Expression of the full-length and alternatively spliced equine luteinizing hormone/chorionic gonadotropin receptor mRNAs in the primary corpus luteum and fetal gonads during pregnancy

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Abstract

The full-length equine luteinizing hormone/chorionic gonadotropin (LH/CG) receptor (eLH/CG-R_A) cDNA and two alternatively spliced isoforms (eLH/CG-R_{B,C}) were isolated from luteal tissue and characterized using a combination of reverse transcription-polymerase chain reaction (RT-PCR) and 5'-rapid amplification of cDNA ends. The 680-amino acid full sequence of eLH/CG-R_A displayed 87–92% homology with other mammalian LH/CG-Rs. The eLH/CG-R_B and eLH/CG-R_C cDNA isoforms were truncated from the 3'-end of exon X: eLH/CG-R_B spliced out of frame into the last exon whereas eLH/CG-R_C contained an in-frame stop codon within a divergent sequence. Consequently, both eLH/CG-R_B and eLH/CG-R_C cDNA isoforms encoded putative proteins without transmembrane and intracellular domains.

In order to study the responsiveness of the primary corpus luteum (CL) and fetal gonads to eCG, the expression of eLH/CG-R mRNAs was examined by RT-PCR and Northern blot analysis during early and mid-pregnancy. All three eLH/CG-R cDNA isoforms (eLH/CG-R_{A,B,C}) were expressed from day 14 to day 83 of pregnancy in the primary CL and from day 44 to day 222 in fetal gonads. Interestingly, the primary CL at days 89 and 151 expressed only truncated eLH/CG-R cDNA isoforms. The relative values of Northern hybridized major 7, 5.7, 3.9 and 1.8 kb eLH/CG-R mRNA transcripts tended to decrease in the primary CL whereas the unique major 1.8 kb eLH/CG-R mRNA was steadily expressed in fetal gonads during pregnancy. These results show that the expression of eLH/CG-R mRNAs occurs in the fetal gonads before ceasing in the primary CL and suggest that eCG may be involved in the gradual transition from a luteal to a feto-placental output of steroids during equine pregnancy.

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Introduction

In the mare, luteal and feto-placental steroids are successively necessary to maintain pregnancy. Initially, stimulation of the primary corpus luteum (CL) or CL of conception by pituitary luteinizing hormone (LH) results in a transient increase in the progesterone level, similar to that which occurs during the estrous cycle (Ginther 1992a). From day 35 of pregnancy, coincident with the secretion of the fetal hormone equine chorionic gonadotropin (eCG), the primary CL increases in size and secretes increasing levels of progesterone and newly synthesized estrogens (Bergfelt *et al.* 1989, Daels *et al.* 1991). Thereafter, from day 40 to day 160–180 of gestation, the primary CL is maintained and continues to secrete progesterone and estrogens (Squires *et al.* 1974). The fetal

gonads, on the other hand, enlarge slowly until approximately day 100 post-ovulation, then very rapidly between days 100 and 200 of gestation, exceeding the weight of maternal ovaries around day 150 of pregnancy (Ginther 1992b). From days 60 to 80, the fetal gonads in both sexes provide precursor steroids for the placental synthesis of estrone and equine-specific estrogens, equine and equilenin, and contribute to the production of large amounts of estrogens by the feto-placental unit until the end of pregnancy (Pashen & Allen 1979, Raeside *et al.* 1979). The equine placenta also produces progesterone and 5 α -reduced metabolites starting around day 70, but using maternal sources of cholesterol (Pashen & Allen 1979). Thus, a gradual transition from a luteal to a feto-placental output of steroids occurs, after which the maternal ovaries

become small and completely inactive for the remainder of gestation (Holtan *et al.* 1979). There is convincing evidence that eCG is responsible for the resurgence and support of the primary CL during early pregnancy in the mare (Urwin & Allen 1982, Bergfelt *et al.* 1989, Daels *et al.* 1998). Furthermore, the primary CL has been shown to be responsive to eCG during the time of eCG secretion (Saint-Dizier *et al.* 2003). However, whether or not eCG plays a role in the initial development and steroid production of fetal gonads remains unknown.

In pregnant mares and pony mares, the fetal hormone eCG, secreted by the endometrial cups, is first detectable in plasma on days 35–40 of gestation, rises rapidly to reach a peak between days 55 and 70, then decreases slowly to low or undetectable levels by days 120 to 150 of pregnancy (Ginther 1992a, Squires 1993). Whereas the endometrial cups act mainly as endocrine glands in the early stages of cup development, they also act from day 42 as exocrine glands since considerable amounts of eCG pour out of the uterine glands into the space between the cups and the allantochorion (Ginther 1992a). The large amounts of eCG secreted into the uterine lumen raise the possibility that this hormone enters the fetoplacental circulation and stimulates the initial hypertrophy and steroidogenesis of fetal gonads between days 60 and 120–150 of pregnancy. Nevertheless, it is still unknown whether the fetal gonads are responsive to eCG or to fetal pituitary LH, if present.

The protein structure of eCG is fully identical to that of equine LH (Bousfield *et al.* 1996) and both hormones bind to the putative LH/CG receptor in equine tissues (eLH/CG-R) (Stewart & Allen 1979, 1981, Guillou & Combarnous 1983). We have previously reported the presence of several eLH/CG-R mRNA transcripts in the primary CL by Northern blot (Saint-Dizier *et al.* 2003). Some of this diversity in transcript size seemed to be due to differences in mRNA sequence since a probe for the transmembrane domain only hybridized to a subset of these transcripts. However, the differences in eLH/CG-R mRNA splicing involved in this process remain unknown. Thus, the aims of this study were first to clone and sequence the full-length eLH/CG-R cDNA and secondly to examine the expression of eLH/CG-R mRNAs in the primary CL and in fetal gonads during early and mid-pregnancy.

Materials and Methods

Animals

Light breed mares and Welsh pony-mares (3–17 years old) with no known reproductive pathology were used in this study. Mares were maintained on pasture and supplemented with hay, minerals, and water available *ad libitum*. The reproductive tracts of the mares were monitored daily during estrus and every other day from ovulation until CL collection using palpation and rectal ultrasonography. The day of ovulation was designated as day 0 of pregnancy. Mares were fertilized by artificial insemination.

Sample collection and RNA isolation

Luteal tissues were obtained from one cyclic mare in dioestrus as control (day 8 post-ovulation) and from pregnant mares before the onset of eCG secretion (days 14, 15, 26, 27, 28 and 31; $n = 1$ for each day), during eCG secretion (days 42, 43, 44, 45, 46, 56, 60, 61, 62, 83 and 89; $n = 1$ for each day) and after the end of eCG secretion (day 151, $n = 1$). The ovaries were collected by hemiovariectomy or within 10 min following sodium pentobarbital-induced euthanasia as previously described (Saint-Dizier *et al.* 2003). Fetal gonads (both males and females) were collected following induced euthanasia or slaughter of the pregnant mares. The fetal gonads were obtained during eCG secretion (days 44, 45, 47, 62, 70, 81 and 101; $n = 1$ for each day) and after the end of eCG secretion (days 151, 202 and 222; $n = 1$ for each day). After dissection, pieces of tissue were immediately snap-frozen in liquid nitrogen then stored at -70°C .

Total RNA was extracted from fragments of frozen tissue using Trizol reagent (Invitrogen Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. The resulting RNA was quantified spectrophotometrically, aliquoted into smaller volumes, and stored at -80°C .

Isolation and characterization of eLH/CG-R cDNA isoforms

Total cellular RNA ($2\text{ }\mu\text{g}$) from a pool of corpora lutea (at dioestrus and days 15, 42 and 61 of pregnancy) was reverse transcribed into first-strand cDNA using oligo(dT) primer and Rnase H-reverse transcriptase (Superscript II; Invitrogen Life Technologies, Carlsbad, CA, USA) in the presence of deoxynucleotides according to the manufacturer's recommendations. A 646-base pair (bp) cDNA fragment encoding a part of the extracellular domain of the LH/CG-receptor was first generated using primer pair P1 and P2 based on cDNA sequences similar among the bovine (Lussier *et al.* 1996), porcine (Loosfelt *et al.* 1989), human (Minegishi *et al.* 1990) and murine (McFarland *et al.* 1989) LH/CG-Rs (Table 1). First-strand cDNA was subjected to 30 cycles of PCR amplification using Advantage 2 polymerase mix (BD Biosciences, Palo Alto, CA, USA) and primer pair P1 and P2. Reaction times were 1 min denaturation at 95°C for the first cycle and 15 s per cycle thereafter, 40 s annealing at 60°C and 1 min extension at 72°C for the first 29 cycles, and 10 min extension on the final cycle at 72°C . The amplified PCR product was resolved on a 1.5% agarose gel, isolated, purified and subcloned into the TA cloning vector pCR II-TOPO (Invitrogen Life Technologies) for nucleic acid sequence determination (Genome Express, Meylan, France).

The 3'-end of the eLH/CG-R cDNA was subsequently isolated using sense primer P3 designed from the 646-bp PCR product described above (Table 1) and a degenerate antisense primer (P4) based on cDNA sequences in bovine, porcine and human LH/CG-R (Table 1) or an

Table 1 Nucleic acid sequences of primers used for the isolation of the equine LH/CG-R cDNA and for RT-PCR. Location of nucleotides, when specified, corresponds to the eLH/CG-R_A cDNA sequence on Fig. 1. Primer P4 is a degenerate primer where B = T,G,C; Y = C,T; K = T,G; R = A,G.

Primer name	Location	Sequence
Forward primer P1	146–167	5'-CTTTCAGAGGACTTAATGAGGT-3'
Reverse primer P2	772–792	5'-TCTAAAAGCACAGCAGTGGCT-3'
Forward primer P3	570–594	5'-GCTGGAGAAGATGCACAACGGAGCC-3'
Reverse primer P4	2019–2047	5'-CAGTTAACABTCYKTRTAGCRAGTCTTG-3'
Reverse primer P5	536–560	5'-TCCTTTAGCTCCAGGAAATCAGTG-3'
Reverse primer P6	516–535	5'-TCGTCCCGTTGAATGCATGA-3'
Reverse primer P7	444–468	5'-TTCGTTATTCATCCCTTGAAGCA-3'
Forward primer P8	689–710	5'-TTGCCACATCATCTATTCTCT-3'
Reverse primer P9	1984–2005	5'-TATATTGGCAGTGAATGTGGT-3'
Reverse primer P10		5'-CTCCAATTCCTTCATGATAA-3'
Forward β actin		5'-CGTGACATTAAGGAGAAGCTGTGC-3'
Reverse β actin		5'-CTCAGGAGGAGCAATGATCTTGAT-3'

oligo(dT) as antisense primer. First-strand cDNA was amplified for 30 cycles using Advantage 2 polymerase mix (BD Biosciences) and primer pair P3 and P4 or primer pair P3 and oligo(dT) as described above.

As degenerate sense 5' primers based on cDNA sequences in bovine, porcine and human LH/CG-Rs did not anneal to first strand equine LH/CG-R cDNA, we eventually used the rapid amplification of cDNA ends (RACE) technique to isolate the eLH/CG-R cDNA in the 5' direction. This was accomplished essentially as described by the manufacturer (Invitrogen Life Technologies). The gene-specific primers P5, P6 and P7 were designed from the 646-bp PCR product described above and were used in this procedure (Table 1). Briefly, first-strand cDNA was synthesized from luteal total RNA using the primer P5 and the Rnase H-reverse transcriptase Superscript II at 50 °C, and then purified. An oligo-dC tail was added in the 3'-end of the cDNA using terminal deoxynucleotidyl transferase and dCTP. The dC-tailed cDNA was then amplified for 30 cycles using primer P6 and the Abridged Anchor Primer under the following conditions: 3 min at 95 °C for one cycle, 30 s at 95 °C, 40 s at 55 °C and 1 min at 72 °C for 30 cycles, 10 min at 72 °C for one cycle. A second amplification was performed with the primer P7 and Abridged Universal Amplification Primer as internal primers using one-tenth of the first reaction volume as template and under amplification conditions described above.

All amplified PCR products were subcloned into the TA cloning vector pCR II-TOPO (Invitrogen Life Technologies) and sequenced on both strands (Genome Express). Sequence analyses were done on the Infobiogen web site (<http://www.infobiogen.fr>).

RT-PCR

Single-strand cDNA was synthesized from 5 μ g total cellular RNA using Rnase H-reverse transcriptase (Superscript II; Invitrogen Life Technologies) and 250 ng oligo(dT) primer as recommended by the manufacturer. PCR amplifications were performed on a geneAmp PCR System 9700 (Perkin-Elmer, Norwalk, CT, USA) and reaction

mixtures contained 10 pmol of each primer, 0.4 mmol dNTPs l⁻¹, 1 $\times$ PCR buffer, Advantage 2 polymerase mix (BD Biosciences) and cDNA template. A primer pair encoding the housekeeping gene β -actin was used as positive control (Table 1). PCR amplifications with RNAs without reverse transcription (RT) as templates were performed in parallel as negative controls. The amplification profile consisted of 5 min at 94 °C followed by 25–35 cycles of 1 min at 94 °C, 1 min at 61 °C and 1 min at 72 °C. The final cycle included a further 10 min at 72 °C to complete extension. The amplified products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized on a UV transilluminator. Bands were individually dissected and DNA was extracted using a DNA purification kit (Wizard PCR Preps, Promega, Madison, WI, USA), subcloned into the TA cloning vector pCR II-TOPO (Invitrogen Life Technologies) and sequenced (Genome Express).

Northern blot analysis

A cDNA probe covering the extracellular domain of the eLH/CG-R (EC-probe) was generated by RT-PCR with primers EC1 and EC2 (see arrows in Fig. 1) as previously described (Saint-Dizier *et al.* 2003) and used for Northern blot analysis. Total cellular RNA (15–20 μ g) was separated by agarose gel electrophoresis in the presence of 17% formaldehyde, transferred overnight by capillary blot to a nylon membrane (Nytran Super Charge, Schleicher and Schuell, Dassel, Germany) then fixed by UV cross-linking. Blots were prehybridized for 2 h at 42 °C in a buffer containing 50% formamide, 5 $\times$ Denhardt's solution, 1% SDS, 5 $\times$ sodium saline citrate (SSC), and 16 μ l/ml denatured salmon sperm DNA (Invitrogen). Blots were then hybridized with the α -³²P-eLH/CG-R cDNA probe overnight at 42 °C in a buffer containing 50% formamide, 2.5 $\times$ Denhardt's solution, 1% SDS, 5 $\times$ SSC, 10 $\times$ dextran sulfate, and 16 μ l/ml denatured salmon sperm DNA. Blots were next washed in 1 $\times$ SSC plus 0.5% SDS at room temperature for 20 min, followed by three 20-min washes in 0.2 $\times$ SSC plus 0.5% SDS at 68 °C. Membranes

eLH/CG-R_A :

CTG	CTG	CCG	CAA	GCG	CTG	CGC	GGG	GCG	CCC	TGC	CCC	GAG	CCC	TGC	CGC	TGC	CCG	CCC	GAC	GGC	GCC	CTG	AGC	TGC	CCC	78	
L	L	P	Q	A	L	R	G	A	P	Q	P	E	P	Q	R	Q	P	P	D	G	A	L	S	Q	P	26	
																									→EC1		
GGC	CGG	CGG	AGG	AAC	CTC	TCC	CGA	CTA	TCA	CTC	ACC	TAT	CTT	CCT	ATC	AAA	GTA	ATC	CCA	TCT	CAA	GCT	ATC	AGA	GGA	156	
G	P	R	R	N	L	S	R	L	S	L	T	Y	L	P	I	K	V	I	P	S	Q	A	F	R	G	52	
CTT	GAT	GAA	GTT	GTA	AAA	ATT	GAA	ATC	TCT	CAG	AGT	GAT	TCC	CTG	GAA	AAG	ATA	GAA	GCT	AAT	GCC	TTT	GAC	AAC	CTC	234	
L	D	E	V	V	K	I	E	I	S	Q	S	D	S	L	E	K	I	E	A	N	A	F	D	N	L	78	
CTC	AAT	TTG	TCT	GAA	ATA	CTG	ATC	CAG	AAC	ACC	AAA	AAC	CTA	GTG	TAC	ATC	GAG	CCT	GGA	GCA	TTT	ACA	AAT	CTC	CCC	312	
L	N	L	S	E	I	L	I	Q	N	T	K	N	L	V	Y	I	E	P	G	A	F	T	N	L	P	104	
CGG	TTA	AAA	TAC	CTA	AGC	ATC	TGT	AAC	ACA	GGC	ATC	CGA	AAG	CTT	CCA	GAT	GTT	ACC	AAG	ATC	TTC	TCC	TCT	GAA	ATT	390	
R	L	K	Y	L	S	I	Q	N	T	G	I	R	K	L	P	D	V	T	K	I	F	S	S	E	I	130	
AAT	TTT	ATT	CTG	GAA	ATT	TGC	GAT	AAC	TTA	CAC	ATA	ACC	ACC	ATA	CGA	GGA	AAT	GCT	TTT	CAA	GGG	ATG	AAT	AAC	GAA	468	
N	F	I	L	E	I	Q	N	L	H	I	T	T	I	P	G	N	A	F	Q	G	M	N	N	E	E	156	
TCC	ATA	ACA	CTC	AAA	CTA	TAT	GGA	AAT	GGA	CTT	GAA	GAA	ATA	CAA	AGT	CAT	GCA	TTC	AAC	GGG	ACG	ACA	CTG	ATT	TCC	546	
S	I	T	L	K	L	Y	G	N	G	L	E	E	I	Q	S	H	A	F	N	G	T	T	L	I	S	182	
CTG	GAG	CTA	AAG	GAA	AAT	GCC	CAG	CTG	GAG	AAG	ATG	CAC	AAC	GGA	GCC	TTC	CAG	GGG	GCC	ACA	GGG	CCC	AGC	ATC	TTG	624	
L	E	L	K	E	N	A	Q	L	E	K	M	H	N	G	A	F	Q	G	A	T	G	P	S	I	L	208	
GAT	ATT	TCT	TCC	ACC	AAA	CTG	CAG	GCC	CTA	CCT	AGC	TAT	GGG	CTG	GAG	TCC	ATT	CAG	ACG	CTA	ATT	GCC	ACA	TCA	TCC	702	
D	I	S	S	T	K	L	Q	A	L	P	S	Y	G	L	E	S	I	Q	T	L	I	A	T	S	S	234	
TAT	TCT	CTA	AAA	AAA	TTA	CCG	TCA	AGA	GAA	AAA	TTT	ACC	AAT	CTC	CTG	GCT	GCC	ACA	CTG	ACT	TAC	CCA	AGC	CAC	TGC	780	
Y	S	L	K	K	L	P	S	R	E	K	F	T	N	L	L	A	A	T	L	T	Y	P	S	H	Q	260	
																									EC2←	→P8	
TGT	GCT	TTT	AGA	AAC	TTG	CCA	ACA	AAA	GAA	CAG	AAT	TTT	TCA	TTT	TCC	ATT	TTT	GAA	AAC	TTT	TCC	CAA	CAA	TGT	GAA	858	
Q	A	F	R	N	L	P	T	K	E	Q	N	F	S	F	S	I	F	E	N	F	S	Q	Q	Q	E	286	
																									Q		
AGC	ACA	GCA	AGG	AAA	CCA	AAT	AAT	GGA	ACA	CTT	TAT	TCT	GCC	AGC	TTF	GCT	GAG	AGT	GAA	CTG	AGT	GGC	TGG	GAT	TAT	936	
S	T	A	R	K	P	N	N	G	T	L	Y	S	A	S	F	A	E	S	G	E	L	S	G	W	D	Y	312
GAC	GAT	GGT	TTC	TGC	CCA	CAC	AAG	ACA	CTC	CGA	TGT	GCT	CCT	GAA	CCA	GAT	GCT	TTT	AAT	CCC	TGT	GAA	GAT	ATT	ATG	1014	
D	D	G	F	Q	P	H	K	T	L	R	Q	A	P	E	P	D	A	F	N	P	Q	E	D	I	M	338	
GGC	TAT	GAC	TTC	CTT	AGG	GTC	CTG	ATT	TGG	CTG	ATT	AAT	ATC	CTA	GCC	ATC	ATG	GGA	AAT	GTG	ACT	GTC	CTC	TTT	GTT	1092	
G	Y	D	F	L	R	V	L	I	W	L	I	N	I	L	A	I	M	G	N	V	T	V	L	F	V	364	
CTC	CTG	ACC	AGT	CAT	TAC	AAA	CTA	ACA	GTG	CCC	CGT	TTT	CTC	ATG	TGC	AAT	CTC	TCT	TTT	GCA	GAC	TTT	TGC	ATG	GGG	1170	
L	L	T	S	H	Y	K	L	T	V	P	R	F	L	M	Q	N	L	S	F	A	D	F	Q	M	G	390	
CTC	TAT	CTG	CTG	CTC	ATT	GCC	TCA	GTT	GAT	TCC	CAA	ACC	AAA	GGC	CAG	TAT	TAT	AAC	CAT	GCC	ATA	GAT	TGG	CAG	ACA	1248	
L	Y	L	L	L	I	A	S	V	D	S	Q	T	K	G	Q	Y	Y	N	H	A	I	D	W	Q	T	416	
GGG	AGT	GGG	TGT	AGT	GCT	GCT	GGC	TTT	TTC	ACT	GTA	TTT	GCA	AGT	GAA	CTT	TCT	GTC	TAC	ACC	CTC	ACA	TGC	ATC	ACA	1326	
G	S	G	Q	S	A	A	G	F	F	T	V	F	A	S	E	L	S	V	Y	T	L	T	V	I	T	442	
CTA	GAA	AGA	TGG	CAC	ACC	ATC	ACC	TAT	GCT	ATT	CAG	CTG	GAC	CAA	AAA	CTA	CGA	TTA	AGA	CAT	GCC	ATT	CCA	ATT	ATG	1404	
L	E	R	W	H	T	I	T	Y	A	I	Q	L	D	Q	K	L	R	L	R	H	A	I	P	I	M	468	
CTT	GGA	GGA	TGG	CTC	TTT	TCT	ACT	CTA	ATT	GCT	ATG	TTG	CCC	CTT	GTG	GGT	GTC	AGC	AAT	TAC	AGG	AAG	GTC	AGC	ATT	1482	
L	G	G	W	L	F	S	T	L	I	A	M	L	P	L	V	G	V	V	S	N	Y	R	K	V	S	I	494
TGC	CTC	CCC	ATG	GAT	ATA	GAA	ACC	ACT	CTC	TCA	CAA	GTC	TAC	ATA	TTA	ACC	ATC	CTG	ATA	CTC	AAT	GTG	GTA	GCC	TTC	1560	
Q	L	P	M	D	I	E	T	T	L	S	Q	V	Y	I	L	T	I	L	I	L	N	V	V	A	F	520	
TTC	ATC	ATT	TGT	GCT	TGC	TAC	ATT	AAA	ATT	TAT	TTT	GCA	GTT	CAA	AAT	CCA	CAA	CTG	ATA	GCT	ACC	AAC	AAA	GAC	ACA	1638	
F	I	I	Q	A	Q	Y	I	K	I	Y	F	A	V	Q	N	P	Q	L	I	A	T	N	K	D	T	546	
AAG	ATT	GCT	AAG	AAA	ATG	GCA	GTC	CTC	ATC	TTC	ACC	GAT	TTC	ACC	TGC	ATG	GCA	CCT	ATT	TCT	TTT	TTT	GCC	ATT	TCA	1716	
K	I	A	K	K	M	A	A	V	L	I	F	T	D	F	T	Q	M	A	P	I	S	F	A	I	S	572	
GCT	GCC	TTC	AAA	GTG	CCC	CTT	ATC	ACA	GTA	ACC	AAT	TCT	AAA	GTT	TTA	CTG	GTT	CTC	TTT	TAT	CCT	GTC	AAT	TCT	TGT	1794	
A	A	F	K	V	P	L	I	T	V	T	N	S	K	V	L	L	V	L	F	Y	P	V	N	S	Q	598	
GCC	AAT	CCA	TTT	CTG	TAC	GCG	ATT	TTC	ACA	AAG	GCA	TTT	CGA	AGA	GAT	TTC	TTT	CTG	TTG	CTG	AGC	AAA	TTT	GGC	TGC	1872	
A	N	P	F	L	Y	A	I	F	T	K	A	F	R	R	D	F	F	L	L	L	S	K	F	G	Q	624	
TGT	AAA	CAT	CGG	GCT	GAA	CTT	TAT	AGA	AGG	AAG	GAT	TTT	TCA	GCT	TAT	ACC	TCC	AAG	TGG	AAG	AAT	GGC	TTC	ACT	GGA	1950	
Q	K	H	R	A	E	L	Y	R	R	K	D	F	S	A	Y	T	S	K	W	K	N	G	F	T	G	650	
																									P9←		
TCA	AAT	AAG	TCT	TCC	CAG	CCC	ACC	TTG	AAG	CTG	ACC	ACA	TTG	CAC	TGC	CAA	TAT	ACA	GCT	GTC	CCA	GAC	AAG	ACT	TGC	2028	
S	N	K	S	S	Q	P	T	L	K	L	T	T	L	H	Q	Q	Y	T	A	V	P	D	K	T	C	676	
TAT	ACA	GAG	TGT	TAA	CTGT																				2047		
Y	T	E	C	*																				68			

$$\text{eLH/CG-R}_B :$$

ACA CTG CAT TCC GAA GAG ATT TCT TTC TGT TGC TGA
T L H S E E I S F C C *

eLH/CG-R_C :

ACA CTG GAG ACA GCA AGA TAC CAG ATG AAT GGC CTG GTC ACA TGA
T L E T A R Y O M N G L V T *

were exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA, USA) at room temperature for 16–18 h before quantification. Membranes were then washed three times for 20 min with a boiling solution of $2 \times \text{SSC}$ plus 0.2% SDS to remove the probe and rehybridized with the human RNA 18S probe from Ambion, Inc. (Austin, TX, USA). Membranes hybridized with the 18S probe were exposed for 1 h to a PhosphorImager screen. All hybridization signals were quantified using ImageQuant software (Molecular Dynamics). Each RNA sample was analyzed 2 to 5 times on different blots. The RNA sample of one CL at the diestrous stage (day 8 post-ovulation) was used as internal control in each blot. The intensities for LH/CG-R signals were adjusted with 18S signal values in each blot and the LH/CG-R:18S ratio values were normalized between blots according to the LH/CG-R:18S ratio value of the internal control.

Statistical analysis

The concentrations of mRNA levels are shown as means $\pm$ S.E.M. Three stages of pregnancy were considered according to the known pattern of eCG secretion: before the onset of eCG secretion (days 14–31 for CL), during eCG secretion (days 42–89 for CL and days 44–101 for fetal gonads) and after eCG secretion (day 151 for CL and days 151–222 for fetal gonads). The relative levels of mRNAs were compared between stages of pregnancy, irrespective of other variables, with the nonparametric Kruskal–Wallis test in the primary CL and with the nonparametric Wilcoxon–Mann–Whitney in the fetal gonads. Tests were all performed using StatXact 5 (CYTEL, Cambridge, MA, USA; <http://www.cytel.com/>). Differences were considered to be significant when $P < 0.05$.

Results

Nucleotide sequence analysis of eLH/CG-R cDNA isoforms

Based on the conserved sequences of the human, porcine, bovine and murine LH/CG-R cDNAs, the primer pair P1 and P2 was designed and used in RT-PCR reaction using total RNA from a pool of corpora lutea as template. A 646-bp PCR product was obtained, subcloned, then sequenced and found to be homologous with known mammalian LH/CG-R cDNAs. The sequence information from the 646-bp fragment combined with RT-PCR and

5'-RACE products generated a 2047-bp eLH/CG-R cDNA (eLH/CG-R_A; Fig. 1), spanning over a part of the signal peptide plus the entire extracellular, transmembrane and intracellular domains of the eLH/CG-R. The cDNA sequence (excluding primer sequence) of eLH/CG-R_A (accession number AY464091 to GenBank) showed 92.3, 91.6, 88 and 85.7% homologies with reported cDNA sequences of porcine (Loosfelt *et al.* 1989), bovine (Lussier *et al.* 1996), human (Minegishi *et al.* 1990) and rat (McFarland *et al.* 1989) LH/CG-R respectively, and showed 64.6% homology with the equine follicle-stimulating hormone (FSH) receptor (FSH-R) cDNA sequence (Robert *et al.* 1994). The deduced 680-amino acids sequence of eLH/CG-R_A cDNA was similarly conserved among mammals (pig 92.5%, cow 91.6%, human 89.1% and rat 87.4%) and showed 58% homology with the equine FSH-R. Alignment with the porcine sequence suggests a putative cleavage site of the signal peptide between Gly⁸ and Ala⁹ (Loosfelt *et al.* 1989). Thus, the eLH/CG-R_A cDNA would encode a mature protein of 672 amino acids. The putative 335-amino acid extracellular domain of eLH/CG-R contains 12 cysteine residues conserved in all species and seven potential N-linked glycosylation sites (N-X-S/T), i.e. one more than in the human, porcine, bovine and rat LH/CG-R sequences (Fig. 1). The transmembrane domain is composed of 266 amino acid residues including seven hydrophobic segments potentially spanning the cytoplasmic membrane and 8 conserved cysteine residues. The 71-amino acid COOH-terminal intracellular domain contains three conserved cysteine residues. The putative intracellular loops as well as the intracellular domain display several serine, threonine and tyrosine residues that are potential sites for phosphorylation.

Two other types of clones were isolated by RT-PCR. Using primer pair P3 and P4, seven of the eleven sequenced clones coded for a splice variant of the eLH/CG-R_A cDNA and was named eLH/CG-R_B. The eLH/CG-R_B cDNA spliced out of frame from the 3'-end of exon X (at bp 891) into the coding region of exon XI (at bp 1829), thus encoding a putative protein of 306 amino acids without transmembrane and intracellular domains (Fig. 1). Using primer pair P3 and an oligo(dT), all three sequenced clones encoded a third eLH/CG-R splice variant, named eLH/CG-R_C. This variant spliced from the 3'-end of exon X (at bp 891) into a divergent nucleotide sequence, which was not found in the eLH/CG-R_A cDNA,

Figure 1 Nucleic acid and deduced amino acid sequences of intact (eLH/CG-R_A) and truncated (eLH/CG-R_B and eLH/CG-R_C) equine LH/CG-R cDNA. The putative cleavage site of the peptide signal after alignment with the porcine LH/CG-R is indicated by an arrow ($\Downarrow$). Conserved cysteine residues are highlighted by gray boxes. The equine-specific N-glycosylation site is indicated in bold letters double underlined, and other double underlines indicate the six N-linked glycosylation sites conserved among other mammalian LH/CG-Rs. Single underlines indicate the seven predicted transmembrane-spanning domains. The point of divergence between intact and truncated eLH/CG-R cDNA isoforms is indicated by an arrowhead (∇) and the point of junction for eLH/CG-R_B is indicated by an arrow ($\downarrow$). The sites of the primers P8, P9 and P10, used in RT-PCR, are indicated on the cDNA sequence. The primer pair EC1 and EC2, also indicated, was used to generate the eLH/CG-R cDNA EC-probe for Northern blot analysis. (Accession number AY464091 to GenBank.)

and contained an in-frame TGA stop codon (eLH/CG-R_C; Fig. 1). This divergent sequence is 590-bp long and contains a poly-A tail at its 3'-end. The eLH/CG-R_C cDNA isoform encoded a putative protein of 310 amino acids without transmembrane and intracellular domains.

Expression of eLH/CG-R mRNA isoforms detected by RT-PCR

The expression of intact (eLH/CG-R_A) and truncated (eLH/CG-R_B and eLH/CG-R_C) eLH/CG-R mRNA isoforms was examined in the primary CL and in fetal gonads at different stages of early and mid-pregnancy using qualitative RT-PCR. A primer pair encompassing exon IX through the end of exon XI (primers P8 and P9; see Table 1 and Fig. 1) revealed the expression of both eLH/CG-R_A (1318-bp band) and eLH/CG-R_B (380-bp band) mRNAs in the primary CL from day 14 to day 83 of pregnancy whereas only the eLH/CG-R_B form was detected at day 89 and faintly detected at day 151 (Fig. 2a). A non-specific 500-bp product was also weakly amplified with primer pair P8 and P9 in some samples. The sense primer P8 combined with an antisense primer located in the non coding region of eLH/CG-R_C mRNA (primer P10; see Table 1 and Fig. 1) revealed the expression of the eLH/CG-R_C mRNA isoform (528-bp band) from day 14 to day 89 of pregnancy. However, no luteal expression of eLH/CG-R_C mRNA could be seen at day 151 of pregnancy (Fig. 2b).

In fetal gonads, the expression of eLH/CG-R_A, eLH/CG-R_B and eLH/CG-R_C mRNAs was detected as early as day 44 of pregnancy and at the following days examined until day 222 of pregnancy (Fig. 3a and 3b). The signal for eLH/CG-R_A mRNA was weak at day 202 of pregnancy

although it could not be determined whether this change was an individual variation or a genuine developmental change. The β -actin signal was detected in all samples (374-bp band; Fig. 2c and 3c) and no signal could be seen with RNAs without RT as templates.

Expression of eLH/CG-R mRNA isoforms detected by Northern blot analysis

Northern blot analysis with a cDNA probe covering the major part of the extracellular domain of the eLH/CG-R (EC-probe) revealed seven eLH/CG-R mRNA transcripts at 7, 5.7, 4.9, 3.9, 2.8, 1.8 kb and 0.6 kb in the primary CL (Fig. 4), as shown previously (Saint-Dizier *et al.* 2003). The number and apparent size of mRNA transcripts did not change from day 14 to day 89 in the primary CL, whereas no hybridization signal was observed in the day 151 primary CL. Northern blot analysis of total RNA from fetal gonads with the EC-probe revealed two transcripts at 3.9 and 1.8 kb on days 44 and 62, and six transcripts at 7, 5.7, 4.9, 3.9, 2.8 and 1.8 kb from day 70 to day 222 of pregnancy (Fig. 5). Furthermore, whereas four major transcripts at 7, 5.7, 3.9 and 1.8 kb were observed in the primary CL, only the 1.8 kb hybridization signal was predominant in the fetal gonads. However, it is not known if these transcripts are translated. No hybridization signal was observed with RNA samples from adult lung, kidney, spleen or liver (data not shown).

Semiquantitative analysis of eLH/CG-R mRNA isoforms

The relative value of the major 1.8 kb mRNA transcript expressed in fetal gonads was quantified and compared with relative values of major eLH/CG-R transcripts

Figure 2 Representative amplification of the alternatively spliced eLH/CG-R_A, eLH/CG-R_B (a) and eLH/CG-R_C (b) isoforms by RT-PCR in the primary corpus luteum from one cyclic mare at day 8 post-ovulation (Dioestrus) and from mares at days 14–151 of pregnancy. Expression of the β -actin gene was used as positive control (c). –RT corresponds to a PCR carried out without reverse transcriptase.

Figure 3 Representative amplification of the alternatively spliced eLH/CG-R_A, eLH/CG-R_B (a) and eLH/CG-R_C (b) isoforms by RT-PCR in fetal gonads at days 44–222 of pregnancy. Expression of the β-actin gene was used as positive control (c). –RT corresponds to a PCR carried out without reverse transcriptase.

Figure 4 Representative Northern blot analysis of total RNA from one CL at the dioestrous stage (Dioes) as internal control and CL from pregnant mares before the onset of eCG secretion (days 14–31), during eCG secretion (days 42–89) and after eCG secretion (day 151) hybridized with the cDNA EC-probe (top panel) and the RNA 18S-probe (bottom panel) for normalization.

expressed in the primary CL during pregnancy (Fig. 6). The major 1.8 kb mRNA transcript was 1.2 to 3 times less expressed in fetal gonads than in the primary CL or in the dioestrous CL used as internal control in each blot. The relative intensity of the 1.8 kb major transcript tended to increase in fetal gonads at days 70, 81 and 101 of pregnancy. However, there was no significant change in the 1.8 kb mRNA intensity between stages of pregnancy from day 44 to day 222 in fetal gonads. In the primary CL, the relative values of the major 7, 5.7, 3.9 and 1.8 kb mRNA transcripts tended to decrease ($P=0.08$, 0.2, 0.06 and 0.14 respectively) between stages of pregnancy from day 14 to day 151 of pregnancy (Fig. 6).

Discussion

Three different eLH/CG-R cDNA isoforms (eLH/CG-R_{A,B,C}) were isolated from a pool of equine corpora lutea and identified by nucleotide sequence analysis of RT-PCR and 5'-RACE products. The full-length eLH/CG-R cDNA

Figure 5 Representative Northern blot analysis of total RNA from equine fetal gonads during eCG secretion (day 44–101) and after eCG secretion (day 151–222), and from one CL at the dioestrous stage (Dioes) as internal control, hybridized with the cDNA EC-probe (top panel) and the RNA 18S-probe (bottom panel) for normalization.

(eLH/CG-R_A) as well as truncated forms encoding receptors without transmembrane and intracellular domains (eLH/CG-R_{B,C}) were expressed from day 14 to day 83 in the primary CL and from day 44 to day 222 of pregnancy in fetal gonads. In contrast, the primary CL at days 89 and 151 expressed only truncated forms of the eLH/CG-R cDNA. The relative values of major mRNA transcripts tended to decrease in the primary CL whereas the unique major 1.8 kb mRNA in fetal gonads was steadily expressed during pregnancy. These results show that the expression of eLH/CG-R mRNAs occurs in fetal gonads before it ends in the primary CL and thus parallels the gradual change from a luteal to a feto-placental steroidogenesis during equine pregnancy.

This study reports the first cloning and sequencing of the full-length equine LH/CG-R cDNA. The deduced 680-amino acids sequence showed high homology with reported LH/CG-R sequences in other mammals, especially with the porcine (92.3% homology) and the bovine (91.6%) LH/CG-R proteins. In contrast to all other

known mammalian LH/CG-Rs, which contain six conserved consensus sequences for N-linked carbohydrates, the eLH/CG-R displays a seventh putative N-glycosylation site in the N-terminal region of the extracellular domain, created by a Gly31Asn replacement (Gly23Asn replacement in the mature protein) (Loosfelt *et al.* 1989, McFarland *et al.* 1989, Minegishi *et al.* 1990, Lussier *et al.* 1996). Interestingly, while most mammalian FSH-Rs contain three highly conserved potential sites for N-glycosylation, the equine FSH-R also displays an additional N-glycosylation site in its extracellular domain (Robert *et al.* 1994). It has been hypothesized that this fourth N-glycosylation site could be involved in preventing eLH/CG binding to the eFSH-R (Richard *et al.* 1997). Although it is known that five of the six N-glycosylation sites in the porcine LH/CG-R (Vu-Hai *et al.* 2000) and all six sites in the rat LH/CG-R (Davis *et al.* 1997) are indeed glycosylated, the potential roles of these glycosidic chains remain unclear. Indeed, the nonglycosylated rat LH/CG-R can be properly folded and expressed at the cell surface, and can bind hormone and transduce signals (Davis *et al.* 1997). It would be of interest to determine whether all seven N-glycosylation sites of the eLH/CG-R contain carbohydrates. Furthermore, eCG binds to the eLH/CG-R with only one tenth or less the affinity of pituitary eLH in equine tissues (Stewart & Allen 1979, 1981, Guillou & Combarous 1983). The possible implication of the equine LH/CG-R extra N-glycosylation site in this differential binding affinity should also be examined.

Northern blot analysis using a cDNA probe encoding the extracellular domain of the eLH/CG-R revealed seven mRNA transcripts at 7, 5.7, 4.9, 3.9, 2.8, 1.8 and 0.6 kb in the primary CL. In a previous study, the presence of multiple eLH/CG-R transcripts seemed to arise in part from alternate splicing of the eLH/CG-R primary transcript since a probe covering the transmembrane domain of the receptor hybridized to only four of these seven transcripts (at 7, 4.9, 3.9 and 1.8 kb) (Saint-Dizier *et al.* 2003). The present work shows that the alternate splicing of the LH/CG-R primary transcript indeed occurs in the primary CL and in fetal gonads and gives rise to at least two splicing variants (eLH/CG-R_B and eLH/CG-R_C) in addition to the full-length eLH/CG-R mRNA (eLH/CG-R_A). The point of divergence between the full-length eLH/CG-R cDNA and the two truncated cDNA isoforms is the same as the one described for the porcine LH/CG-R cDNA and corresponds to the 3' end of exon X (Loosfelt *et al.* 1989). One of the three splicing variants described for the porcine LH/CG-R (the D form) has been shown to splice in frame at the transmembrane to intracellular sequence junction and thus contained a putative intracellular domain.

Figure 6 Relative intensities of eLH/CG-R major mRNA transcripts in the primary CL and in fetal gonads (FG) before eCG secretion (CL: days 14–31, $n = 6$), during eCG secretion (CL: days 42–89, $n = 11$; FG: days 44–101, $n = 7$) and after eCG secretion (CL: day 151, $n = 1$; FG: days 151–222, $n = 3$). Values are means $\pm$ S.E.M.

In contrast, the eLH/CG-R_B form displayed a frameshift at approximately the same point of junction as the porcine D variant and was thus truncated for the putative transmembrane and intracellular domains. The eLH/CG-R_C variant, which completely lacks the exon XI encoding the transmembrane and intracellular domains, was similar to the truncated form found in the turkey ovary (You *et al.* 2000). Nevertheless, such a variant had not previously been described in mammalian species. The three eLH/CG-R transcripts that have been shown to lack the transmembrane domain by Northern blot analysis were at 5.7, 2.8 and 0.6 kb (Saint-Dizier *et al.* 2003). The eLH/CG-R_B and eLH/CG-R_C cDNA isoforms could correspond to the 5.7 kb and/or to the 2.8 kb transcripts observed on Northern blots. Nevertheless, as the open reading frame of the full eLH/CG-R is approximately 2.1 kb, other processes like alternate transcriptional start sites and/or multiple sites and lengths of polyadenylation are probably involved in large differences in LH/CG-R mRNAs sizes.

The detection of eLH/CG-R mRNA isoforms by RT-PCR showed that the expression of the eLH/CG-R_A isoform ceased in the primary CL between days 83 and 89 of pregnancy. At days 89 and 151 of pregnancy, only truncated forms of eLH/CG-R mRNAs without transmembrane sequence (eLH/CG-R_B and eLH/CG-R_C) were detected by RT-PCR. This change in eLH/CG-R mRNA alternative splicing occurs while the fetoplacental steroidogenesis is sufficient to support pregnancy (Holtan *et al.* 1979). However, the primary CL is maintained and continues to secrete progesterone and estrogens until days 160–180 of pregnancy, probably stimulated by eCG (Squires *et al.* 1974, Ginther 1992a). In a previous study, ¹²⁵I-eLH saturation binding assays performed on luteal membranes showed that the primary CL at days 83–101 of pregnancy bound ¹²⁵I-eLH with high affinity and displayed a substantial level of membrane eLH/CG binding sites, which was 24.7% of the level measured at days 14–31 of pregnancy (Saint-Dizier *et al.* 2003). Furthermore, luteal membranes at day 151 also bound ¹²⁵I-eLH (data not shown). It is assumed that luteal cells of the primary CL still have membrane eLH/CG-Rs despite only truncated eLH/CG-R mRNAs being synthesized between days 89 and 151 of pregnancy. It is thus supposed that luteal eLH/CG-Rs exhibit long half-lives over this period of time.

As shown by RT-PCR, the eLH/CG-R_A isoform was expressed in fetal gonads as early as day 44 of pregnancy and in all gonads until day 222 of pregnancy, which was the last time point examined. However, the 1.8 kb transcript was largely predominant in Northern blots performed on the same samples, whereas three additional mRNA transcripts with larger sizes (7, 5.7 and 3.9 kb) were predominantly expressed in the adult primary CL. Since the size of the 1.8 kb transcript is less than the 2.1 kb open reading frame of the full sequence eLH/CG-R, this 1.8 kb transcript must be an incomplete eLH/CG-R mRNA. Furthermore, it is not yet known if this 1.8 kb

transcript encodes a functional receptor. In rats, the expression of truncated LH/CG-R mRNA transcripts is first detectable in fetal ovaries and testes as early as embryonic day 13.5 (Sokka *et al.* 1996) but the full-length LH/CG-R mRNA appears thereafter on embryonic day 15.5 in the testis (Zhang *et al.* 1994) and on postnatal day 7 in the ovary (Sokka *et al.* 1992). While the different eLH/CG-R cDNA isoforms were observed at all stages examined in horse fetal gonads, our results do not exclude a possible change in alternative splicing of the eLH/CG-R primary transcript before day 44 and/or after day 222 of pregnancy in fetal ovaries and testes. Furthermore, the major 1.8 kb eLH/CG-R mRNA transcript remained 1.2 to 3 times less expressed in fetal gonads than in the dioestrous or in the primary CL of adult mares during early and mid-pregnancy, which would indicate that a differential regulation of the eLH/CG-R gene transcription occurs between the fetal and the adult life in the mare.

Although gene expression does not necessarily imply that transcripts are translated in proteins or that the receptors are functionally involved in signal transduction, the presence of eLH/CG-R transcripts in horse fetal gonads indicates that the eLH/CG-R, and therefore fetal pituitary eLH and/or eCG themselves, may have a physiological role in early development and steroidogenesis of gonads in this species. Fetal content of pituitary eLH has been found to be low at day 90 of pregnancy and has been shown to increase slowly between days 90 and 150 of pregnancy (Wesson & Ginther 1980). In contrast, the fetal blood concentration of eLH measured by radioimmunoassay was highest between days 100 and 150 of pregnancy, parallel with a rapid growth of fetal gonads (Wesson & Ginther 1980). Since plasma eLH cannot be distinguished from plasma eCG by immunological methods, the high eLH/CG level measured in fetal blood between days 100 and 150 of pregnancy could be due, in part, to the presence of eCG in the fetal circulation. Most of the increasing weight of fetal gonads results from the hyperplasia of the medullar interstitial cells, histologically similar in fetal ovaries and testes (Cole *et al.* 1933). These interstitial cells are analogous to luteal cells and contain all the organelles normally associated with steroid biosynthesis (Hay & Allen 1975). Since fetal gonads also express the full-length eLH/CG-R cDNA as early as day 44 of gestation, one can hypothesize that eCG, fetal in origin, would stimulate the initial steroidogenesis of fetal gonads.

In conclusion, the presence of the full-length eLH/CG-R cDNA isoform in the primary CL and in fetal gonads during eCG secretion suggests that eCG may be involved in the progressive transition from a luteal to a fetoplacental output of steroids during equine pregnancy.

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