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Human steroidogenic acute regulatory protein: Functional activity in COS-1 cells, tissue-specific expression, and mapping of the structural gene to 8p11.2 and a pseudogene to chromosome 13

(steroidogenesis/cAMP/pregnenolone/cholesterol side-chain cleavage)

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ABSTRACT **Steroidogenic acute regulatory protein (StAR) appears to mediate the rapid increase in pregnenolone synthesis stimulated by tropic hormones. cDNAs encoding StAR were isolated from a human adrenal cortex library. Human StAR, coexpressed in COS-1 cells with cytochrome P450scc and adrenodoxin, increased pregnenolone synthesis >4-fold. A major StAR transcript of 1.6 kb and less abundant transcripts of 4.4 and 7.5 kb were detected in ovary and testis. Kidney had a lower amount of the 1.6-kb message. StAR mRNA was not detected in other tissues including placenta. Treatment of granulosa cells with 8-bromo-adenosine 3′,5′-cyclic monophosphate for 24 hr increased StAR mRNA 3-fold or more. The structural gene encoding StAR was mapped using somatic cell hybrid mapping panels to chromosome 8p. Fluorescence *in situ* hybridization placed the StAR locus in the region 8p11.2. A StAR pseudogene was mapped to chromosome 13. We conclude that StAR expression is restricted to tissues that carry out mitochondrial sterol oxidations subject to acute regulation by cAMP and that StAR mRNA levels are regulated by cAMP.**

Cholesterol side-chain cleavage in the adrenals and gonads and 1α-hydroxylation of vitamin D in the kidney are acutely stimulated by tropic hormones that act through the intermediacy of cAMP (1, 2). It has been recognized that proteins are required for the translocation of sterol substrates to the inner mitochondrial membrane where the P450 enzymes that catalyze these reactions reside (3–5). A 30-kDa phosphorylated protein rapidly appears in mitochondria of steroidogenic cells following tropic stimulation (6–10). This protein, named steroidogenic acute regulatory protein (StAR), has recently been purified from a murine Leydig cell tumor line (MA-10 cells) and its cDNA has been cloned (11). The cDNA encodes a protein with an N-terminal mitochondrial targeting sequence. Transient expression of the StAR cDNA in MA-10 cells resulted in enhanced steroidogenesis. These findings are strong evidence that StAR is the factor responsible for the rapid movement of substrate from the outer to the inner mitochondrial membranes.

To explore the role of StAR in human tissues and its potential involvement in disorders of steroidogenesis, we isolated cDNAs encoding human StAR, examined the pattern of StAR expression, and mapped the StAR structural gene.¶

MATERIALS AND METHODS

Isolation of Human StAR cDNA Clones and DNA Sequence Analysis. A human adrenal cortex cDNA library in λgt22A was kindly provided by Andre Lacroix, Alain Belanger, and Yves Tremblay (University of Laval, Quebec). The library was screened with a partial-length mouse StAR cDNA (11). More than 50 positive clones were detected in the screening of 600,000 plaques. Two plaque-purified phage clones were selected for sequence analysis. Each contained an insert of ≈1.6 kb. Both inserts were subcloned into pSPORT (GIBCO/BRL) and sequenced utilizing an automated DNA sequencer (Applied Biosystems) employing Taq DyeDeoxy sequencing reagents. Ambiguities were corrected by manual sequencing.

Expression of StAR cDNA in COS-1 Cells. COS-1 cells were transfected with various expression vectors with Lipofectamine (GIBCO/BRL) using 10 μl per dish (12). The vectors included pSPORT without cDNA insert, pSPORT with the 1.6-kb StAR cDNA (pStAR), and expression vectors for bovine P450scc (pCDP450scc) and adrenodoxin (pCDADX), kindly provided by Michael Waterman (Vanderbilt University, Nashville, TN). Forty-eight hours after transfection, medium was collected for radioimmunoassay of pregnenolone as described (12). In one experiment, the hydroxysterol, 20α-hydroxycholesterol, was added (5 μg/ml) to the incubation medium. This hydroxysterol is a more soluble pregnenolone precursor and an intermediate in the cholesterol side-chain cleavage reaction. Hydroxysterols bypass the regulated translocation mechanism of cholesterol movement and, therefore, generally provide an index of maximal cholesterol side-chain cleavage activity (13).

Expression of StAR mRNA. Northern blots containing 2 μg of poly(A)$^+$ RNA from various human tissues were purchased from Clontech and probed with the 1.6-kb StAR cDNA and a β-actin cDNA according to the supplier's protocol.

Total RNA was also isolated from cultures of human granulosa cells obtained from women undergoing *in vitro* fertilization/embryo transfer or from purified human cytotrophoblast cells. The human granulosa cells were cultured for 4 days and then treated with 1.5 mM 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) for 24 hr. The cytotrophoblast cells were cultured for 24 hr in the absence or presence of 1.5 mM 8-Br-cAMP. Detailed protocols for the preparation, culture, and isolation of total RNA from the granulosa cells and trophoblast cells have been described (14,

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Abbreviations: StAR, steroidogenic acute regulatory protein; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; FISH, fluorescence *in situ* hybridization; YAC, yeast artificial chromosome.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession No. U17280).

15). Northern blots were probed with the StAR cDNA and a cDNA encoding human 28S rRNA.

Mapping of the StAR Structural Gene and Pseudogene. The StAR gene and its pseudogene were mapped by hybridization to Southern blots of DNA from somatic cell hybrids (16) and by polymerase chain reaction (PCR) analyses using primers specific for the structural gene or pseudogene. Genomic DNAs from human × hamster and human × mouse somatic cell hybrid lines were obtained from the NIGMS Human Genetic Mutant Cell Repository (1992/1993 Catalog of Cell Lines, National Institutes of Health), and DNAs from human × hamster somatic cell hybrids were purchased from Bios (New Haven, CT).

Regional mapping of the StAR structural gene was accomplished with a chromosome 8 regional mapping panel consisting of hybrids 9HL10, ISHL27, and 20XP0435-2, generously supplied by M. Wagner (17), 8q−, 21q+, and clone 17 (17–19), and VErec8-7a1, which is a hybrid produced by the fusion of the GlyB CHO-K1 mutant with cells from a patient suffering from recombinant 8 syndrome (20).

Southern Blotting. Ten to 12 μg of genomic DNAs from 24 somatic cell hybrids, human, hamster (RJK88), and mouse (GM C1 1-D) was digested with *Hin*dIII and subjected to Southern blotting (16).

PCR Analyses. The StAR structural gene and pseudogene were mapped by PCR analysis of somatic cell hybrid DNA with sequence-specific primers. For the structural gene the forward primer used was 5′-GTGAGCAAAGTCCAGGTGCG-3′ and the reverse primer was 5′-TGTGGCCATGCCAGCCAGCA-3′. These sequences span a small intron and yield an product of 300 nt. Primers derived from the DNA sequence of the PCR-amplified pseudogene, the sequence of which will be reported elsewhere, were used to determine the pseudogene location. The forward primer was 5′-AGCCTCACCGGCGT-TGGCGG-3′ and the reverse primer was 5′-CTGCAAGAC-CTTGATCGCCTTG-3′. These primers yield an 800-nt pseudogene-specific product. The PCR conditions were denaturation at 94°C for 5 min followed by a cycle of denaturation at 94°C for 45 sec, annealing at 65°C for 45 sec, and extension at 72°C for 2 min for 30 cycles with 10 pM of the primers in a buffer containing 2 mM $MgCl_2$. The PCR products were analyzed by electrophoresis in 1% agarose gels.

We also analyzed the regional mapping panel for several genes known to map to chromosome 8p, including the clustrin gene (*CL1*), the lipoprotein lipase gene (*LPL*), and the squalene synthase gene (*SS*) (21–26). PCR primers were designed from the published sequences.

Fluorescence *in Situ* Hybridization (FISH) Mapping. An individual yeast artificial chromosome (YAC) colony containing the StAR structural gene was isolated from the St. Louis library by PCR screening using StAR-specific primers corresponding to the 3′ untranslated sequences. The sense primer was 5′-CCTACTGGAAGCCTGCAAGTCTAAG-3′. The antisense primer was 5′-TGGTTTTAGGTGGGTACATA-AGGG-3′. StAR sequences in YAC DNA were amplified in a standard PCR. The reaction products were analyzed for the presence of the expected 240-nt amplification product.

YAC FISH was performed as described (27, 28) with the following modifications. The biotin-labeled probe was denatured at 75°C for 5 min, preannealed with human Cot-1 DNA for 1 hr at 37°C, and applied to human chromosome slide preparations that had been previously denatured and dehydrated. In some experiments, a chromosome 8 centromere-specific probe (D8Z2; Oncor) was added to the hybridization mixture. Detection was by avidin-fluorescein isothiocyanate, with one amplification by the manufacturer's directions (Oncor).

Twelve metaphase spreads were G-banded by trypsin (GTG) and photographed prior to FISH, which was performed after destaining and dehydration of the slides. Metaphase spreads were relocated and banding patterns were compared with probe signal to determine chromosomal location. Fractional length measurements confirmed the assignment (27).

RESULTS AND DISCUSSION

Human StAR cDNA. The two human StAR cDNAs that were characterized by DNA sequence analysis had identical 126-nt 5′ untranslated regions. Both clones contained an 855-nt open reading frame encoding a 285-amino acid protein and a 623-nt 3′ untranslated sequence that ended in a poly(A)$^+$ tail preceded 23 nt upstream by an AATAAA sequence.

The deduced human StAR amino acid sequence is 87% identical to that of mouse StAR (11). It contains an N-terminal sequence that is composed of basic and hydrophobic amino acids that are characteristic of mitochondrial targeting sequences. Seven consensus sites for phosphorylation by cAMP-dependent protein kinase and three protein kinase C phosphorylation sites are present in the sequence.

Expression of StAR in Engineered COS-1 Cells Increases Steroidogenesis. To document that human StAR has steroidogenesis enhancing activity, we cotransfected expression vectors for StAR and cholesterol side-chain cleavage enzyme and adrenodoxin into COS-1 cells and determined pregnenolone synthesis from endogenous substrates. COS-1 cells do not express StAR protein or mRNA (unpublished observations).

COS-1 cells did not secrete pregnenolone when transfected with the pSPORT vector lacking a cDNA insert or the pSPORT vector harboring the StAR cDNA (Table 1). However, cotransfection of the cells with plasmids directing expression of bovine P450scc and adrenodoxin endowed the cells with steroidogenic activity. Triple transfection of the COS-1 cells with P450scc, adrenodoxin, and StAR expression plasmids consistently increased steroid secretion 4- to 20-fold over

Table 1. Stimulation of steroidogenesis by StAR in COS-1 cells transfected with cholesterol side-chain cleavage enzyme and adrenodoxin

Treatment	Pregnenolone secretion, ng per dish			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Mock transfection	<5			
pSPORT	<5			
pStAR	<5			
+ 20α-OH-C				<5
pCDP450scc + pCDADX				
+ pSPORT	26 ± 6	14 ± 1.0	10 ± 0.01	20 ± 0.5
+ pSPORT + 20α-OH-C				157 ± 10
+ pStAR	545 ± 50	78 ± 4	41 ± 2.0	175 ± 10
+ pStAR + 20α-OH-C				137 ± 8

COS-1 cells were transfected with the indicated plasmids (2 μg of plasmid per 35-mm dish) with Lipofectamine. The media were collected after 48 hr and assayed for pregnenolone by radioimmunoassay. 20α-Hydroxycholesterol (20α-OH-C; 5 μg/ml) was added to some cultures. The results of four separate experiments are presented. Values are means ± SE. n = 3 or 4 replicates per experiment.

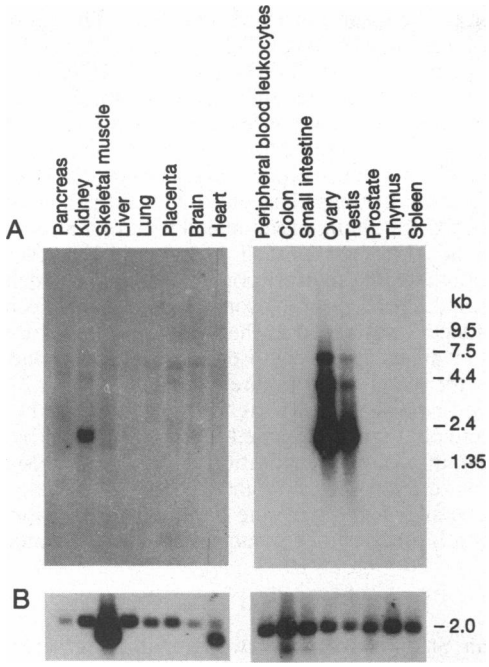


FIG. 1. Expression of StAR mRNA in various human tissues. Northern blots containing 2 μg of poly(A)$^+$ RNA isolated from the indicated tissues were probed sequentially with StAR and β-actin cDNAs. (*A*) The autoradiogram on the left for StAR was exposed for 24 hr; the autoradiogram on the right for StAR was exposed for 4 hr. (*B*) The blots were both exposed for 2 hr for actin.

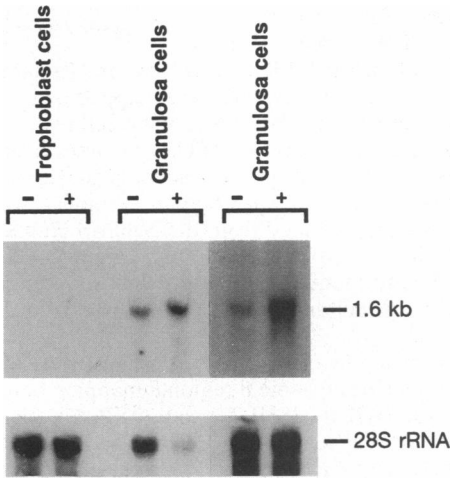


FIG. 2. Regulation of StAR mRNA expression in human granulosa cells by cAMP. Human granulosa cells were established in culture for 4 days and then 8-Br-cAMP (1.5 mM) was added to some dishes (+) for a 24-hr period. Results from two separate experiments are presented. Primary cultures of human trophoblast cells were also established in the absence (−) or presence (+) of 1.5 mM 8-Br-cAMP for 24 hr. Total RNA was subjected to Northern blotting (5 μg of RNA per lane) and the blots were probed sequentially with StAR and 28S rRNA cDNAs. Autoradiograms were analyzed with an image analysis system (Resource Technology, Nashville, TN) to determine the increase in StAR mRNA in the human granulosa cells relative to 28S rRNA. The increase was 3-fold in one experiment and 7-fold in the other.

cells transfected with P450scc, adrenodoxin, and the control pSPORT plasmid. Incubation of cells transfected with pP450scc, pADX, and pSPORT with 20α-hydroxycholesterol, a relatively soluble intermediate of the cholesterol side-chain cleavage reaction, stimulated pregnenolone secretion to the same extent as pStAR but did not augment the pStAR response in COS cells cotransfected with P450scc and adrenodoxin plasmids. In the absence of P450scc and adrenodoxin expression, there was no detectable pregnenolone synthesis in the presence of 20α-hydroxycholesterol. These findings document that the pSPORT plasmid "control" did not interfere with expression of the steroidogenic enzymes. The fact that an exogenous hydroxycholesterol did not augment steroid production stimulated by StAR also suggests that StAR promotes nearly maximal steroidogenic activity in the transfected COS cells.

The more than 4- to 20-fold increase in pregnenolone secretion promoted by expression of StAR in the COS cell system provides unambiguous proof that StAR stimulates steroidogenesis at a step prior to 3β-hydroxysteroid dehydrogenase. Moreover, the fact that StAR works in a nonsteroidogenic host cell (COS-1 cells) indicates that it does not require additional steroidogenic cell-specific factors to function beyond the side-chain cleavage system.

StAR mRNA Expression in Human Tissues. StAR mRNA was detected in human ovary, testis, and kidney. The most abundant transcript was 1.6 kb and less abundant mRNAs of 4.4 and 7.5 kb were observed in ovary and testis (Fig. 1). The ovarian sample contained the most StAR mRNA followed by the testis and then

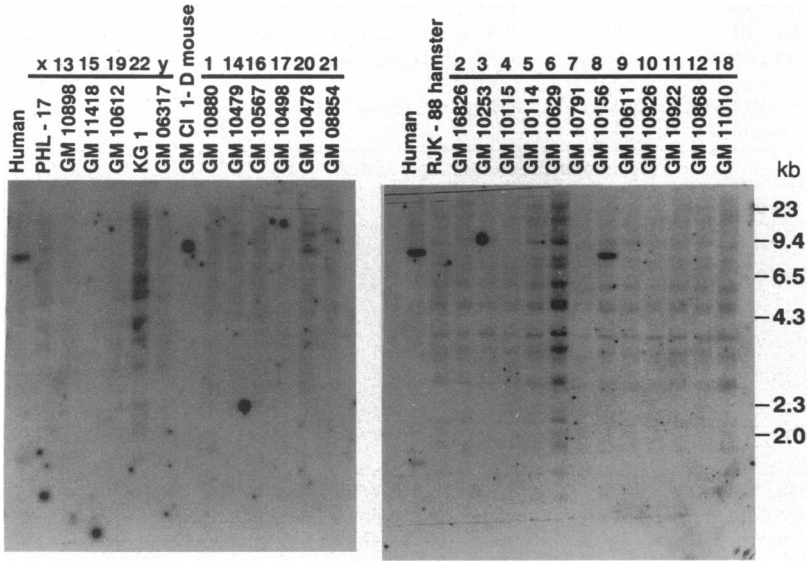
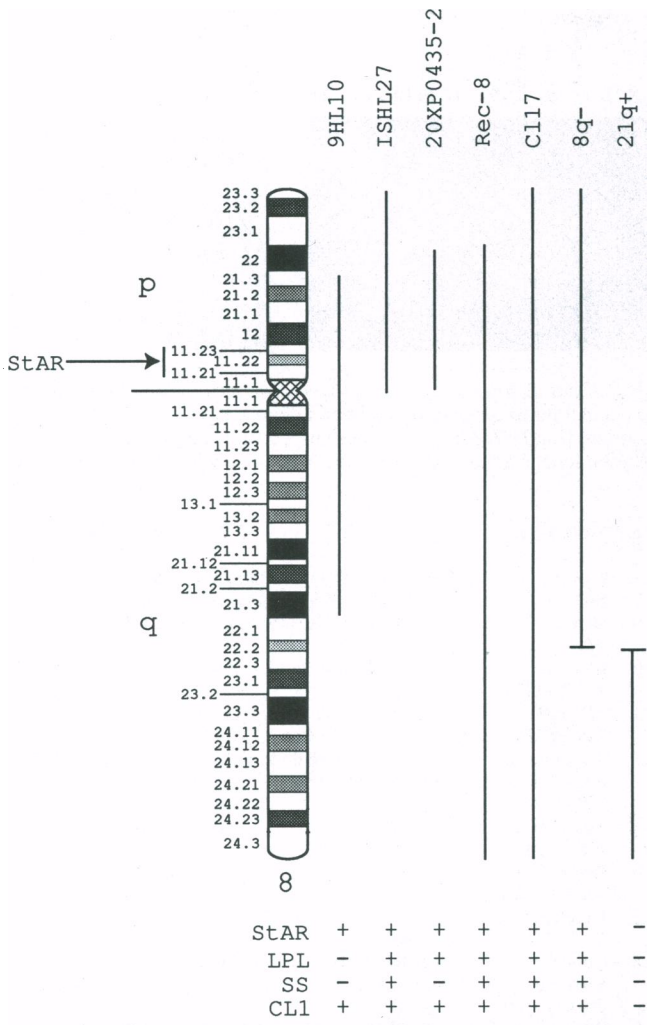


FIG. 3. Assignment of the StAR gene to human chromosome 8. Genomic DNA was digested with *Hin*dIII and subjected to Southern blotting. The hybrid designation and the human chromosome that predominates, which in some cases is the only human chromosome present in the hybrid, are indicated. A hybridization band corresponding to that found in human genomic DNA was found in a hybrid containing only human chromosome 8. A weaker band was found in hybrid GM 10478, which in addition to chromosome 20 is known to contain 8p. A faint band at 1.3 kb seen in human genomic DNA and hybrids containing chromosome 13 represents the StAR pseudogene.



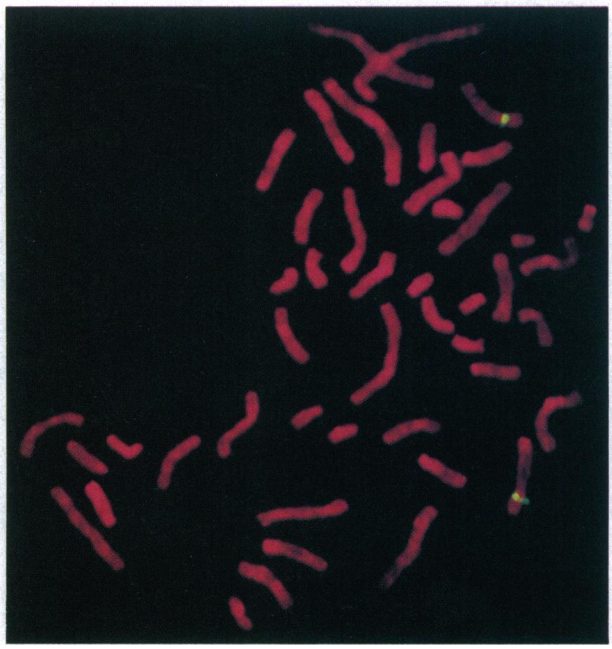


FIG. 4. (*Upper*) Regional mapping of the StAR gene to 8p by somatic cell hybrid mapping. The chromosome 8 idiogram is modified according to Francke (32). The right side of the idiogram shows a diagrammatic representation of the portion of human chromosome 8 present in the respective cell lines. The localizations of the boundaries of these DNAs on the cytogenetic map of the chromosome are approximate. Presence of a gene is denoted by a + and its absence is denoted by a −. A negative control cell line, CHO-K1, which contains only hamster DNA, was also included in these experiments. The localizations of *LPL*, *SS*, and *CL1* are consistent with previously published data (26, 33, 34). (*Lower*) YAC FISH localization of the StAR functional gene locus to 8p11.2. The probe was detected with avidin-fluorescein isothiocyanate (yellow) and chromosomes were counterstrained with propidium iodide (red). The arrow to the left of the idiogram in *Upper* indicates the FISH location of the A 10 G5 YAC to the 8p11.2 region.

the kidney. In the Northern blots shown in Fig. 1, probed simultaneously with the same preparation of ^{32}P-labeled cDNA, the blot containing ovary and testis RNA was exposed for 6 hr for StAR, whereas the blot containing the kidney sample was exposed for 24 hr for StAR. Longer exposures of both blots failed to reveal StAR mRNA in other tissues, including placenta. However, β-actin mRNA was readily detected in all of these tissues on the same blots. StAR expression in human adrenal cortex is inferred from the fact that multiple StAR phage clones were detected in the library used to isolate the human StAR cDNA. The nature of the multiple transcripts detected in the testis and ovary remains to be clarified. Because the predominant 1.6-kb mRNA is approximately the same size as the cDNAs we isolated, it is likely that they represent full-length copies of the primary StAR mRNA.

Our observations suggest that StAR expression is restricted to organs that carry out mitochondrial sterol hydroxylation reactions that are under acute regulation by tropic hormones that act via the intermediacy of cAMP. This is true for the adrenals and gonads, which respond to their respective pituitary tropic hormones, corticotropin and luteinizing hormone, with enhanced cholesterol side-chain cleavage, and to the kidney, which increases 1α-hydroxylation of vitamin D in response to parathyroid hormone. It is notable that another steroidogenic organ, the placenta, does not appear to express StAR. However, placental progesterone does not seem to be under acute regulation by cAMP. The reported stimulatory effect of agents that raise placental trophoblast cAMP levels or cAMP analogs is most likely related to increased expression of genes encoding steroidogenic enzymes, a process that takes hours or days (15). The brain, which is also a site of steroidogenesis (29), did not appear to express StAR either. The absence of StAR expression in the placenta, which produces substantial amounts of progesterone, and the brain implies that steroid hormone synthesis in these organs is regulated by other mechanisms, as suggested by Lieberman and Prasad (30).

cAMP Regulates StAR mRNA Expression in Granulosa Cells. Culture of human granulosa cells in the presence of 1.5 mM 8-Br-cAMP for 24 hr increased StAR mRNA 3- to 7-fold relative to 28S rRNA (Fig. 2). In contrast, StAR mRNA was not detectable in primary cultures of human trophoblast cells incubated for 24 hr without or with the cAMP analog. StAR mRNA was also not detected in Northern blots of poly(A)$^+$ RNA isolated from JEG-3 choriocarcinoma cells cultured for 24 hr without or with 8-Br-cAMP (data not shown), a treatment that up-regulates P450scc and adrenodoxin gene expression (31). These observations suggest that tropic hormones acting through a cAMP second messenger system may control levels of StAR in part by increasing the mRNA encoding the protein and hence its synthesis. The fact that StAR mRNA is not detectable in JEG-3 cells with or without 8-Br-cAMP stimulation is consistent with the absence of StAR mRNA from placenta and isolated trophoblast cells.

Mapping of the StAR Structural Gene to Chromosome 8p11.2 and a Pseudogene to Chromosome 13. When genomic DNA from the hybrid panel was digested with *Hin*dIII and subjected to Southern blotting, a strong hybridization band of about 8 kb was detected in the human genomic DNA control and in hybrid GM 10156, which contains only human chromosome 8 (Fig. 3). A faint band was also detected in GM

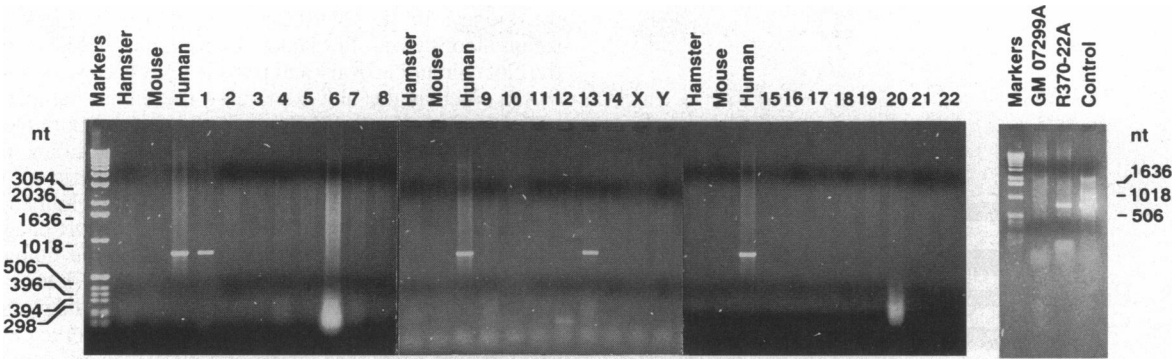


FIG. 5. Assignment of StAR pseudogene to human chromosomes 13. PCR analysis of somatic cell hybrid DNA was carried out with primers specific for the StAR pseudogene. The numbers above the lanes in the lefthand panel refer to the hybrids analyzed in Fig. 4. Hybrid "1" (GM 10880) contains human chromosomes X and 1. R370-22A contains human chromosomes 1 and 13. The hybrid designated "13" contains only human chromosome 13. Control is the cloned pseudogene sequences. The 800-nt StAR pseudogene amplification product is seen only in hybrids containing human chromosome 13.

10478, which in addition to containing human chromosome 20 also contains a fragment of human chromosome 8p. These findings indicated that the StAR gene resides on chromosome 8.

We examined somatic cell hybrid DNA by PCR with primers that specifically amplify the structural gene. Hybrids containing chromosome 8 gave a positive signal, whereas all other hybrids, including those known to contain human chromosome 20 but not chromosome 8, did not yield a specific amplification product (data not shown).

Analysis of a human chromosome 8 regional mapping panel placed the StAR gene on 8p (Fig. 4 *Upper*). Confirmation and refinement of the regional mapping of the functional StAR gene was carried out by isolating a YAC containing the StAR functional gene and using this YAC as a probe in FISH (Fig. 4 *Lower*). Regional mapping was done by sequential banding followed by FISH. By this method the StAR locus was assigned to 8p11.2. Simultaneous FISH with the StAR YAC and an 8 centromere-specific probe as well as fractional length measurements confirmed this assignment.

PCR analysis of reverse transcribed RNA from human testis and PCR analysis of human genomic DNA suggested the existence of a pseudogene. DNA sequences of the amplified pseudogene product did not contain introns and differed in a large number of positions from the functional StAR gene sequence in terms of nucleotide insertions, deletions, and substitutions. The amplified sequences differed among several individuals, suggesting significant polymorphism. Using primers specific for the pseudogene sequences, we determined that a StAR pseudogene resides on chromosome 13 (Fig. 5). Knowledge of their existence should prevent confusion in the molecular studies of the mRNA transcribed from the structural StAR gene.

The availability of the human StAR cDNA will facilitate studies on the structure of the StAR locus and regulation of steroidogenesis in human adrenals and gonads. Moreover, the cDNA probe can be used to explore the molecular pathology of diseases in which steroidogenesis is disordered.

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