In vivo effects of adrenocorticotrophin on the expression of the hamster steroidogenic acute regulatory protein

A Fleury, L Ducharme and J-G LeHoux

Department of Biochemistry, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada, J1H 5N4

(Requests for offprints should be addressed to J-G LeHoux)

ABSTRACT

In this study, we report the cDNA cloning of hamster adrenal steroidogenic acute regulatory (StAR) protein and the effect of adrenocorticotrophin (ACTH) on its expression *in vivo*. A hamster adrenal cDNA library was screened using an 852 bp fragment obtained by polymerase chain reaction; this fragment corresponds to the entire coding sequence (CDS) of the hamster adrenal StAR cDNA. Ten clones of different lengths were isolated and sequenced. The longest clone was 1564 bp and contained 34 bp in the 5'-untranslated region, 852 bp in the CDS, and 678 bp in the 3'-untranslated region (3'-UTR). Two polyadenylation signal sequences were found in the 3'-UTR. The CDS of the ten isolated clones was identical, but six of these lacked the last 132 nucleotides in the 3'-UTR, thus indicating that they had used the first polyadenylation signal. The hamster StAR protein

contains 284 amino acid residues, and is 91.9% homologous to mouse, 90.5% to rat, 86.4% to human, 85% to porcine, and 82.5% to bovine StAR protein. Southern blot analysis indicated the presence of only one StAR gene in the hamster genome. Northern blotting analysis revealed the presence of the StAR mRNA in male and female steroidogenic tissues, namely adrenals and gonads, but not in the liver or in the kidneys of either sex. Three mRNA species of 1.7, 3.1 and 5.3 kb were found in whole hamster adrenals. Administration of ACTH to hamsters provoked increases (two- to threefold) in the adrenal content of the StAR mRNA within 1 h *in vivo*. Western blotting analysis on adrenal mitochondria showed that the level of StAR protein was also significantly elevated (1.5-fold) 1 h after ACTH treatment.

Journal of Molecular Endocrinology (1998) **21**, 131–139

INTRODUCTION

Adrenal steroidogenesis involves numerous products all from one common precursor, cholesterol (LeHoux *et al.* 1996a). The transformation of cholesterol to pregnenolone is catalysed by the cholesterol side-chain cleavage enzyme complex (P450 scc) located in the inner mitochondrial membrane. This reaction was long believed to be the rate-limiting step in steroidogenesis; however, it is the transfer of cholesterol from the outer to the inner mitochondrial membrane that is rate-limiting (Crivello & Jefcoate 1980, Jefcoate *et al.* 1987). This transfer requires *de novo* synthesis of a regulatory protein which has recently been identified as the steroidogenic acute regulatory (StAR) protein (Clark *et al.* 1994).

The cDNA encoding the StAR protein was initially cloned from MA-10 mouse Leydig tumour

cells (Clark *et al.* 1994). Since then, StAR cDNAs have been cloned for many animal species, namely human (Gradi *et al.* 1995, Sugawara *et al.* 1995a), ovine (Juengel *et al.* 1995), bovine (Hartung *et al.* 1995), rat (Sandhoff & McLean 1996, Selvaraj *et al.* 1996, Lee *et al.* 1997, Miyamoto *et al.* 1997) and porcine (Pilon *et al.* 1997). The general effects of gonadotrophins (Pon & Orme-Johnson 1986) and adrenocorticotrophin (ACTH) (Pon *et al.* 1986, Epstein & Orme-Johnson 1991) on StAR protein expression have been well documented by studies with cell suspensions and cell cultures. The hormonal regulation of StAR expression *in vivo* has been less studied. Sandhoff & McLean (1996) found that StAR mRNA levels in the rat ovary were regulated by pregnant mare serum gonadotrophin and human chorionic gonadotrophin (CG) treatment. Preliminary work on the regulation of the hamster adrenal StAR mRNA levels was presented

at the Seventh Conference on the Adrenal Cortex (Fleury *et al.* 1996). We now report on how StAR mRNA and protein levels respond *in vivo* to ACTH stimulation in hamster adrenal, and on the cloning of hamster adrenal StAR cDNA. The hamster is an excellent model for such studies because, like humans, cortisol is the major glucocorticoid (LeHoux *et al.* 1992). Also hamster cytochrome P450 17 α -hydroxylase can transform pregnenolone to dehydroepiandrosterone (Cloutier *et al.* 1997), as in human adrenals.

MATERIALS AND METHODS

Materials and animals

[³²P] α -dCTP was purchased from Amersham Life Science Inc. (Oakville, ON, Canada), ACTH (Synacthen Depot) was obtained from Ciba Pharmaceuticals, Division of Ciba-Geigy Canada Ltd (Mississauga, ON, Canada) and [³⁵S]DNA markers were from Amersham International plc (Little Chalfont, Bucks, UK). Adult female and male Syrian golden hamsters were purchased from Charles River Canada Inc. (St-Constant, QC, Canada). Purina rat chow and tap water were available *ad libitum*. Prior to and during experimentation the animals were kept in an isolated room with a controlled light–darkness cycle (lights on from 0600–1800 h).

Treatment

Five groups of four male hamsters received intramuscular (i.m.) injections of ACTH in the gluteal region (1 IU Synacthen Depot (equivalent to 12 μ g peptide) diluted in 0.3 ml 0.9% NaCl/100 g body weight), at 5-h intervals for up to 20 h. Two groups of animals received one injection and were killed at 1 or 5 h after treatment; one group received 2 injections and was killed 10 h after the first treatment; another group received 3 injections and was killed 15 h after the first treatment; the last group received 5 injections and was killed 24 h after the first treatment. One additional group of hamsters was injected at 0 and 5 h and this group was killed 6 h after the first injection. Other groups of male hamsters were injected with 1 IU ACTH and then killed at 1, 3 or 5 h after treatment. Controls were injected with 0.9% NaCl and were killed immediately after administration. For all groups, the injection schedule was organised in order to kill animals between 0800–1000 h. Animals were killed by decapitation in accordance with the ethical standards of the institutional review committee.

Cloning of hamster StAR cDNA

Polymerase chain reaction (PCR), using *Taq* DNA polymerase enzyme (Pharmacia Biotech Inc. Baie d'Urfé, QC, Canada), was performed using primers based on a consensus between human and mouse StAR coding sequence (CDS). The first oligonucleotide, 5'-GGAATTCATGTTTCCTCGCTACGTTCAAGC-3', contained an EcoRI site and the start codon (ATG). The second oligonucleotide, 5'-GCTCTAGAGACACTGGGCCTCAGAGGCAGG-3', contained an XbaI site and a stop codon (TAG). From this, an 852 bp PCR product was amplified from a hamster adrenal Uni-ZAP XR custom cDNA library (Stratagene, La Jolla, CA, USA). This fragment was randomly labelled with [³²P] α -dCTP and used to screen the same cDNA library. The screening procedures were performed as previously described (Cloutier *et al.* 1997). Several clones were obtained, upon which an *in vivo* excision of pBluescript SK[−] from the Uni-ZAP XR bacteriophage was performed using Stratagene's ExASSIST/SOLR System (Stratagene). The isolated clones were then sequenced using Pharmacia's T7 sequencing kit (Pharmacia Biotech Inc.).

Southern blotting analysis

Genomic DNA was prepared from Syrian golden hamster kidneys. Thirty microgrammes DNA were digested with EcoRI, BamHI, HindIII, PstI, XbaI, BglII and KpnI, separated on 0.8% agarose gel and blotted on Hybond N nylon membrane (Amersham International plc). The PCR fragment corresponding to the CDS of hamster StAR cDNA was radiolabelled for hybridisation. Radioactivity on the membrane was detected using an optical imager (PhosphorImager SF, Molecular Dynamics, Sunnyvale, CA, USA).

RNA extraction from tissues and Northern blotting analysis

Total RNA from hamster gonads, liver, kidneys and adrenals was extracted using the Tri-Reagent protocol (Molecular Research Center, Cincinnati, OH, USA). The poly(A)⁺ fraction was obtained using an oligo (dT)-cellulose spun column (Pharmacia Biotech Inc.). Total RNA (15 μ g) samples were denatured with glyoxal (Tremblay *et al.* 1991), and then fractionated by electrophoresis on a diethyl pyrocarbonate-treated 1% agarose gel in 0.01 M phosphate buffer, pH 7.0. The fractionated RNA was transferred to positively charged nylon membranes (Boehringer Mannheim, Mannheim,

Germany), which were then hybridised for 16 h at 42 °C with a ^{32}P -labelled hamster adrenal StAR PCR fragment containing the entire CDS. The blots were also analysed with an 18S cDNA probe for standardisation of quantities of mRNA. Autoradiograms were obtained by exposing the blots to Kodak X-Omat RP films with Hyperscreen intensifying screens (Amersham International plc, Little Chalfont, Bucks, UK). The intensity of bands on the films was determined using a LKB 2222-020 Ultrascan XL laser densitometer (Pharmacia Biotech Inc.). For some experiments, radioactivity was detected using the above mentioned optical imager.

Immunoblotting

Hamster whole adrenals were homogenised in 50 mM Tris, pH 7.4, 0.25 M sucrose, 5 mM EDTA, 1 mM phenylmethyl-sulfonylfluoride, 0.1 mM leupeptin, 30 mM iodoacetamide and 0.125 μM aprotinin using a Potter-Elvehjem homogeniser with a loosely fitting Teflon pestle. A portion of the homogenate was centrifuged for 10 min at 900 g , and the supernatant was then centrifuged for 15 min at 9500 g . The mitochondrial pellet was resuspended in homogenisation buffer. Cholate and sodium dodecyl sulphate (SDS) were added to the homogenate and mitochondrial fractions to final concentrations of 1% and 0.1% respectively. Preparations were frozen in liquid nitrogen, thawed twice and solubilised in Laemmli sample buffer (Laemmli 1970). They were passed through a 26-gauge needle, then boiled for 5 min and finally centrifuged at 12 000 g for 2 min. Soluble proteins, 80 μg homogenate and 50 μg mitochondrial preparations were electrophoresed on 10% polyacrylamide gel in the presence of 0.1% SDS (SDS-PAGE) and analysed by immunoblotting as previously described (LeHoux *et al.* 1992, 1996b) using a rabbit polyclonal anti-mouse StAR protein antibody kindly given by Dr Dale Buchanan Hales (Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL, USA); this antibody was raised against a GST-fusion protein from a mouse StAR cDNA fragment from bp 208–1467 (Clark *et al.* 1997). Immunoreactive proteins were detected using ECL light emitting reagents (Amersham International plc). The relative intensities were determined with the laser densitometer mentioned above.

Cortisol determination

Plasma cortisol was analysed by radioimmunoassay as previously described (LeHoux *et al.* 1992).

RESULTS

Cloning of hamster StAR cDNAs and amino acid sequence alignment

StAR cDNAs were obtained by screening a cDNA library from hamster adrenals with an 852 bp PCR fragment corresponding to the entire hamster StAR CDS. Clone 3-2 has a total length of 1564 bp, of which 852 bp are in the open reading frame giving rise to a protein of 284 amino acids, with a predicted molecular mass of 31.5 kDa. The 5'-untranslated region (5'-UTR) contains 34 bp, and the 3'-untranslated region (3'-UTR) contains 678 bp including two putative polyadenylation signals (Fig. 1). Nine other clones were sequenced and they were found to share 100% identity within the original CDS, but six of these lack the last 132 nucleotides in their 3'-UTR. Thus six clones utilised the first polyadenylation signal, and four utilised the second. Figure 2 shows amino acid sequence alignments between the hamster StAR protein and that of other species. The boxes indicate consensus between at least five species demonstrating a strong identity among the studied species. Proposed cleavage sites for the matrix-processing protease and the mitochondrial intermediate-processing peptide are designated as C1 and C2 in Fig. 2. This region of the protein contains an amino acid motif that is highly conserved in presequences that undergo a two-step cleavage (Hendrick *et al.* 1989). The predicted molecular mass of the cleaved hamster protein is 26.3 kDa. The hydrophobicity profile (Kyte & Doolittle 1982) obtained for the hamster StAR protein is very similar to those of the other species studied; most of the conserved regions are hydrophilic (results not shown).

Genomic DNA Southern blotting

Southern blot analysis was performed on hamster genomic DNA digested with different restriction enzymes. Results obtained suggest the presence of only one gene for StAR in the hamster genome (Fig. 3). Indeed, digestion with EcoRI, HindIII and KpnI produced single genomic fragments of 8.2 kb, 9.3 kb, and 11.4 kb respectively, specifically hybridising with the StAR PCR fragment; this suggests that the whole CDS of hamster StAR protein was included in these fragments. BamHI, XbaI and BglII enzymes that do not cut in the StAR CDS produced two fragments, suggesting that these enzymes have restriction sites in intronic sequences of the StAR gene.

cacaagtgcaagtgcctgccacatctgcaggaagcATGTTGCTAGCCACGTTTAAGCTGTGTGCTGGC	67
MetLeuLeuAlaThrPheLysLeuCysAlaGly	(11)
AAGTCCTACAGACATCTGCGGAACATGAAAGGACTGAGACACCAAGCTGTGCTGGCCATTGGCCAGGAA	136
SerSerTyrArgHisLeuArgAsnMetLysGlyLeuArgHisGlnAlaValLeuAlaIleGlyGlnGlu	(34)
CTCAACAGAAGAACTGGGAGACTCCAGCCCTGGATGGATCAGTCAGGTCGGCGTCGGAGCTCTCTG	205
LeuAsnArgArgThrLeuGlyAspSerSerProGlyTrpIleSerGlnValArgArgSerSerLeu	(57)
CTTGTTCTCAGCTGGAAGCGGCACTCTACAGTGAGCAGGAGCTGTCTATATCCAGCAGGGAGAGGTG	274
LeuGlySerGlnLeuGluAlaAlaLeuTyrSerGluGlnGluLeuSerTyrIleGlnGlnGlyGluVal	(80)
GCTATGCAGAAGGCCTTGAGTATCCTCAGCAACCAGGAAGGCTGGAAGAAAGAGAACCAGCAGGAAAAC	343
AlaMetGlnLysAlaLeuSerIleLeuSerAsnGlnGluGlyTrpLysLysGluAsnGlnGlnGluAsn	(103)
GGGGATGAGGTGCTGAGTAAAGTGGTCCCTGATGTGGGCAAGGTATTTCCGCTGGAGGTGGTGGTAGAC	412
GlyAspGluValLeuSerLysValValProAspValGlyLysValPheArgLeuGluValValValAsp	(126)
CAGCCCATGGACAGACTCTATGCAGAACTTGTGGACCGCATGGAGGCAATGGGAGAGTGGAAACCCAAAT	481
GlnProMetAspArgLeuTyrAlaGluLeuValAspArgMetGluAlaMetGlyGluTrpAsnProAsn	(149)
GTCAAGGAGATCAAGGTCTCTGCAAAAGATTGGGAAAGACACAGTCATCACCCTAGAGCTGGCTGCAGCA	550
ValLysGluIleLysValLeuGlnLysIleGlyLysAspThrValIleThrHisGluLeuAlaAlaAla	(172)
GCAGCAGGAAACCTGGTGGGACCCCGTGACTTTGTGAGCGTACGCTGTGCCAAGCGCGAGGCTCCACC	619
AlaAlaGlyAsnLeuValGlyProArgAspPheValSerValArgCysAlaLysArgArgGlySerThr	(195)
TGTGTGCTGGCAGGCATAGCCACACATTTTGGGGAGATGCCTGAACAAAGCGGTGTTATCAGAGCTGAA	688
CysValLeuAlaGlyIleAlaThrHisPheGlyGluMetProGluGlnSerGlyValIleArgAlaGlu	(218)
CAGGGTCCACCTGCATGGTGTCTTACCCACTGGCTGGGAGTCCCTCGAAGACTAAATTCACCTTGGCTG	757
GlnGlyProThrCysMetValLeuHisProLeuAlaGlySerProSerLysThrLysPheThrTrpLeu	(241)
CTCAGTATTGACCTCAAGGGTGGCTGCCGAAAAGCATCATCAATCAGGTCTTATCACAAACCCAGATG	826
LeuSerIleAspLeuLysGlyTrpLeuProLysSerIleIleAsnGlnValLeuSerGlnThrGlnMet	(264)
GAGTTTGCAACACCTGCGCAAGCGCTGGAGTCCAGCTCTGCCTCTGAGGCCCGGTGTTaagactgt	895
GluPheAlaAsnHisLeuArgLysArgLeuGluSerSerSerAlaSerGluAlaArgCys	(284)
accactacatcaacctgcatgcatcagcagcctcccgaggaagcctgcaagtctgtttcaaatctc	964
cagttgatgactacgggaggggtagctacttatcagacactaggactgactggcgaaacatgaccatcag	1033
gaaattagcaatgaggctgagcagaagagagcctcagcctctcccacaagattagctgtgaagtgggct	1102
aagagatagacatgtaaaccttttatgtaggcctgtgtatgctcacctaaaaaacctactcaaaacgc	1171
aagtagttagttgacaaaggggggtgttttaggaactgtgtctcttgagctcaaaagccactgcctgaa	1240
gcagtggtgtgcacacagcaaggtctcccagggacttcagtgacctctgttctccataaaaggccaa	1309
gaacatcaaacactcacaacagaccttccagagtacactccaagttctctctaaggagaagggaaa	1378
A	
ctaaatattacgttggcttcctactattacatcacaaataaagtaccacacatgaaatttcagcctat	1447
acttgtaagtccttatggcacagaatctgtgctgggagccacagcttcacagactctggatcaagac	1516
B C	
tgtgggttccaatttccatgttcattaaagggaacaaaacttcacattt	1564

FIGURE 1. Nucleotide and deduced amino acid sequences of the StAR cDNAs isolated from a Syrian hamster adrenal library. Numbers underlined indicate nucleotide positions, those in parentheses indicate amino acid positions. The two putative polyadenylation signals 'ataaa' and 'attaaa' are doubly underlined. Six clones utilised the first polyadenylation signal and terminated at nucleotide 1432 (A). Four clones utilised the second polyadenylation signal, two terminated at nucleotide 1557 (B) and two others at nucleotide 1564 (C). Both 5'- and 3'-UTR are in lower case; the capitalised letters indicate the nucleotides of the open reading frame. The longest cDNA sequence was deposited in the GenBank database under the accession number U66490.

Tissue distribution of hamster StAR mRNAs

The tissue distribution of hamster StAR mRNAs was assessed by Northern blotting analysis (Fig. 4). Total RNA was extracted from female and male hamsters of the same age, from ovary, testis, liver, kidneys and adrenals. Poly(A)⁺ RNA was also pre-

pared from adrenals. Hybridisation with a ³²P-labelled PCR fragment containing the entire CDS of hamster StAR protein revealed the presence of StAR mRNA in adrenal tissues and gonads of both sexes, but not in liver or kidneys, where no signals were detected even after 72 h of film exposure (results not shown). Two major mRNA species of 1.7 kb and

		C1		
HAM	M L L A T F K L C A G S S Y R H I	R N M K G L R H	Q A Y L A I G Q E L N R R A L G	41
RAT	M L L A T F K L C A G S S Y R H I	R N M K G L R H	Q A Y L A I G Q E L N R R A L G	37
MOU	M L L A T F K L C A G S S Y R H I	R N M K G L R H	Q A Y L A I G Q E L N R R A L G	41
HUM	M L L A T F K L C A G S S Y R H I	R N M K G L R H	Q A Y L A I G Q E L N R R A L G	41
POR	M L L A T F K L C A G S S Y R H I	R N M K G L R H	Q A Y L A I G Q E L N R R A L G	41
BOV	M L L A T F K L C A G S S Y R H I	R N M K G L R H	Q A Y L A I G Q E L N R R A L G	41
OVI	M L L A T F K L C A G S S Y R H I	R N M K G L R H	Q A Y L A I G Q E L N R R A L G	0
		C2		
HAM	D S S P G - M I S O Y R R R S S L L G S Q L E A A L Y S E Q E L S Y I Q G G E V A			81
RAT	D S S P G - M M G Q Y R R R S S L L G S Q L E A T L Y S D Q E L S Y I Q G G E V A			77
MOU	D S S P G - M M G Q Y R R R S S L L G S Q L E A T L Y S D Q E L S Y I Q G G E V A			81
HUM	D S S P G - M M G Q Y R R R S S L L G S Q L E A T L Y S D Q E L S Y I Q G G E V A			82
POR	G P T S G W I N O Y R R R S S L L G S Q L E D T L Y S D Q E L A H I Q G G E V A			82
BOV	G P A D A A W I N O Y R R R S S L L G S Q L E D P L Y S D Q E L A H I Q G G E V A			82
OVI				0
HAM	M Q K A L S I L S N Q E G W K K E S I Q E N G D E Y L S K V Y P D Y G K Y F R L E			122
RAT	M Q K A L G I L N N Q E G W K K E S I Q E N G D E Y L S K V Y P D Y G K Y F R L E			118
MOU	M Q K A L G I L N N Q E G W K K E S I Q E N G D E Y L S K V Y P D Y G K Y F R L E			122
HUM	M Q K A L G I L N N Q E G W K K E S I Q E N G D E Y L S K V Y P D Y G K Y F R L E			123
POR	M Q K A L G I L N N Q E G W K K E S I Q E N G D E Y L S K V Y P D Y G K Y F R L E			123
BOV	M Q K A L G I L N N Q E G W K K E S I Q E N G D E Y L S K V Y P D Y G K Y F R L E			123
OVI				27
HAM	Y Y Y D Q P M D R L Y A E L Y D R M E A M G E W N P N Y K E I K Y L Q K I G K D T			163
RAT	Y Y Y D Q P M D R L Y E E L Y D R M E A M G E W N P N Y K E I K Y L Q K I G K D T			159
MOU	Y Y Y D Q P M D R L Y E E L Y D R M E A M G E W N P N Y K E I K Y L Q K I G K D T			163
HUM	Y Y Y D Q P M E R L Y E E L Y E R M E A M G E W N P N Y K E I K Y L Q K I G K D T			164
POR	Y Y Y D Q P M E R L Y E E L Y E R M E A M G E W N P N Y K E I K Y L Q K I G K D T			164
BOV	Y Y Y D Q P M E R L Y E E L Y E R M E A M G E W N P N Y K E I K Y L Q K I G K D T			164
OVI				68
HAM	V I T H E L A A A A A G N L Y G P R D F Y S V R C A K R R G S T C V L A G L I A T H			204
RAT	V I T H E L A A A A A G N L Y G P R D F Y S V R C T K R R G S T C V L A G M A T H			200
MOU	V I T H E L A A A A A G N L Y G P R D F Y S V R C T K R R G S T C V L A G M A T H			204
HUM	V I T H E L A A A A A G N L Y G P R D F Y S V R C A K R R G S T C V L A G M A T D			205
POR	V I T H E L A A A A A G N L Y G P R D F Y S V R C T K R R G S T C V L A G M A T D			205
BOV	V I T H E L A A A A A G N L Y G P R D F Y S V R C T K R R G S M C V L A G M A T L			205
OVI				109
HAM	F G E M P E Q S G Y I R A E H G P T C M Y L H P L A G S P S K T K L T W L L S I D			245
RAT	F G E M P E Q S G Y I R A E H G P T C M Y L H P L A G S P S K T K L T W L L S I D			241
MOU	F G E M P E Q S G Y I R A E H G P T C M Y L H P L A G S P S K T K L T W L L S I D			245
HUM	F G N M P E Q K G Y I R A E H G P T C M Y L H P L A G S P S K T K L T W L L S I D			246
POR	F G E M P E Q K G Y I R A E H G P T C M Y L H P L A G S P S K T K L T W L L S I D			246
BOV	F G E M P E Q K G Y I R A E H G P T C M Y L R P L A G S P S R T K L T W L L S I D			246
OVI	Y E M P Q Q K G Y I R A E H G P T C M Y L R P L A G S P S R T K L T W L L S I D			121
HAM	L K G W L P K T I I N Q Y L S Q T Q I E F A N H L R K R L E S S P A S E A R C			284
RAT	L K G W L P K T I I N Q Y L S Q T Q I E F A N H L R K R L E S S P A S E A R C			280
MOU	L K G W L P K T I I N Q Y L S Q T Q I E F A N H L R K R L E S S P A S E A R C			284
HUM	L K G W L P K S I I N Q Y L S Q T Q Y D F A N H L R K R L E S H P A S E A R C			285
POR	L K G W L P K T I I N Q Y L S Q T Q Y D F A N H L R K R L E S R P A L E A R C			285
BOV	L K G W L P K T I I N Q Y L S Q T Q Y D F A N H L R K R L E S C P A L E A R C			285
OVI				121

FIGURE 2. Comparison of the deduced amino acid sequence of the hamster (HAM) StAR protein with that of other species. The compared species were rat (RAT), mouse (MOU), human (HUM), pig (POR), bovine (BOV) and ovine (OVI). Boxes indicate consensus between at least five species. The ovine sequence is incomplete. For mouse and hamster, the proposed cleavage sites for the mitochondrial matrix-processing protease and the mitochondrial intermediate-processing peptide are indicated by C1 and C2 respectively.

3.1 kb were found in adrenals and gonads. Minor mRNA species of 5.3 kb in adrenals and 6 kb in gonads were also found. The 6 kb band was more intense in the testis than in the ovary. Hybridisation of the ^{32}P -labelled PCR fragment with the adrenal poly(A)⁺ RNA indicated that the three StAR mRNA species were polyadenylated, and consequently were not pre-mRNA. Based on poly(A)⁺ RNA results, in adrenals of control hamsters, the relative abundance of 1.7 kb, 3.1 kb and 5.3 kb StAR mRNA was 55%, 42%, and 3%.

ACTH-stimulated expression of hamster StAR mRNA *in vivo*

We have studied the short term (one injection) and long term (up to five injections) effects of ACTH on

FIGURE 3. Genomic Southern blot for hamster StAR gene. Genomic DNA (30 µg) prepared from hamster kidney was digested with EcoRI, BamHI, HindIII, PstI, XbaI, BglII and KpnI, lanes 1 to 7 respectively. After electrophoresis and transfer on nylon membrane, digested DNA was hybridised with a hamster StAR PCR fragment corresponding to the entire CDS. S, [^{35}S]DNA markers used for estimation of the length of genomic fragments; kb, kilobases.

StAR mRNA levels. We previously reported (LeHoux *et al.* 1992) that one single injection of ACTH to hamsters could not sustain elevated plasma cortisol for more than 5 h, and that serial ACTH injections at 5-h intervals resulted in sustained high plasma cortisol levels. Northern blotting analysis (Fig. 5) showed that the administration of ACTH to hamsters provoked an increase in the intensity of the two main StAR mRNA bands at 1.7 and 3.1 kb in the adrenal; indeed, within 1 h after ACTH administration, the level of these two mRNA bands was increased by 192% and 299% respectively. For the 1.7 kb band the greatest increase was observed when hamsters were sacrificed at time 5 h (291%), whereas the increase for the 3.1 kb band was 293%. Moreover, these increases were sustained at times 10, 15, and 24 h. When animals were injected at times 0 and 5 h

FIGURE 4. Tissue distribution of hamster StAR mRNAs. Lanes 1' and 2': 15 µg total RNA from ovary and testis with a film exposure time three times longer than for lanes 1 to 9 (film exposure time is 24 h). Lanes 1–8: 15 µg total RNA from ovary, testis, female kidney, male kidney, female liver, male liver, female adrenal and male adrenal respectively. Lane 9: 1 µg poly(A)⁺ RNA from male hamster adrenals. Electrophoresis was carried out in 1% agarose gel. The kb scale on the right hand side is for lane 9 only. [³⁵S]DNA markers were used for estimation of the length of transcripts.

and then killed 6 h after the first injection, the adrenal level of StAR mRNA was further increased (1.7 kb, 412%; 3.1 kb, 364%), compared with animals which had received only one injection at time 0 and were killed at time 5 h. The 5.3 kb band was also increased, with a peak intensity 5 h after ACTH administration, and decreased thereafter. Membranes were stripped and then hybridised with a ³²P-labelled 18S cDNA probe (results not shown) and results obtained were used to normalise the relative quantity of RNA between wells.

Western analysis on hamster adrenal under *in vivo* ACTH treatment

Western blotting analysis on hamster adrenal mitochondrial preparations showed the presence of three immunoreactive protein bands of about 32, 30 and 29 kDa. The levels of the 30 and 32 kDa bands were already significantly elevated 1 h after ACTH treatment, with increases of $147 \pm 13\%$ and $135 \pm 10\%$ (means $\pm$ S.E.M., $n=3$, $P<0.05$) respectively, and remained elevated at 5 h; the apparent increase of the 29 kDa band was not statistically significant (Fig. 6). Similar results were obtained with homogenate preparations (results not shown). Hamster plasma cortisol levels in nmol/l (means $\pm$ S.E.M., $n=3$) were 8.3 ± 1.3 for controls and 127 ± 30 ($P=0.017$), 88 ± 24 ($P=0.028$), and 42 ± 34 (not significant) at times 1, 3 and 5 h after ACTH treatment.

FIGURE 5. The effects of ACTH on the level of hamster adrenal StAR mRNA. Groups of male hamsters received one, two, three or five i.m. injections of ACTH at 5-h intervals, as described in Materials and Methods. These animals were killed at 1, 5, 10, 15 or 24 h after the first injection. One additional group of hamsters (6*) was injected at 0 and 5 h and this group was killed 6 h after the first injection. Controls were injected with 0.9% NaCl. Electrophoresis was carried out in 1% agarose gel. [³⁵S]DNA markers were used for estimation of the length of transcripts. Film exposure time was 72 h.

FIGURE 6. Western blotting analyses on hamster adrenal mitochondrial preparations. Groups of three male hamsters were injected i.m. with 1 IU ACTH; they were killed 1, 3 or 5 h after treatment. Controls were injected with 0.9% NaCl. Analyses were performed on 50 µg mitochondrial protein on SDS-PAGE.

DISCUSSION

The deduced amino acid sequence of the hamster StAR protein is composed of 284 residues and thus falls in a distinct sub-group with the mouse and the

rat. In comparison, the deduced amino acid sequence of the human, porcine and bovine StAR protein possesses 285, one more amino acid than that of rodents (Fig. 2). The hamster StAR protein sequence is highly homologous with that of the mouse (91.9%) and only slightly less with that of the rat, human, porcine, and bovine proteins. The hamster StAR protein shares 100% identity with the putative mitochondrial signal sequence cleavage site described for the mouse sequence (Stocco & Clark 1996). This sequence follows the consensus motif for two cleavages (Hendrick *et al.* 1989), whereas only one cleavage is predicted for sequences of other species (Fig. 2). Furthermore, the primary structure of the hamster StAR protein is very similar to that of the mouse for the first 25 residues (23/25) at the amino terminus and the predicted secondary structure is an amphipathic α helix (Clark *et al.* 1994), a characteristic consistent with mitochondrial targeting sequences.

The hamster StAR protein possesses many consensus motifs for different putative phosphorylation sites. Many putative phosphorylation sites are conserved between species, which strongly suggests that they could be the sites of those post-translational modifications necessary for the transfer of cholesterol to P450 scc in the mitochondria. In agreement with this, Arakane *et al.* (1997) reported that phosphorylation of serine 195 on the human StAR protein increases the biological activity of StAR which accounts, in part, for the immediate effects of cAMP on steroid production. Moreover, it has been shown that phosphoproteins were generated following ACTH (Krueger & Orme-Johnson 1983, Pon *et al.* 1986) or cAMP stimulation of rat adrenal cells (Elliott *et al.* 1997).

In agreement with reports on other species, StAR mRNA was found in hamster steroidogenic tissues, namely adrenals and gonads. No StAR mRNA was found in the liver or in the kidneys by Northern analysis. In humans, a 1.6 kb StAR mRNA was detected in a kidney preparation (Sugawara *et al.* 1995a). In agreement with our preliminary report (Fleury *et al.* 1996), in non-stimulated hamster adrenals, two major StAR mRNA species were revealed by Northern analysis at 1.7 kb and 3.1 kb, and a minor species at 5.3 kb (Fig. 4). These three mRNA species were also found after chromatography of total adrenal RNA on an oligo-dT column, indicating that they were polyadenylated. After a long exposure time, a transcript of 0.65 kb was also observed, but this mRNA species was not stimulated by ACTH treatment (Fleury *et al.* 1996) and may not be related to StAR. Hartung *et al.* (1995) have also found a weak low molecular weight mRNA band in bovine adrenal hybridising with a

bovine StAR cDNA probe, which they tentatively identified as a tissue inhibitor of metalloproteinases (TIMP-1) mRNA. The two main StAR mRNA species were also found in hamster testis and ovary with the 1.7 kb species predominating. In gonads, the 5.3 kb mRNA band was absent and after a long exposure time, a 6 kb species was revealed. The level of StAR mRNA in the gonads was much lower than that in the adrenals, probably due to the preparations containing fewer steroidogenic cells.

In this study we sequenced ten StAR cDNAs isolated from a hamster adrenal cDNA library. Although the CDS of these cDNA was identical, the 3'-end differed in length and two polyadenylation signal sites were identified. These cannot explain the difference in length of mRNA species observed by Northern analysis. Various explanations have been proposed for the different lengths of StAR mRNAs in different species. For example other 3'-end polyadenylation sites could be present in the hamster StAR gene. In agreement with this hypothesis, Hartung *et al.* (1995) have cloned a bovine StAR transcript from a downstream polyadenylation site which gives rise to a long 3'-UTR. In the rat, the very long exon seven explains the synthesis of the 3.5 kb mRNA (Kim *et al.* 1997). Longer mRNA species could also come from alternative splicing. Indeed, Sugawara *et al.* (1995b) isolated and sequenced one structural human StAR gene which they predicted to be 1642 nucleotides, in agreement with the size of the primary StAR mRNA observed by Northern blotting analysis. Since the human gene spans 8 kb and consists of seven exons and six introns, they speculated that the larger StAR mRNA species of 4.4 kb and 7.5 kb observed in human adrenals may represent nascent transcripts of partially or alternatively spliced mRNAs.

The stimulating effect of ACTH on the production of hamster adrenal StAR mRNA was seen 1 h after its administration (Fig. 5), and maximal stimulation occurred within 5 h. However, when animals were killed at 6 h, 1 h after the second injection, a further enhancement of the StAR mRNA levels was observed. This suggests that ACTH injection initially increases StAR mRNA levels over the first hour, and that these then fall back to lower levels but can be increased again by subsequent injection which increased circulating ACTH. Although ACTH increased the intensity of the three mRNA bands (1.7, 3.1 and 5.3 kb), the 1.7 and 3.1 kb mRNA bands were the most affected. In agreement with our results, Kim *et al.* (1997) reported that in hypophysectomised rats, ACTH stimulated 1.6 and 3.5 kb mRNA levels in whole adrenals.

In confirmation of results from Northern blot analysis, increases in the levels of immunoreactive StAR protein bands of 32 and 30 kDa were observed 1 h after ACTH administration and remained elevated at 3 h and 5 h. The increase in the level of a 29 kDa immunoreactive band after ACTH administration was not statistically significant. This species was produced in smaller quantities than the other two. The same antibody used by Clark *et al.* (1997) to analyse StAR protein in MA-10 mouse Leydig tumor cells, specifically immunoprecipitated two proteins of 35.5 and 29.1 kDa. This antibody was also successfully used for immunofluorescence histochemical studies and immuno-electron microscopy in rat adrenal (Ronen-Fuhrmann *et al.* 1998).

Although maximally increased after 1 h (1524%), hamster plasma cortisol levels decreased thereafter to values of 1056% and 504% after 3 h and 5 h of ACTH treatment respectively. At the present time we do not know why the plasma cortisol level was declining 5 h after ACTH administration while the adrenal StAR protein content was still maximally elevated. We can only speculate that, in the hamster adrenal *in vivo*, the quantity of StAR protein may not be the only rate-limiting component of steroidogenesis and that cholesterol availability, post-translational modifications, such as protease processing and phosphorylation which result in the formation of specific StAR species, may also be important.

In conclusion, in this study we have cloned the hamster adrenal StAR cDNA and we have shown that StAR protein is expressed in gonads and adrenals. We have also shown that administration of ACTH induces a rapid increase (within 1 h) in adrenal StAR mRNA and protein, showing the importance of this component under acute ACTH stimulation *in vivo*.

ACKNOWLEDGEMENTS

We thank Andrée Lefebvre for her assistance. We also thank Dr Dennis Shapcott for reviewing this article. This work was supported by a grant from the Medical Research Council of Canada (MT10983), and the Heart and Stroke Foundation of Canada.

REFERENCES

- Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM & Strauss JF 1997 Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. *Journal of Biological Chemistry* **272** 32656–32662.
- Clark BJ, Wells J, King SR & Stocco DM 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumour cells. Characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry* **269** 28314–28322.
- Clark BJ, Combs R, Hales KH, Hales DB & Stocco DM 1997 Inhibition of transcription affects synthesis of steroidogenic acute regulatory protein and steroidogenesis in MA-10 mouse Leydig tumor cells. *Endocrinology* **138** 4893–4901.
- Cloutier M, Fleury A, Courtemanche J, Ducharme L, Mason JI & LeHoux JG 1997 Characterization of the adrenal cytochrome P450C17 in hamster, a small animal model for the study of dehydroepiandrosterone. *DNA and Cell Biology* **16** 357–368.
- Crivello JF & Jefcoate CR 1980 Intracellular movement of cholesterol in rat adrenal cells. *Journal of Biological Chemistry* **255** 8144–8151.
- Elliott ME, Goodfriend TL, Ball DL & Jefcoate CR 1997 Angiotensin-responsive adrenal glomerulosa cell proteins: characterization by protease mapping, species comparison, and specific angiotensin receptor antagonists. *Endocrinology* **138** 2530–2536.
- Epstein LF & Orme-Johnson NR 1991 Regulation of steroid hormone biosynthesis: identification of precursors of a phosphoprotein targeted to the mitochondrion in stimulated rat adrenal cortex cells. *Journal of Biological Chemistry* **266** 19739–19745.
- Fleury A, Cloutier M, Ducharme L, Lefebvre A, LeHoux J & LeHoux JG 1996 Adrenocorticotropin regulates the level of the steroidogenic acute regulatory (StAR) protein mRNA in hamster adrenals. *Endocrine Research* **22** 515–520.
- Gradi A, Tangwai R, McBride HM, Chu LL, Shore GC & Pelletier J 1995 The human steroidogenic acute regulatory (StAR) gene is expressed in the urogenital system and encodes a mitochondrial polypeptide. *Biochimica et Biophysica Acta* **1258** 228–233.
- Hartung S, Rust W, Balvers M & Ivell R 1995 Molecular cloning and *in vivo* expression of the bovine steroidogenic acute regulatory protein. *Biochemical and Biophysical Research Communications* **215** 646–653.
- Hendrick JP, Hodges PE & Rosenberg LE 1989 Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: leader peptides cleaved by two matrix proteases share a three-amino acid motif. *Proceedings of the National Academy of Sciences of the USA* **86** 4056–4060.
- Jefcoate CR, DiBartolomeis MJ, Williams C & McNamara BC 1987 ACTH regulation of cholesterol movement in isolated adrenal cells. *Journal of Steroid Biochemistry* **27** 721–729.
- Juengel JL, Meberg BM, Turzillo AM & Niswender GD 1995 Hormonal regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in ovine corpora lutea. *Endocrinology* **136** 5423–5429.
- Kim YC, Ariyoshi N, Artemenko I, Elliott ME, Bhattacharyya KK & Jefcoate CR 1997 Cytochrome P450 scc in rat adrenal cells mediated by regulation of the steroidogenic acute regulatory protein. *Steroids* **62** 10–20.
- Krueger RJ & Orme-Johnson NR 1983 Acute adrenocorticotrophic hormone stimulation of adrenal corticosteroidogenesis. *Journal of Biological Chemistry* **258** 10159–10167.
- Kyte J & Doolittle RF 1982 A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157** 105–132.
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.

- Lee HK, Ahn RS, Kwon HB & Soh J 1997 Nucleotide sequence of rat steroidogenic acute regulatory protein complementary DNA. *Biochemical and Biophysical Research Communications* **230** 528–532.
- LeHoux JG, Mason JI & Ducharme L 1992 *In vivo* effects of adrenocorticotropin on hamster adrenal steroidogenic enzymes. *Endocrinology* **131** 1874–1882.
- LeHoux JG, Bernard H, Ducharme L, Lefebvre A, Shapcott D, Tremblay A & Véronneau S 1996a The regulation of the formation of glucocorticoids and mineralocorticoids *in vivo*. In *Advances in Molecular and Cell Biology*, vol 14, pp 149–201. Ed CR Jefcoate. London, England: JAI Press Inc.
- LeHoux JG, Lefebvre A, Ducharme L, LeHoux J, Martel D & Brière N 1996b Some effects of a low sodium intake on the expression of P450 aldosterone synthase in the hamster adrenal cortex: immunoblotting, immunofluorescent and immuno-gold electron microscopic studies. *Journal of Endocrinology* **149** 341–349.
- Miyamoto K, Mizutani T & Sonoda Y 1997 In GenBank, Accession Number AB001349.
- Pilon N, Daneau I, Brisson C, Éthier JF, Lussier JG & Silversides DW 1997 Porcine and bovine steroidogenic acute regulatory protein (StAR) gene expression during gestation. *Endocrinology* **138** 1085–1091.
- Pon LA & Orme-Johnson NR 1986 Acute stimulation of steroidogenesis in corpus luteum and adrenal cortex by peptide hormones. Rapid induction of a similar protein in both tissues. *Journal of Biological Chemistry* **261** 6594–6599.
- Pon LA, Hartigan JA & Orme-Johnson NR 1986 Acute ACTH regulation of adrenal corticosteroid biosynthesis. Rapid accumulation of a phosphoprotein. *Journal of Biological Chemistry* **261** 13309–13316.
- Ronen-Fuhrmann T, Timberg R, King SR, Hales KH, Hales DB, Stocco DM & Orly J 1998 Spatio-temporal expression patterns of steroidogenic acute regulatory protein (StAR) during follicular development in the rat ovary. *Endocrinology* **139** 303–315.
- Sandhoff TW & McLean MP 1996 Hormonal regulation of steroidogenic acute regulatory (StAR) protein messenger ribonucleic acid expression in the rat ovary. *Endocrine* **4** 259–267.
- Selvaraj N, Israeli D & Amsterdam A 1996 Partial sequencing of the rat steroidogenic acute regulatory protein message from immortalized granulosa cells: regulation by gonadotropins and isoproterenol. *Molecular and Cellular Endocrinology* **123** 171–177.
- Stocco DM & Clark BJ 1996 Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Reviews* **17** 221–244.
- Sugawara T, Holt JA, Driscoll D, Strauss III JF, Lin D, Miller WL, Patterson D, Clancy KP, Hart IM, Clark BJ & Stocco DM 1995a 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. *Proceedings of the National Academy of Sciences of the USA* **92** 4778–4782.
- Sugawara T, Lin D, Holt JA, Martin KO, Javitt NB, Miller WL & Strauss III JF 1995b Structure of the human steroidogenic acute regulatory protein (StAR) gene: StAR stimulates mitochondrial cholesterol 27-hydroxylase activity. *Biochemistry* **34** 12506–12512.
- Tremblay A, Waterman MR, Parker KL & LeHoux JG 1991 Regulation of rat adrenal messenger RNA and protein levels for cytochrome P-450 s and adrenodoxin by dietary sodium depletion or potassium intake. *Journal of Biological Chemistry* **266** 2245–2251.

REVISED MANUSCRIPT RECEIVED 16 March 1998