Structure of the Human Steroidogenic Acute Regulatory Protein (StAR) Gene: StAR Stimulates Mitochondrial Cholesterol 27-Hydroxylase Activity[†,‡]

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ABSTRACT: Steroidogenic acute regulatory protein (StAR) plays a key role in steroid hormone synthesis by enhancing the metabolism of cholesterol into pregnenolone. We determined the organization of the StAR structural gene, mapped to 8p11.2. The gene spans 8 kb and consists of seven exons interrupted by six introns. The 1.3 kb of DNA upstream from the transcription start site directed expression of a luciferase reporter gene in mouse Y-1 adrenal cortical tumor cells but not in BeWo choriocarcinoma cells. Reporter gene expression in the Y-1 cells was increased more than 2-fold by 8-Br-cAMP, indicating that the 1.3 kb DNA fragment contains sequences that confer tissue-specific expression and cAMP regulation. The sequence of a related StAR pseudogene, mapped to chromosome 13, lacks introns and has an insertion, numerous substitutions, and deletions. Expression of StAR in COS-1 cells cotransfected with cholesterol 27-hydroxylase (P450c27) and adrenodoxin resulted in a 6-fold increase in formation of 3β-hydroxy-5-cholestenoic acid, demonstrating that StAR's actions are not specific to steroidogenesis but extend to other mitochondrial cholesterol-metabolizing enzymes.

The rate-limiting step in steroid hormone synthesis is the formation of pregnenolone from cholesterol, catalyzed by the cholesterol side-chain cleavage enzyme (P450scc), which resides with its associated electron transport chain in the inner mitochondrial membranes [for review see Miller (1988)]. This first committed reaction in the biosynthesis of steroid hormones is acutely stimulated by tropic hormones (ACTH in the adrenal cortex; LH in the gonads) acting through the intermediacy of cAMP. It has been known for two decades that the acute steroidogenic response to tropic stimulation involves the translocation of cholesterol from the outer to inner mitochondrial membranes. This translocation process is believed to be mediated by a short-lived, cycloheximide-sensitive protein (Ferguson, 1963; Garren et al., 1968; Simpson et al., 1978; Toaff et al., 1979).

Steroidogenic acute regulatory protein (StAR) plays an important role in the process of steroidogenesis and seems to be the factor that causes the movement of cholesterol within mitochondria (Clark et al., 1994; Lin et al., 1995). The expression of StAR protein is directly correlated with steroidogenic activity in adrenal and gonadal cells (Epstein & Orme-Johnson, 1991; Stocco & Ascoli, 1991; Stocco & Sodeman, 1991). Interestingly, the StAR gene is not expressed in the placenta (Sugawara et al., 1995). Congenital lipoid adrenal hyperplasia (lipoid CAH) is an autosomal recessive disease in which synthesis of adrenal and gonadal steroids is undetectable [for review see Hauffa et al. (1985)]. Although gonadal and adrenal steroidogenesis is severely impaired in affected fetuses, placental production of progesterone persists (Saenger et al., 1995). We have recently shown that lipoid CAH is caused by mutations in the StAR gene (Lin et al., 1995). The mechanisms by which StAR promotes increased steroidogenesis remain poorly understood with the exception of the knowledge that it enters mitochondria by virtue of a mitochondrial targeting sequence in the amino terminus which is subsequently cleaved to yield the mature protein (Clark et al., 1994). The factors that govern the tissue-specific pattern of StAR expression are also unknown.

The goals of the present work were the following: (1) to elucidate the organization of the StAR gene and its promoter as a first step to understanding the regulation of StAR expression and (2) to test the hypothesis that StAR's actions are specific for steroid hormone synthesis by examining the effects of StAR on metabolism of cholesterol by another mitochondrial P450 enzyme, cholesterol 27-hydroxylase (P450c27) (Andersson et al., 1989).

MATERIALS AND METHODS

Isolation and Characterization of Genomic Clones. A human genomic library in bacteriophage λ-fix II was screened as previously described with a 1.6 kb human StAR cDNA (Ohba et al., 1994; Sugawara et al., 1995). The λ clones were analyzed by restriction endonuclease digestion and Southern blotting with the StAR cDNA. Genomic fragments were isolated and subcloned into pBluescript

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[‡] Sequences reported in this paper have been deposited in GenBank, U29098-U29105 for the StAR structural gene and U29106 for the StAR pseudogene.

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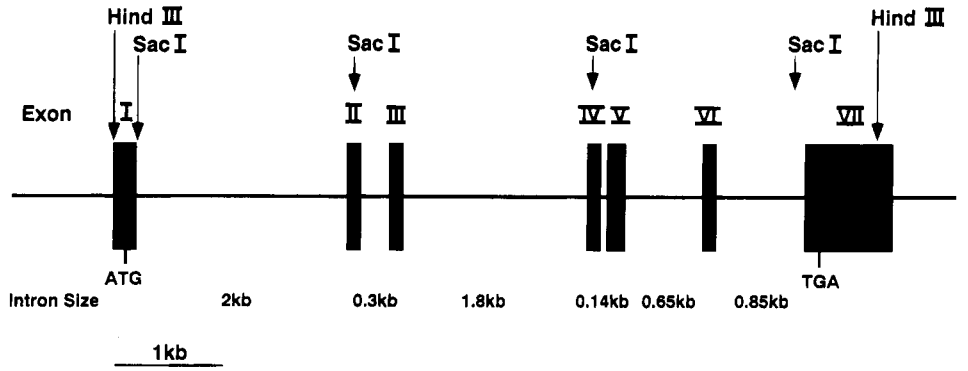


FIGURE 1: Organization of the StAR structural gene. The map identifies the seven exons, the six introns, and the *Hin*dIII and *Sac*I restriction sites. The locations of the initiation and stop codons are indicated in Exons I and VII, respectively.

Table 1: Exon−Intron Junctions of the Human StAR Gene

exon no.	nucleotide position	length (nt)	sequence at the exon−intron junctions: 5′-splice donor		intron size	3′-splice acceptor	
I	1−203	203	CATGAAGG	gtgagcgctgcgggaaggaggcga	2 kb	aacaagggttattcccttctgcag	GGCTGAGG
II	204−318	114	TCTACTCG	gtaagtgctgaggcttctgggctc	0.3 kb	gtctctcctcggctgtgtatccag	GTTCTCGG
III	319−445	126	GTCAGCAG	gtaagtgtcggggagaagcctgtg	1.8 kb	tctgggggctcctttctctgacag	GACAATGG
IV	446−605	159	AGATCAAG	gtgagcaaagtccaggtgcgggtg	0.14 kb	ttctggttccccatggcctggtag	GTCCTGCA
V	606−791	185	GTCATCAG	gtaatacgggcagcaggctccaaac	0.65 kb	gacttgacttgctccatttgccag	GGCGGAGC
VI	792−886	94	ACCTCAAG	gtgaagggcatgggaggggggacct	0.85 kb	aaattctcctacctcctactgcag	GGGTGGCT
VII	897−1634	747					

Table 2: StAR Gene Promoter Activity in Y-1 Adrenal Cortical Tumor Cells and BeWo Choriocarcinoma Cells[a]

plasmid/treatment	Y-1 cells (%)	BeWo cells (%)
pGL_2 control	100	100
PGL_2 control + cAMP	109 ± 6	118 ± 14
pGL_2 basic	0.8 ± 0.1	0.17 ± 0.02
pGL_2 basic + cAMP	1.1 ± 0.9	0.4 ± 0.04
pGLStAR 1.3 kb	17.8 ± 4	0.8 ± 0.1
pGLStAR 1.3 kb + cAMP	42.8 ± 8	1.5 ± 0.1

[a] The indicated plasmids were transfected into Y-1 or BeWo cells with the control plasmid pCH110 as described in Materials and Methods. Some cultures were incubated with 1 mM 8-Br-cAMP for 24 h prior to harvest. Luciferase and β-galactosidase activities were determined, and the luciferase/β-galactosidase ratios were used to estimate promoter activities relative to the pGL_2 control vector whose activity was arbitrarily set to 100%. The results presented are the mean ± SE of four separate transfections carried out with triplicate cultures for each treatment for Y-1 cells and three separate transfections carried out with triplicate cultures for BeWo cells.

Table 3: Effect of StAR on Mitochondrial Cholesterol 27-Hydroxylase Activity[a]

group	plasmid: P450c27	StAR	adrenodoxin	3β-hydroxy-5-cholestenoic acid[b] (pmol/mL)
1	−	−	+	0.90 ± 0.42 (6)
2	−	+	+	0.44 ± 0.32 (5)
3	+	−	+	3.25 ± 0.70 (6)
4	+	+	+	20.64 ± 3.79 (6)

[a] COS-1 cells were transfected with the indicated expression plasmids for bovine adrenodoxin, StAR, or the control vector pSV-SPORT-1, introduced when the StAR plasmid was not included, as previously described (Sugawara et al., 1995). Media were collected 48 h after transfection for quantification of 3β-hydroxy-5-cholestenoic acid. [b] Values presented are means ± SE of determinations from the indicated number of dishes from three separate experiments.

(Stratagene) for manual (Sequenase version 2.0) or automated (Applied Biosystems Inc.) sequence analysis as previously described (Ohba et al., 1994).

Mapping of Transcription Start Sites by RNase Protection Analysis. To identify the transcription start sites of the StAR gene, total RNA isolated from human fetal adrenal cortex and testis was analyzed by RNase protection as described by Lin et al. (1990). Yeast tRNA was used as a control. A 310 bp *Sac*I−*Hae*III fragment of the 5′-end of the StAR structural gene was cloned into *Sac*I/*Sma*I sites in pBluescript (SKII) and *Eco*RI-digested plasmid was transcribed with T3 polymerase and [^{32}P]UTP to generate a 354 nt riboprobe. A 15 μg amount of RNA was mixed with 5 × 10^5 cpm of riboprobe in 80% formamide/40 mM PIPES, pH 6.7/0.4 M NaCl/1 mM EDTA, denatured by boiling for 5 min, and then incubated for 12 h at 58 °C. After hybridization, the mixture was digested with RNase A and RNase T as described by Lin et al. (1990), and the products were analyzed by electrophoresis on 5% acrylamide/7 M urea gels.

Plasmid Constructions for Analysis of Promoter Activity. A 1.3 kb *Hin*dIII fragment of the StAR gene (nt −1293 to +25) was cloned in the correct and reverse orientation into the plasmid vector pGL_2 (Promega) which contains firefly luciferase as a reporter gene. Other plasmids used in these experiments included the pGL_2 basic vector, which contains no promoter sequences; pGL_2 control, which places the luciferase gene under the control of the SV40 promoter and enhancer; and pCH110, a plasmid in which the *lac*Z gene is under control of the early SV40 promoter (Pharmacia).

Characterization of StAR Promoter Activity. Murine Y-1 adrenal tumor cells and BeWo choriocarcinoma cells were grown in 35 mm plastic dishes in a culture medium consisting of Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 50 μg/mL of gentamycin. Plasmids used for transfection were purified using the Maxiprep reagent system (Qiagen). Cell cultures at 40%−60% confluence were washed twice with serum-free medium before adding 1 mL of serum-free medium

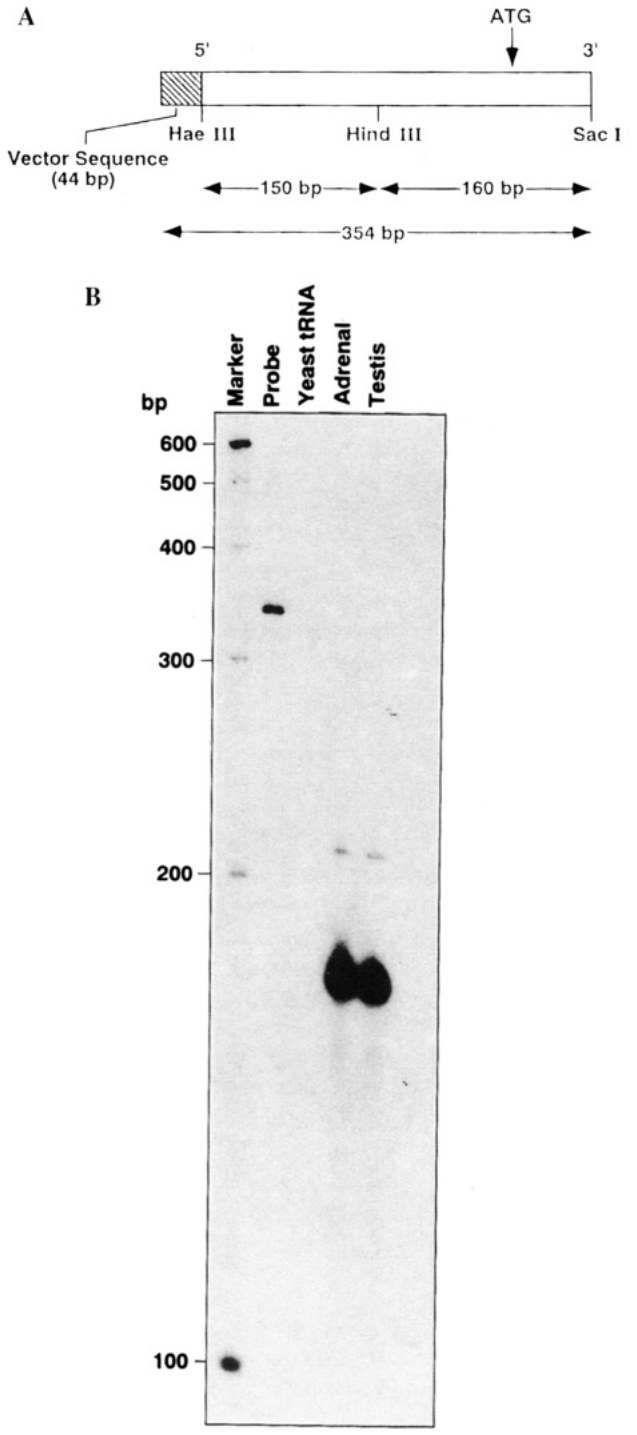


FIGURE 2: Mapping of transcription start sites by RNase protection analysis. Human adrenal and testis RNA and yeast tRNA were analyzed using a 354 nt riboprobe shown in A. The RNase protection assay revealed no protected fragments with yeast tRNA (B). A major fragment (180 bp) was protected in both adrenal and testis RNA, indicating a primary transcription start site 154 bp upstream of the initiation codon. A minor fragment (215 bp) indicating a second transcription start site about 35 bp upstream of the major site was also identified.

containing 1 μg of pGL_2 plasmid constructs and 1 μg of pCH110 plasmid with 10 μL of Lipofectamine (GIBCO/BRL). After 5 h of incubation, the medium was replaced with 1 mL of medium with 20% serum. Medium was changed after 18 h, and 2 mL of medium was supplemented with 10% serum. Cells were harvested after 48 h of culture. In some experiments, 8-Br-cAMP (1 mM) was added to the medium for the final 24 h of culture.

Luciferase and β-Galactosidase Assays. Cells were harvested 48 h after transfection, and extracts were made in Promega lysis buffer. One aliquot (40 μL out of 400 μL total extract volume) was used for luciferase assays with Promega reagents, and another 150 μL was taken for β-galactosidase assays with Promega reagents. The "blank" luciferase value measured in untransfected cell extracts was subtracted from luciferase readings of transfected cell extracts. The luciferase assay results were normalized to β-galactosidase activity to compensate for variations in transfection efficiency. In each experiment the activity of the pGL_2 control vector was defined as 100%. Each treatment group contained at least triplicate cultures, and each experiment was repeated two or three times.

Transfection Studies and Analysis of 3β-Hydroxy-5-cholestenoic Acid. COS-1 cells were transfected with the rat P450c27 cDNA in pCMV4 (Su et al., 1990) and a bovine adrenodoxin expression plasmid kindly provided by Dr. Michael Waterman (Vanderbilt University) with either the pSV-SPORT-1 empty vector (BRL, Bathesda, MD) or the vector containing the human StAR cDNA as previously described (Sugawara et al., 1995).

Formation of 3β-hydroxy-5-cholestenoic acid, the end-product of cholesterol metabolism by P450c27 (Andersson et al., 1989), was analyzed by isotope ratio mass spectrometry as previously described (Reiss et al., 1994). In brief, deuterated standard (500 ng) was added to 1 mL aliquots of medium, and after acidification and extraction into ethyl acetate the product was isolated by thin-layer chromatography. After methylation of the C_{27} acid, the eluates were acetylated and injected into a Hewlett-Packard GLC−MS onto a fused silica column (CP-sil 19CB, 0.25 mm i.d., 25 m length; Chrompack, Raritan, NJ). Using an isotope ratio program, the areas for *m/z* 412/418 [3β-hydroxy-5-cholestenoic acid methyl ester acetate, molecular ion = 472 − 60 (acetate)] were monitored and the respective areas determined by integration for calculation of the amount of endogenous sterol.

RESULTS AND DISCUSSION

Organization of the StAR Structural Gene. The StAR gene sequence comprised approximately 8 kb. On the basis of a comparison with the human StAR cDNA sequence, the gene was divided into seven exons (Figure 1). The six introns range in size from 141 nt to 2 kb. The exon−intron junctions of the gene (Table 1) all obey the GT/AG rule (Mount, 1982). The spliced StAR gene transcript is predicted to be 1642 nt long, which is in agreement with the size of the primary StAR mRNA detected in Northern hybridization analyses (Sugawara et al., 1995). Larger StAR RNA species of 4.4 and 7.5 kb which we previously observed may represent nascent transcripts or partially or alternatively spliced RNAs.

The major transcription start site, determined by RNase protection analysis (Figure 2) and confirmed by primer extension analysis (data not shown), lies 154 bp in front of the translation start site (Figures 3 and 4). A minor site was identified about 35 bp further upstream of the major site. The DNA sequence upstream from the major transcription start site contains a TATA-like element (TTTAA) at −24 to −20 bp. Several putative Sp1 binding sites are also present in the promoter region as well as repeats of the sequence ATTT and $(T)_nCCT$. A stretch of trinucleotide

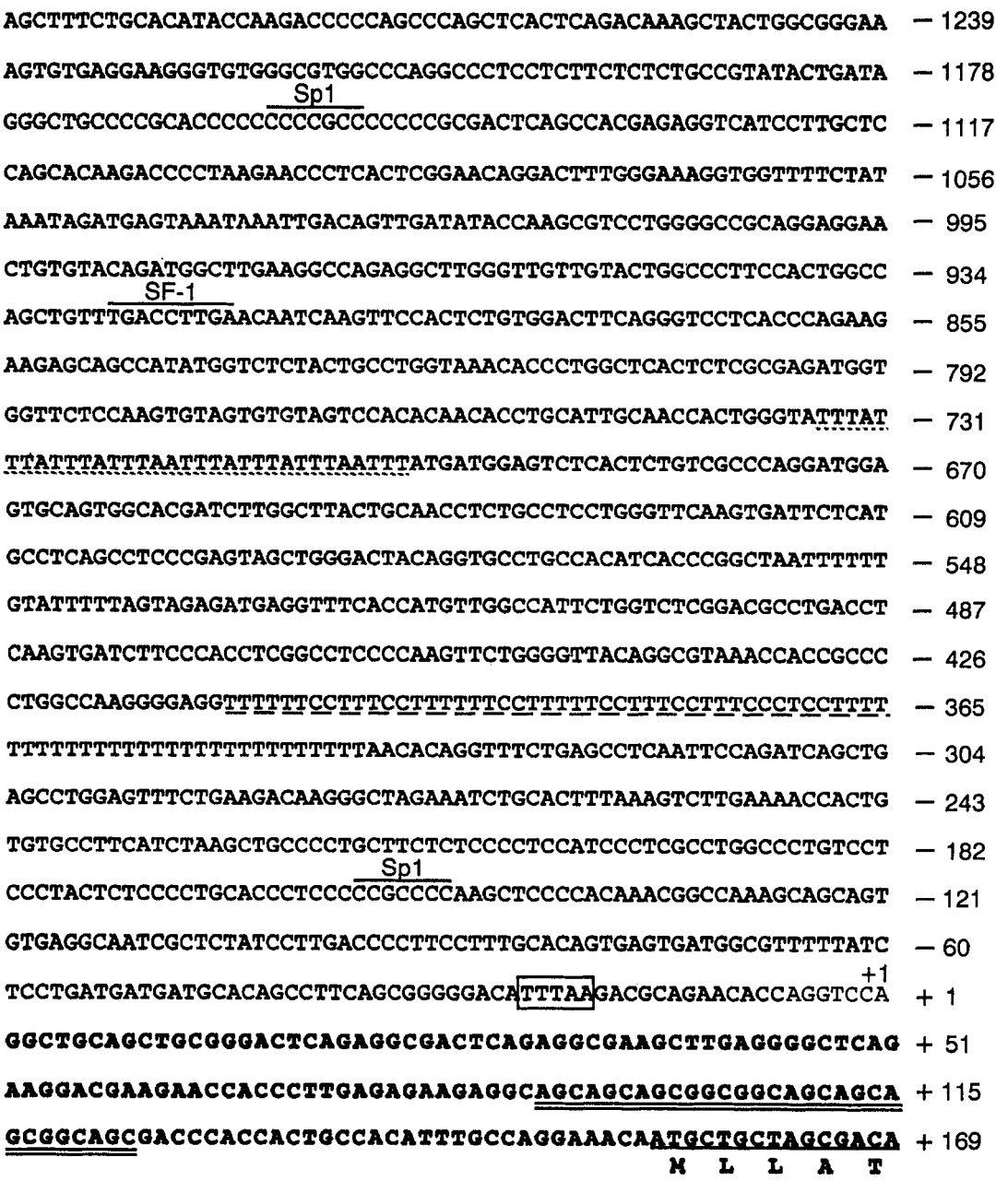
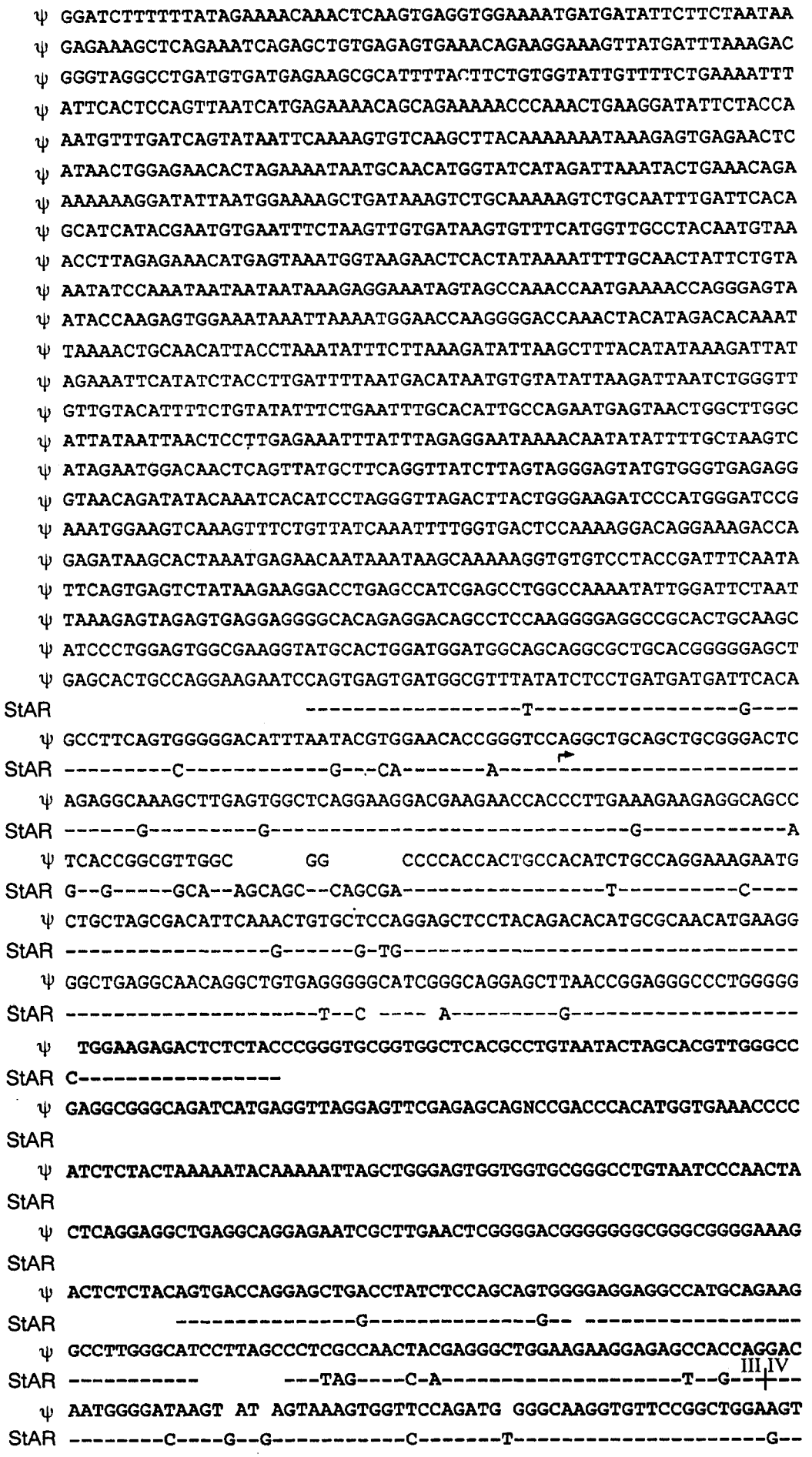


FIGURE 3: DNA sequence of the StAR gene promoter. The transcribed sequences from the major transcription start site are indicated in bold letters. Translated sequences are underlined and the amino acids given in single-letter codes. Putative Sp1 and SF-1 binding sites are indicated. The TATA-like element is boxed. Repetitive sequences are noted with underlines.

repeats was found in the transcribed 5′-untranslated region. We located one consensus sequence on the opposite strand (TGACCTTGA) that could be recognized by the orphan nuclear receptor, steroidogenic factor-1 (SF-1, also designated AdBP4), which appears to be essential for the expression of a number of steroidogenic enzyme genes (Honda et al., 1990; Rice et al., 1991).

StAR Pseudogene. A 3.4 kb genomic fragment containing the StAR pseudogene identified in our previous studies (Lin et al., 1995; Sugawara et al., 1995) was cloned and sequenced. This pseudogene, mapped to chromosome 13, is highly homologous to the StAR cDNA, but it has numerous deletions and substitutions as well as a large insertion. The pseudogene has no introns, lacks a poly(A) tail, and ends in CA repeats. The pseudogene sequence corresponding to that of the StAR cDNA is presented in Figure 4. This pseudogene appears to be transcribed at low levels since pseudogene sequences can be detected in reverse transcriptase PCR experiments that are controlled for possible genomic DNA contamination (Lin et al., 1995; Sugawara et al., 1995). Interestingly, the pseudogene sequence corresponds to the StAR structural gene upstream of the major and minor transcription start sites.

StAR Promoter Activity. The 1.3 kb of DNA upstream from the transcription start site directed expression of the luciferase reporter gene when transfected into mouse Y-1 adrenocortical tumor cells (Table 2), but the same fragment inserted in the opposite orientation drove luciferase expression only to the same extent as the promoterless control plasmid (data not shown). Treatment of the Y-1 cells with 8-Br-cAMP increased StAR promoter activity 2.3-fold ($p < 0.002$ analysis of log-transformed data by the paired t-test), suggesting that this segment of DNA contains cAMP-responsive elements. This increase in StAR promoteractivity


ψ GGATCTTTTTTATAGAAAACAAACTCAAGTGAGGTGGAAAATGATGATATTCTTCTAATAA
ψ GAGAAAGCTCAGAAATCAGAGCTGTGAGAGTGAAACAGAAGGAAAGTTATGATTTAAAGAC
ψ GGGTAGGCCTGATGTGATGAGAAGCGCATTTTACTTCTGTGGTATTGTTTTCTGAAAATTT
ψ ATTCACTCCAGTTAATCATGAGAAAACAGCAGAAAAACCCAAACTGAAGGATATTCTACCA
ψ AATGTTTGATCAGTATAATTCAAAAGTGTCAAGCTTACAAAAAAATAAAGAGTGAGAACTC
ψ ATAACTGGAGAACACTAGAAAATAATGCAACATGGTATCATAGATTAAATACTGAAACAGA
ψ AAAAAAGGATATTAATGGAAAAGCTGATAAAGTCTGCAAAAAGTCTGCAATTTGATTCACA
ψ GCATCATACGAATGTGAATTTCTAAGTTGTGATAAGTGTTTCATGGTTGCCTACAATGTAA
ψ ACCTTAGAGAAACATGAGTAAATGGTAAGAACTCACTATAAAATTTTGCAACTATTCTGTA
ψ AATATCCAAATAATAATAATAAAGAGGAAATAGTAGCCAAACCAATGAAAACCAGGGAGTA
ψ ATACCAAGAGTGGAAATAAATTAAAATGGAACCAAGGGGACCAAACTACATAGACACAAAT
ψ TAAAACTGCAACATTACCTAAATATTTCTTAAAGATATTAAGCTTTACATATAAAGATTAT
ψ AGAAATTCATATCTACCTTGATTTTAATGACATAATGTGTATATTAAGATTAATCTGGGTT
ψ GTTGTACATTTTCTGTATATTTCTGAATTTGCACATTGCCAGAATGAGTAACTGGCTTGGC
ψ ATTATAATTAACTCCTTGAGAAATTTATTTAGAGGAATAAAACAATATATTTTGCTAAGTC
ψ ATAGAATGGACAACTCAGTTATGCTTCAGGTTATCTTAGTAGGGAGTATGTGGGTGAGAGG
ψ GTAACAGATATACAAATCACATCCTAGGGTTAGACTTACTGGGAAGATCCCATGGGATCCG
ψ AAATGGAAGTCAAAGTTTCTGTTATCAAATTTTGGTGACTCCAAAAGGACAGGAAAGACCA
ψ GAGATAAGCACTAAATGAGAACAATAAATAAGCAAAAAGGTGTGTCCTACCGATTTCAATA
ψ TTCAGTGAGTCTATAAGAAGGACCTGAGCCATCGAGCCTGGCCAAAATATTGGATTCTAAT
ψ TAAAGAGTAGAGTGAGGAGGGGCACAGAGGACAGCCTCCAAGGGGAGGCCGCACTGCAAGC
ψ ATCCCTGGAGTGGCGAAGGTATGCACTGGATGGATGGCAGCAGGCGCTGCACGGGGGAGCT
ψ GAGCACTGCCAGGAAGAATCCAGTGAGTGATGGCGTTTATATCTCCTGATGATGATTCACA
StAR ----------T----------G----
ψ GCCTTCAGTGGGGGACATTTAATACGTGGAACACCGGGTCCAGGCTGCAGCTGCGGGACTC
StAR ---------C----------G--CA-------A----------
ψ AGAGGCAAAGCTTGAGTGGCTCAGGAAGGACGAAGAACCACCCTTGAAAGAAGAGGCAGCC
StAR ------G---------G----------G----------A
ψ TCACCGGCGTTGGC GG CCCCACCACTGCCACATCTGCCAGGAAAGAATG
StAR G--G-----GCA--AGCAGC--CAGCGA----------T----------C----
ψ CTGCTAGCGACATTCAAACTGTGCTCCAGGAGCTCCTACAGACACATGCGCAACATGAAGG
StAR ----------G------G-TG----------
ψ GGCTGAGGCAACAGGCTGTGAGGGGGCATCGGGCAGGAGCTTAACCGGAGGGCCCTGGGGG
StAR ----------T--C ---- A---------G----------
ψ TGGAAGAGACTCTCTACCCGGGTGCGGTGGCTCACGCCTGTAATACTAGCACGTTGGGCC
StAR C----------
ψ GAGGCGGGCAGATCATGAGGTTAGGAGTTCGAGAGCAGNCCGACCCACATGGTGAAACCCC
StAR
ψ ATCTCTACTAAAAATACAAAAATTAGCTGGGAGTGGTGGTGCGGGCCTGTAATCCCAACTA
StAR
ψ CTCAGGAGGCTGAGGCAGGAGAATCGCTTGAACTCGGGGACGGGGGGGCGGGCGGGGAAAG
StAR
ψ ACTCTCTACAGTGACCAGGAGCTGACCTATCTCCAGCAGTGGGGAGGAGGCCATGCAGAAG
StAR ----------G----------G-- ----------
ψ GCCTTGGGCATCCTTAGCCCTCGCCAACTACGAGGGCTGGAAGAAGGAGAGCCACCAGGAC
StAR ---------- ---TAG----C-A----------T--G---
III IV
ψ AATGGGGATAAGT AT AGTAAAGTGGTTCCAGATG GGGCAAGGTGTTCCGGCTGGAAGT
StAR --------C----G--G----------C-------T----------G--

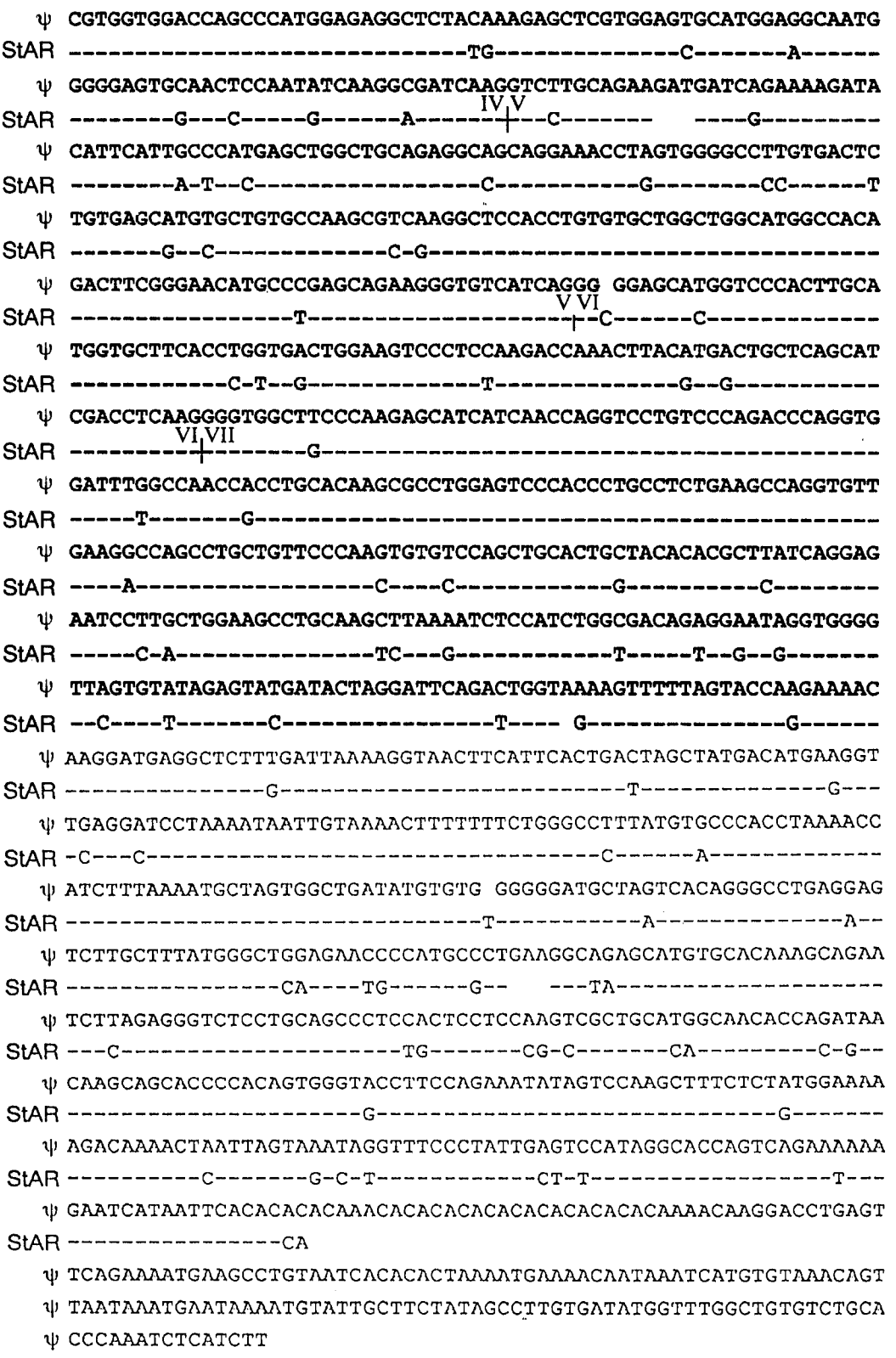


FIGURE 4: Alignment of the StAR pseudogene (Ψ) nucleotide sequence with the StAR cDNA nucleotide sequence and upstream sequences of the StAR structural gene. Identical nucleotides in the StAR cDNA are indicated by a dash, and nucleotide substitutions are noted by the single-letter code. Deletions/insertions in the pseudogene sequence are indicated by gaps in either the pseudogene or the StAR cDNA sequence. The position of the major transcription start site of the StAR gene is indicated by an arrow. The exon−intron junctions are marked in the StAR cDNA sequence by vertical lines.

corresponds to the 3-fold increase in steady state levels of StAR mRNA in human granulosa cells treated for 24 h with cAMP (Sugawara et al., 1995). This suggests that the increase in StAR mRNA in response to cAMP is, at least in part, the result of increased transcription.

In contrast to our findings with Y-1 cells, the StAR promoter did not cause significant reporter gene expression in BeWo choriocarcinoma cells, which do not express the StAR gene. This was true whether BeWo cells were examined in the basal state or after stimulation with 8-Br-

cAMP. This is consistent with the lack of detectable StAR mRNA in placenta and choriocarcinoma cells (Sugawara et al., 1995) and with the persistence of placental steroidogenesis in pregnancies in which the fetus is affected with lipoid CAH (Saenger et al., 1995). Thus, cis elements directing the tissue-specific expression of StAR and regulation by cAMP appear to be located within 1.3 kb of DNA upstream from the cap site.

StAR Stimulates Mitochondrial 27-Hydroxylase Activity. Mitochondrial cholesterol 27-hydroxylase (P450c27) catalyzes the formation of 27-hydroxycholesterol and the C_{27} acid, 3β-hydroxy-5-cholestenoic acid (Andersson et al., 1989; Su et al., 1990). Expression of StAR in COS-1 cells cotransfected with P450c27 and adrenodoxin resulted in a more than 6-fold increase ($p < 0.005$ for comparison of group 3 vs group 4) in the production of 3β-hydroxy-5-cholestenoic acid (Table 3). This StAR-mediated increase in cholesterol metabolism by P450c27 is on the same order of magnitude as we observed for StAR stimulation of cholesterol side-chain cleavage (Sugawara et al., 1995). These observations demonstrate that StAR is capable of enhancing mitochondrial cholesterol metabolism by enzymes other than the steroidogenic cytochrome P450scc. In light of these findings, we conclude that StAR's actions are not specific to steroidogenesis.

P450c27 is found in a number of tissues, including the ovary which expresses StAR (Andersson et al., 1989; Su et al., 1990). Thus, StAR could contribute to the metabolism of cholesterol to 27-hydroxycholesterol and the C_{27} acid in steroidogenic cells. The hydroxysterols so-formed could have roles in governing cellular cholesterol homeostasis (Rennert et al., 1990).

P450c27 is highly expressed in the liver where it plays a role in bile acid synthesis (Andersson et al., 1989). Because the StAR gene is apparently not expressed in the adult liver (Sugawara et al., 1995), other factors or processes must govern cholesterol access to the hepatic 27-hydroxylase. The possibility of introducing the StAR gene into the liver with the goal of enhancing bile acid formation through the 27-hydroxylase pathway, and hence promoting cholesterol disposal could be an attractive approach to gene therapy of hypercholesterolemic states.

NOTE ADDED IN PROOF

Clark et al. (1995) have recently determined the structure of the murine StAR gene which is similar to the human gene, spanning 6.5 kb and consisting of 7 exons.

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