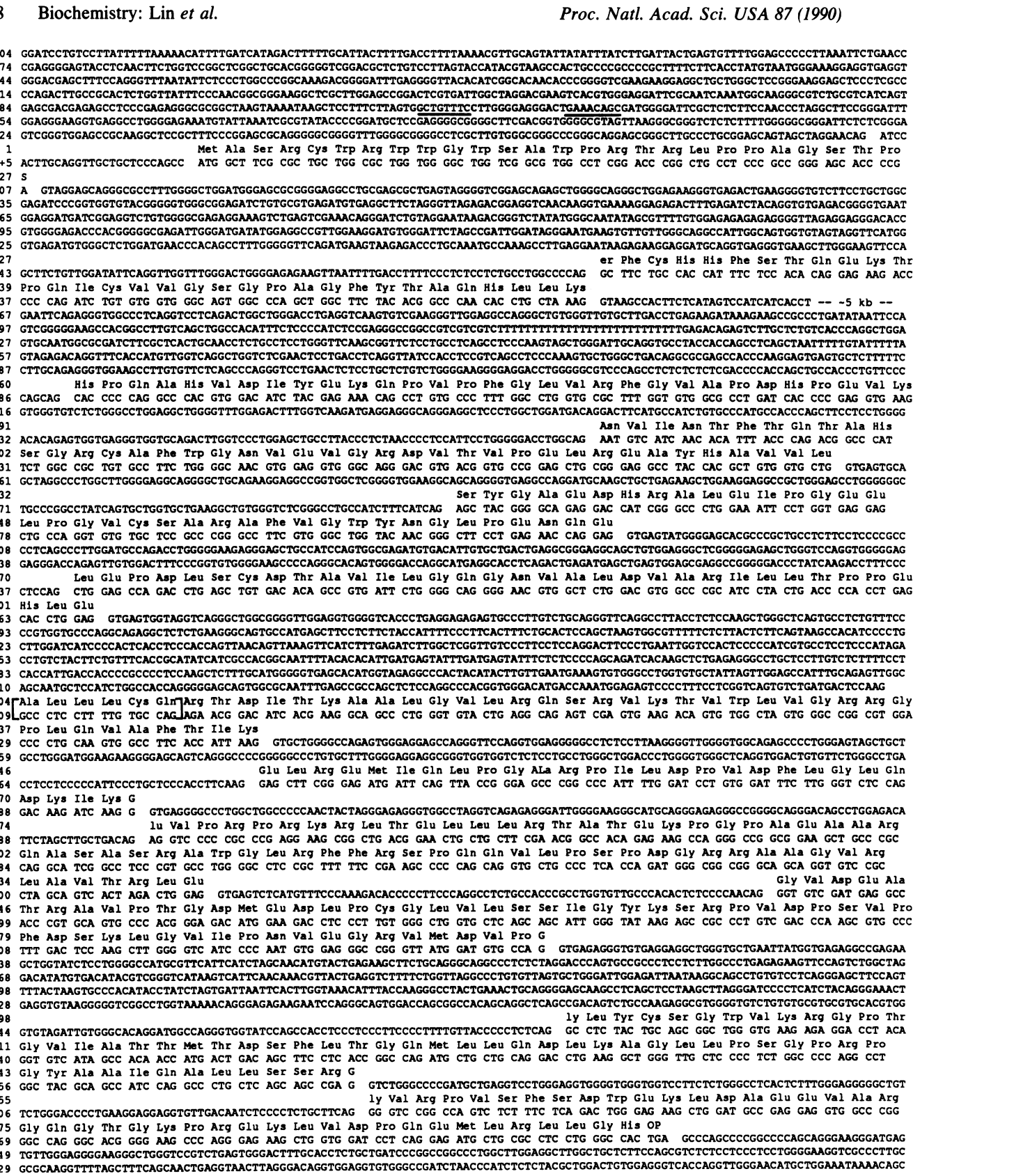

FIG. 2. Sequence of the human adrenodoxin reductase gene. Nucleotides are numbered with the principal cap site, identified in Fig. 3, designated as base 1; no correction is made in the numbering for the ≈5 kb of unsequenced DNA in the second intron. Spaces between introns and exons, between codons, and between the 5′ and 3′ flanking DNA and the cap site are for clarity only and do not represent missing bases. Amino acids are numbered from the translational initiator methionine of the preprotein rather than from the first residue of the mature protein (Ser-33). The inverted repeated sequences at −326 to −319 and at −304 to −297 are underlined. The "extra" 18 nucleotides at the beginning of exon 7 in the alternately spliced form of adrenodoxin reductase are in brackets.

mRNA containing the additional 18 bases accounts for only 10–20% of adrenodoxin reductase mRNA (14).

Mapping the Cap Site. We considered that the sequence TATTAAA (bases −229 to −235 in Figs. 2 and 3) might serve

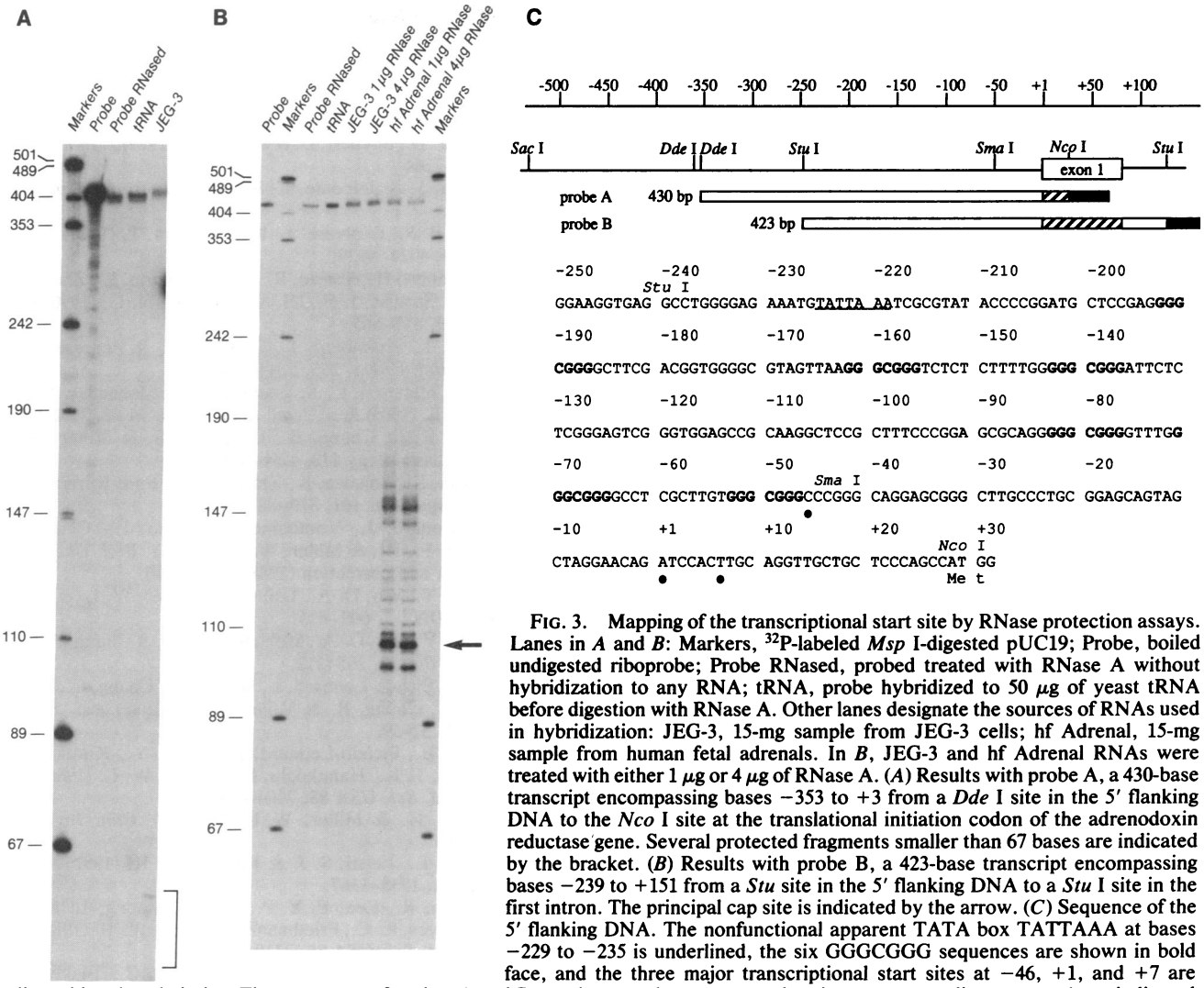

FIG. 3. Mapping of the transcriptional start site by RNase protection assays. Lanes in *A* and *B*: Markers, ^{32}P-labeled *Msp* I-digested pUC19; Probe, boiled undigested riboprobe; Probe RNased, probed treated with RNase A without hybridization to any RNA; tRNA, probe hybridized to 50 μg of yeast tRNA before digestion with RNase A. Other lanes designate the sources of RNAs used in hybridization: JEG-3, 15-mg sample from JEG-3 cells; hf Adrenal, 15-mg sample from human fetal adrenals. In *B*, JEG-3 and hf Adrenal RNAs were treated with either 1 μg or 4 μg of RNase A. (*A*) Results with probe A, a 430-base transcript encompassing bases −353 to +3 from a *Dde* I site in the 5′ flanking DNA to the *Nco* I site at the translational initiation codon of the adrenodoxin reductase gene. Several protected fragments smaller than 67 bases are indicated by the bracket. (*B*) Results with probe B, a 423-base transcript encompassing bases −239 to +151 from a *Stu* site in the 5′ flanking DNA to a *Stu* I site in the first intron. The principal cap site is indicated by the arrow. (*C*) Sequence of the 5′ flanking DNA. The nonfunctional apparent TATA box TATTAAA at bases −229 to −235 is underlined, the six GGGCGGG sequences are shown in bold face, and the three major transcriptional start sites at −46, +1, and +7 are indicated by closed circles. The structures of probes A and B are shown as bars: protected regions corresponding to exon 1 are indicated by hatched boxes; digested regions corresponding to 5′ flanking DNA or intron 1 are open boxes; and additional sequences at the 3′ end derived from the vector are indicated as filled boxes.

as a TATA box to direct transcriptional initiation. Alternatively, the existence of the six GGGCGGG sequences could result in multiple transcriptional initiation sites, as is often observed for "housekeeping" genes. Therefore, we synthesized two riboprobes for use in RNase protection experiments to distinguish these two possibilities. As shown in Fig. 3*C*, probe A is a 430-base transcript containing only the 5′ flanking region from bases −353 to +3. This would protect mRNA molecules with cap sites close to the suspected TATA box, giving protected bands of about 220 bases. Probe B is a 423-base transcript including 79 bases of exon 1 and 239 bases of the 5′ flanking region and would identify mRNA cap sites further downstream. RNA from JEG-3 cells protected several fragments of probe A smaller than 67 bases (Fig. 3*A*). Since the mRNA cap site usually occurs between 19 and 27 bp downstream from the TATA box (when it is present), the results with probe A indicate that the TATTAAA sequence underlined in Fig. 3 is not used as a TATA box and that such an element is probably absent in the adrenodoxin reductase gene.

This was supported by the protection pattern with RNA from JEG-3 cells and human fetal adrenal tissues using probe B. This probe protected multiple fragments of 100 to 170 bases (Fig. 3*B*), indicating that transcripts were initiated 21 to 91 bases upstream from the AUG codon. These bands correspond to the small bands protected by probe A. Thus, the principal cap site, indicated by the arrow, corresponds to an adenine residue 28 bases upstream from the ATG translational start site and is designated base 1 in Figs. 2 and 3.

Two other cap sites are found at +7 and at −46, with small amounts of transcription initiated from several other bases as well (Fig. 3). Since the same amount of total cellular RNA was used for JEG-3 cells and fetal adrenal tissues, the much stronger signals observed in fetal adrenal tissues indicated a higher level of expression. Longer autoradiographic exposures showed that the same transcriptional start sites are used in both JEG-3 cells and human fetal adrenals. The identity of the pattern of bands for adrenodoxin reductase mRNA from both a normal and a transformed phenotype and the identity of the pattern of bands at different concentrations of RNase A indicate that these were not the result of under- or overdigestion with RNase.

DISCUSSION

Cloning of the adrenodoxin reductase gene was considerably more difficult than anticipated. In cloning three other genes for steroidogenic enzymes, bovine P450c21 (21, 22), human P450c17 (23), and human adrenodoxin (11), we screened an average of 2×10^5 phage plaques for each positive clone identified. This is exactly the number anticipated if the human genome has 2×10^9 bp and the average phage clone contains 15 kb of human genomic DNA. By contrast, we

screened 3.5×10^6 phage clones without identifying a single positive clone, while I. Hanukoglu (personal communication) screened 10^6 plaques in a human genomic DNA library with similar unsuccessful results. However, screening of cosmid libraries readily yielded the human adrenodoxin reductase gene. These results suggest that sequences in or near the human adrenodoxin reductase gene may be toxic to the propagation of λ phage.

A search of currently available data bases (National Biomedical Research Foundation and GenBank) reveals no other proteins having significant amino acid sequence similarity to adrenodoxin reductase. Nevertheless, adrenodoxin reductase maintains two features conserved among oxidoreductases: the flavin (FAD)-binding domain and the NADPH-binding domain. The portion of the FAD-binding domain that interacts with the ADP dinucleotide has a βαβ structure, similar to NAD-binding domains (24–26). The sequence Gly-Xaa-Gly-Xaa-Xaa-Gly is highly conserved in this region at the border between the first β-sheet and the α-helix (25). This entire region is found at the amino-terminal end of the mature form of adrenodoxin reductase (27) and is entirely encoded by a single exon, exon 2, of the adrenodoxin reductase gene, while the second β-sheet in the βαβ structure is encoded by exon 3.

The NADPH binding site has a similar βαβ structure (25), having the consensus sequence Gly-Xaa-Gly-$(Xaa)_2$-Ala-$(Xaa)_3$-Ala-$(Xaa)_6$-(Gly or Pro) first identified in bovine adrenodoxin reductase (27). The validity of this consensus is confirmed by extensive amino acid sequence comparisons and by site-directed mutagenesis (28). This entire 17-amino acid region is also encoded by a single exon, exon 6. Thus, the two crucial functional domains of adrenodoxin reductase are encoded by individual exons, consistent with the hypothesis that exons permit shuffling of functional domains during eukaryotic evolution (29). Similarly, 17β-hydroxysteroid dehydrogenase has this NADPH-binding site on one exon (30), but the sequence is interrupted by an intron in rat malic enzyme (31). The six additional amino acids resulting from alternate splicing of adrenodoxin reductase, Ala-Leu-Leu-Leu-Cys-Gln, would probably disrupt the second β-sheet of the βαβ structure. The functional consequences of this altered NADPH-binding site are not yet known.

RNase protection experiments with two different probes permitted us to establish that transcription of the human adrenodoxin reductase gene is initiated from multiple sites. All of these are downstream from a group of six GGGCGGG sequences, and none is close to TATA or CAAT sequence. This finding is uncommon among genes for steroidogenic enzymes, but is typical of "housekeeping" genes expressed in most tissues (32). While it is attractive to regard adrenodoxin reductase as simply a housekeeping gene because of its broad tissue distribution of expression and its function as a "generic" electron-transport protein for all mitochondrial forms of P450, it should also be noted that adrenodoxin, which has a similar role, is encoded by a gene having a classical TATA box (11). Thus elucidation of the significance of this promoter structure in the adrenodoxin reductase gene will require further experimentation.

The 5′ flanking DNA lacks obvious consensus sequences regulated by cAMP, glucocorticoids, or phorbol esters. However, bases −326 to −319 and −304 to −297 form a perfect 8-base inverted repeat. This could conceivably form a stem structure with a 14-base loop centered on the symmetrical sequence GGGAGGG. Such a stem-and-loop structure would have a free energy (ΔG) of about −10.2 kcal/mol at 25°C (33). The significance of this region in the regulation of the adrenodoxin reductase gene is not yet known.

We thank Israel Hanukoglu (Weizmann Institute, Rehovot, Israel) for productive discussions. This work was supported by National Institutes of Health Grants DK 37922 and DK 42154 and March of Dimes Grant 6-396 to W.L.M.; Y.S. was supported by the University of California, San Francisco Cheng Scholars Program.

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