Linear *SRY* Transcript in Equine Testis

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ABSTRACT. Employing a combination of reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) techniques, the complete coding sequence of cDNA for the equine *SRY* gene was determined. We also attempted to make clear whether the equine *SRY* gene transcript is expressed in the adult testis, and whether the type of transcript is expressed as linear or circular RNA. As a result, in total a 1420 bp cDNA sequence was determined. Accomplishment of 3' RACE infers that equine *SRY* gene was expressed as a linear RNA transcript in testicular tissue just after puberty, in contrast to the situation in mice.—**KEY WORDS:** equine, sex-determination, *SRY*.

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The function of *SRY* gene in inducing male gonadal differentiation was identified by development of a XX transgenic mouse with testes by introducing a single gene into an embryo [11]. The intron-less *SRY* encodes a putative transcriptional protein harboring the HMG motif [1, 17]. The HMG motif is highly conserved despite lesser conservation in other domains [4]. Therefore, the HMG motif must play a critical role in the transcriptional regulation leading to the development of male gonads [6, 10]. However, a non HMG box C-terminal domain of *SRY* protein may also be indispensable for inducing normal testicular development [9]. As with other domestic animals, the inquiry into genetic diagnosis for infertility is increasing in equine clinics. PCR amplification of the *SRY* gene will provide a more powerful tool in addition to karyotyping [12]. Although generic primers are commonly used for this purpose, the generic primers cannot eliminate the possibility of amplifying a contaminating human counterpart. Moreover, since the HMG-box is a common structure for high mobility gene group proteins, many homologues, such as members of the *SOX* family, are cloned by screening genomic libraries with a *SRY* HMG-box probe [4]. Consequently, molecular cloning of equine *SRY* gene is requisite to both specific diagnoses in reproductive disorders and functional analysis of gonadal differentiation in equids. We report the first cloning of equine *SRY* gene transcript expressed in the adult testicular tissue by a combination of RT-PCR and rapid amplification of cDNA ends (RACE) techniques, instead of screening an equine genomic DNA library. Employing 3' RACE technique as a method of cDNA cloning, we also attempted to investigate whether the form of the equine testicular *SRY* transcript is linear or not.

Testicular tissue obtained from a thoroughbred colt (2 years old) by castration was diced into 10 mm cubes, and quickly frozen in liquid nitrogen. Frozen testicular tissue was stored at -80°C until RNA extraction. Total testicular RNA was extracted by guanidinium/cesium chloride ultracentrifugation [2]. Poly(A)⁺ rich RNA was purified with OligoTex dT30 SUPER (Takara, Kyoto). Double

stranded cDNA of equine testis was synthesized from poly(A)⁺ rich RNA with SuperScript Choice System for cDNA Synthesis (GIBCO BRL, Bethesda) using poly(dT) primer. A pair of oligonucleotide primers ESRY-R25E and ESRY-F23E were designed by comparing equine [8, 12] and human [17] *SRY* consensus sequences, and the hypothetical size of the amplification product was 195 bp. In 200 µl MicroAmp tubes (Perkin-Elmer, Urayasu), 100 pmoles of each primer, 400 nmoles of each dNTP, 10 to 30 ng of equine testicular cDNA as template, and 2.5 units of *Taq* PLUS DNA polymerase (Stratagene, San Diego) were mixed in 100 µl low salt reaction buffer (provided with *Taq* PLUS DNA polymerase; Stratagene, San Diego) reaction mixture. Thermal cycling parameters consisted of a preamplification denaturation at 94°C for 5 min followed by 60 shuttle thermo-cycles of 94°C for 30 sec and 55°C for 30 sec, and extension at 72°C for 7 min [15]. The *SRY* conserved region was successfully amplified as an expected size fragment from equine testicular cDNA (Fig. 1, Lane 1). PCR products were analyzed by agarose gel electrophoreses and purified with GENE CLEAN II kit (BIO-101, La Jolla). Purified PCR fragments were ligated with pGEM-T Easy Vector (Promega, Madison) and transformed *E. coli* cells (XL-1 Blue MRF'; Stratagene, San Diego). An A.L.F. automated sequencer (Pharmacia Biotech, Tokyo), fluorescein labeled sequence primers (M4 and RV-M; Takara, Kyoto), and SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison) were used for the determination of nucleotide sequences [16]. The PCR product was 195 bp in sequence, and identical to the previously reported partial equine *SRY* genomic sequences [8, 12]. Three antisense strand primers, R1-*SRY*, R3-*SRY* and R4-*SRY*, for 5' RACE and 2 sense strand primers, F1-*SRY* and F3-*SRY* for 3' RACE (Table 1) were designed from the sequence of PCR product of the *SRY* consensus region. 5' RACE System version 2.0 (GIBCO BRL, Bethesda) and 3' RACE Adapter Primer (GIBCO BRL, Bethesda) were used for rapid amplification of both cDNA ends [7, 13]. RACE procedures were performed according to the manufacturer's instructions, using 1 to 2

Fig. 1. PCR products from equine testicular cDNA and genomic DNA. HMG box (195 bp) fragments were amplified from testicular cDNA (cDNA) and male genomic DNA (Colt 1–4), but not amplified from female genomic DNA (Mare 1–2) and negative control (Water).

Table 1. Oligodeoxynucleotide primers used in this study

Primers	Sequences (5'–3')
ESRY-R25E	GAATTCGGGTATTTCTCTTGATGCATAGCC
ESRY-F23E	ACCCATGAATTCATTCATGGTGTGGTCTCGTGA
poly(dT)AP*	GGCCACGCGTCGACTAC(T) ₁₇
AAP*	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
AUAP*	GGCCACGCGTCGACTAGTAC
R1-SRY	CCTCAAAGAATGGCAATTTTTCGGCTTCCG
R3-SRY	CGCTTGCTGATCTCTGAGTTTTCAGTTGG
R4-SRY	GGGATTCTCTAGAGCGACCTTGCCTGTGA
F3-SRY	GAGAATCCCCAACTGCAAACTCAGAGA
F5-SRY	AATTGCCATTCTTTGAGGAGGCACAGAG

*: Primers provided with RACE System (GIBCO BRL, Bethesda).

μ g of total RNA or 100 ng of poly(A)⁺ RNA as templates.

Amplification products from 3' and 5' RACE procedures were successfully cloned, and sequenced. The product from 3' RACE was 1074 bp in length, and contained a putative poly-adenylation signal "AATAAA" at 19 bases upstream of the 17 nt stretch of the poly(A) tail. The product from 5' RACE was 330 bases in length, and contained 99 bases of

5' untranslated region upstream of a putative *SRY* open reading frame. An in total 1403 bp cDNA sequence of equine testicular *SRY* transcript was reassembled from three fragments (one RT-PCR and two RACEs) of equine testicular cDNA harboring a 672 bp (nucleotides 100 to 771) open reading frame encoding 223 amino acids of the presumed equine sex-determining protein. Both nucleotide

N-Terminal Region

	10	20	30	40	50	57
horse	MSRV	SNSDNYSLAG	QQHTVLGSGR	TSSLLWTSNP	CSHFRSETRG	NGRENGQ
human	MQSYASAMLS	VFNSDDYSPA	VQENIPALRR	SSSFLCTESC	NSKYQCETGE	NSKGNVQ
cattle	MFRV	LNDDVYSPAV	VQQQTTLAFR	KDSSLCTDSH	SANDQCERGE	HVRESSQ
mouse						ME

HMG (High Mobility Group) Domain

	60	70	80	90	100	110	120	130	136
horse	DRV	KRPMNAFMVW	SRDHRRKVAL	ENPOLQNSEI	SKRLGCQWKM	LTEAEKLPPF	EEAQRRLAMH	QEKYPDYKYR	PRRKAK
human	MLPK	NCSLLPADPA	SVLCSEVQLD	NRLYRDDCTK	ATHSRMEHQL	GHLPPINAAS	SPQQRDRYSH	WTKL*	
cattle	H	I	ER	KMK	D	Q	YE	R	D
mouse	GH		GE H L Q Q	SM	T	Q	CR	S	R

C-Terminal Region

	140	150	160	170	180	190	200	210
horse	MPQK	SDKPLPQTPL	LHCAGRRTYT	STSGCPFLIH	GRFLRLATQS	QTGGAVKPFA	AGATAISALQ	QELSQQHRTL
human	MLPK	NCSLLPADPA	SVLCSEVQLD	NRLYRDDCTK	ATHSRMEHQL	GHLPPINAAS	SPQQRDRYSH	WTKL*
cattle	RPQK	SLPADSSILC	NPMHVETLHP	FTYRDGCAKT	TYSQMESQLS	RSQSVIITNS	LLQKEHHSSW	TSLGHNKVTL
mouse	VSQR	SGILQPAVAS	TKLYNLLQWD	RNPHAITYRQ	DWSRAAHLYS	KNQQSFYWQP	VDIPTGHLQQ	QQQQQQQQQF

	220	230	240	250	260	270	280
horse	RCHSGNVGYA	DIRRRSLSL*					
cattle	ATRISADFP	C NKSLEPGLSC	AYFQY*				
mouse	HNHHQQQQQF	YDHHQQQQQF	QQQQQQFHDH	HQQKQQFHDH	HQQQQQFHDH	HHHHQEQQFH	DHHQQQQQFH

mouse	DHQQQQQQQQ	QQQFHDHHQQ	KQQFHDHHHH	QQQQQFHDHQ	QQQQQFHDHQ	QQQHQQFQQFH	DHHQQQQQFH	350
	HDHPQQKQQF	HDHPQQQQQF	HDHHHQQQQK	QQFHDHHQK	QQFHDHHQK	DHHQQQQQQQ	QQQQQQFHDQ	420
	QLTYLLTADI	TGEHTPYQEH	LSTALWLAVS					450

Fig. 2. Comparison of deduced amino acid sequences for the SRY protein in equine, human, bovine and mouse species. Numbering is based on the human sequence. The depicted SRY sequence is divided into the N-terminal region, the HMG box and the C-terminal region. Only nonhomologous sequences for the HMG domain are shown. Stop codons are represented by *.

and amino acid sequences of reconstructed equine *SRY* transcript were compared with the registered data of GenBank and SWISSPROT databases by personal computer based sequence analyzing software (DNASIS-Mac; Hitachi Software, Yokohama). The nucleotide sequence of equine *SRY* transcript, which will appear in the DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number AB004572, and its predicted amino acid sequence were highly conserved in the HMG motif, while sequence similarities with human and cattle were less consistent in other parts of the sequence (Fig. 2, Table 2).

Accomplishment of 3' RACE, the existence of a putative poly-adenylation signal and the 17 nt stretch of the poly(A) tail suggest that the equine *SRY* gene was expressed as a linear RNA transcript in testicular tissue at puberty. In cattle, the *SRY* gene transcribed in mature, but not late fetal or adolescent, bull testicle could be cloned by 3' RACE [3]. On the other hand, linear *Sry* transcripts of mice are detected only in the genital ridge of the early embryo, and the major form of *Sry* transcript is a circular RNA in the

Table 2. Nucleic acid and amino acid similarity (%) of N-terminal, HMG-box and C-terminal regions of equine *SRY* to human, bovine and mouse *SRY/Sry* sequences*

	Region		
	N-terminal	HMG-box	C-terminal
Nucleic acid similarities / Overlap:			
Human	67.5%/156	86.5%/237	49.7%/143
Cattle	67.3%/154	84.0%/237	62.4%/263
Mouse	- %**	78.0%/236	49.1%/110
Amino acid similarities / Overlap:			
Human	39.2%/52	82.3%/79	0%
Bovine	38.5%/52	74.7%/79	0%
Mouse	-% **	69.6%/79	0%

*A homology search was performed using DNASIS-Mac ver.3.6 (Hitachi Software, Yokohama), utilizing a Lipman and Pearson search algorithm [14].

**Mouse N-terminal sequences were too short to compare with equine sequences.

differentiated testis [5]. The function of both types of testicular transcripts, circular *Sry* in mice and linear *SRY* in other species, are still under investigation. In this study, we demonstrate direct evidence for the linear messenger RNA-type *SRY* transcript in the equine differentiated testicle as well as its nucleotide sequence for the complete amino acid coding region. Lesser sequence consistency with other species in the non-HMG-box may contribute to species specific design of diagnostic primers.

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