CHAPTER IV:

Characterization of Sperm Protein 17:

A Primate Model

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1. Abstract

Recent studies of human Sp17 have generated controversy over the Sp17 nucleotide sequence, mRNA expression, translation and the detection of Sp17 gene variants. To further characterize the Sp17 gene, the primate was used as a non-human model. RT-PCR analysis detected the Sp17-1 transcript in multiple non-testes tissues and differentiated between alternative transcriptional start sites, Sp17-1a and Sp17-1b. Similarly, PCR was used to detect the Sp17-2 gene variant. However, northern blot and western blot analyses did not detect Sp17 mRNA or the Sp17 protein in non-testes tissues. Furthermore, RT-PCR analysis revealed the Sp17 coding sequence in the rhesus macaque.

2. Introduction

The human Sp17 gene was previously presumed to be testes specific by northern blot analysis (1, 2). However, the Sp17 gene was detected by differential display in normal sheep mucosa-associated lymphoid tissues, human rheumatoid arthritis, metastatic squamous cell carcinoma and in multiple myeloma cells (3, 4, 5, 6). Although these studies revealed Sp17 gene expression, they did not differentiate between Sp17 gene variants or examine Sp17 mRNA and translation. In addition, recent studies of human Sp17 have generated controversy over the Sp17 nucleotide sequence, mRNA expression, translation and the detection of Sp17 gene variants (Chapter 1, Chapter 2). Thus, an extensive multi-tissue model characterizing the Sp17 gene is warranted.

The primate was selected for these studies because of the high degree of homology to humans. For example, the Sp17 nucleotide sequences identified in the baboon (Accession number U75209) and cynomologous macaque (Accession number AF334809) are 97.7% and 98.5% identical to the human Sp17 sequence (7, 8). In addition, the cynomologous macaque was used as a non-human primate model to study immune response using Sp17 as an antigen (7). Thus, the cynomologous macaque was selected as a suitable model to study the specificity of the Sp17 gene.

Sp17 gene transcription was examined in normal cynomologous macaque somatic tissues. RT-PCR analysis was used to detect the Sp17-1 transcripts and to differentiate between alternative transcriptional start sites, Sp17-1a and Sp17-1b. Similarly, PCR was used to elucidate the Sp17-2 gene variant and detect the Sp17-2 transcript. Moreover, northern blot and western blot analyses were performed to examine Sp17 mRNA expression and detect the Sp17 protein in multiple cynomologous macaque tissues. Furthermore, to identify potential variances among primates, parallel Sp17 studies were conducted using the rhesus macaque.

3. Materials and Methods

3.1 RNA isolation, DNase treatment and Reverse transcription (RT)

Normal primate somatic tissue samples were obtained from the UCD primate center (UCD biological use authorization #0657). The tissues included adrenal,

bladder, brain, colon, esophagus, heart, kidney, liver, lung, lymph node, muscle, pancreas, prostate, salivary, skin, small intestine, spinal column, spleen, synovium, thyroid, uterus, juvenile testes and adult testes.

The tissues were homogenized (50-100 mg) in TRIzol reagent and the total RNA was extracted, analyzed and stored as described in Chapter 1, section 3.1. The total RNA extracts (3ug) were treated with DNase I and converted into cDNA by reverse transcription (RT) as described in Chapter 1, section 3.2 (Chapter 1, Table 1.1).

3.2 Polymerase chain reaction (PCR)

The Sp17 nucleotide sequence was amplified from multiple tissue cDNAs by RT-PCR. Sets of Sp17 specific primers were created to detect the Sp17 transcript and distinguish between the Sp17 transcript variants; Sp17-1a and Sp17-1b (Table 4.1). In addition, Sp17 specific primers were used to amplify the Sp17-2 gene variant from genomic DNA (Table 4.1). All primers were synthesized by Biosynthesis, Inc. (Lewisville, TX) and resuspended to 100pmol in sterile water.

The PCR products were prepared and analyzed as described in Chapter 1, section 3.3 with the following primer and template modifications: 200nM sense primer, 200nM anti-sense primer, 4uL cDNA or genomic DNA template. The PCR products were resolved by gel electrophoresis, visualized with UV light and photographed. As needed to visualize the amplified DNA fragments, a second round of PCR was performed as described above using 2uL of the first PCR cocktail as the template.

The appropriately sized PCR amplified DNA bands were excised, purified and subcloned into a TOPO vector as described in Chapter 1, section 3.6. The positive clones were purified and the DNA inserts were sequenced from the forward (M13R) and reverse (T7) directions using ABI BigDye Terminator chemistry (Applied Biosystems; Foster City, CA) by the DBS Automated DNA Sequencing Facility (University of California, Davis, CA). DNA sequence analysis, assembly and alignment was performed as described in Chapter 1, section 3.7.

3.3 Northern blot analysis

A 10-30 ug total RNA aliquot was precipitated with ethanol and 3M NaOac from multiple normal tissues. The RNA pellets were resuspended in DepC water. The RNA samples and RNA molecular weight marker (Life Technologies, Inc.) were heat denatured in a solution of 5X formaldehyde gel running buffer (0.1M MOPS (3-(N-morpholino)propanesulphonic acid), 40mM NaOAC, 5mM EDTA (pH 8.0), run through a 0.2 micron filter), 12.3M formaldehyde and deionized formamide at 65°C for 15 minutes.

The formaldehyde gel was pre-run in 1X formaldehyde gel running buffer at 80 Volts for 5 minutes. The denatured samples were mixed with 6X gel loading dye and were gel electrophoresed on a 1.5% formaldehyde gel (1.5% agarose, 5X formaldehyde gel running buffer, 12.3M formaldehyde, 1ug/mL ethidium bromide). The formaldehyde gel was run in 1X formaldehyde gel running buffer at 3-4 Volts

overnight. The gel was visualized with UV light and photographed next to a UV labeled ruler.

The gel was transferred to nylon paper (Micron Separations, Inc., Westborough, MA) by capillary movement of DepC treated 20X SSC (3.0M NaCl, 0.3M Sodium citrate, pH 7.0) (9). The transfer was conducted at room temperature overnight.

The wells of the formaldehyde gel were indicated on the nylon blot with a pencil. The nylon membrane was washed with 2X SSC (300mM NaCl, 30mM Sodium citrate, pH 7.0) at room temperature for 15 minutes with constant agitation. The nylon membrane was air dried at room temperature, crosslinked with UV light and vacuum dried at 80°C for 30 minutes. The membrane was stored between two dry pieces of 3MM Whatman paper (Whatman, Inc.) in a plastic bag at -70°C.

The membrane was pre-hybridized at 42°C for 3 hours in hybridization solution (50% deionized foramide, 5X SSC (750mM NaCl, 75mM Sodium citrate, pH 7.0), 0.1% Sodium dodecyl sulfate (SDS), 10% dextran sulfate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 50ug/mL denatured salmon sperm DNA). The blot was hybridized with a Sp17 gene specific, ^{32}P-labelled nick-translated (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) DNA probe (615bp; 10^{6} cpm/mL) in hybridization solution at 42°C for 18 hours (Table 4.2). The DNA probe was amplified from human testis tissue by RT-PCR and spanned Sp17 exon 3-5 and the 3'UTR of the Sp17 nucleotide sequence.

The blot was washed once with 2X SSC at room temperature for 30 minutes and once with 0.1X SSC (15mM NaCl, 1.5mM Sodium citrate, pH 7.0) containing 0.5% SDS at 65°C for two hours. The blot was scanned with a STORM Imaging System (Molecular Dynamics, Sunnyvale, CA) and analyzed with Image Quant v1.2 computer software (Molecular Dynamics).

3.4 Western blot analysis

Normal primate somatic tissues were homogenized (50-100mg) in 1mL 1% SDS and incubated at 50°C for one hour. The supernant (50uL) containing the total protein extracts was combined with 4X SDS-PAGE sample buffer (10% D-Saccharose, 0.1% Bromophenol blue, 200mM Tris-HCl (pH 8.0), 5mM EDTA (pH 8.0), 2% Sodium dodecyl sulfate, 10mM DTT), heat denatured at 95°C for 5 minutes and cooled on ice for 5 minutes. The total protein extracts were separated on a 12% SDS-PAGE polyacrylamide gel adjacent to a 230kda kaleidoscope pre-stained protein marker (BioRad Laboratories, Hercules, CA) (10). The gels were run at 40mAmps for 2.5 hours and transferred to nitrocellulose paper (Micron Separations, Inc., Westborough, MA) overnight.

The blot was blocked overnight with blocking reagent (3% skim milk in PBS). The primary antibody, rabbit anti-Sp17 sera or rabbit preimmune sera (Chapter 3), was diluted (1:200) with blocking reagent and incubated with each blot at room temperature for one hour. The blots were washed four times for 15 minutes with wash

solution. The secondary antibody, HRPO goat anti-rabbit IgG (Caltag, Burlingame, CA), was diluted 1:2000 with blocking reagent and incubated with each blot at room temperature for one hour. The blots were washed four times for 15 minutes with wash solution. The blots were developed using chemiluminescent reagents (SuperSignal, Pierce) and exposed to film.

4. Results

4.1 Coding region RT-PCR

The Sp17 coding region sequence was initially examined in normal adult cynomologous and rhesus macaque testes by RT-PCR (primers Shu:Ahu). RT-PCR and sequence analyses revealed a PCR amplified DNA fragment (482bp) that was 100% identical to the published human Sp17 nucleotide sequence (Figure 4.1). The rhesus macaque Sp17 coding region sequence was submitted to GenBank (Accession number AF334809).

4.2 Sp17-1 gene transcript

The Sp17-1 transcript and the identification of alternative transcriptional start sites were examined in multiple normal cynomologous macaque tissues by RT-PCR (primers Sg1a, Ssp1/2 and Ag1). Amplification of cDNA from normal esophagus, kidney, pancreas, small intestine, spinal column, spleen, thyroid and adult testes revealed a DNA fragment corresponding to the 849bp DNA band expected for Sp17-1a (Figure 4.2A). However, a 902bp DNA band, corresponding to Sp17-1b was not detected in these tissues (Figure 4.2B). All controls were negative for genomic DNA.

4.3 Sp17-2 gene

The Sp17-2 gene was examined in normal cynomologous and rhesus macaque testes by PCR (primer sets Sgr2 and ACDS, Shu and Ag2, Sgr2 and Ag2, Sg2full and ACDS and Shu and A2full). PCR amplification of the Sp17-2 gene was detected in reactions containing a genomic DNA template. Conversely, RT-PCR analysis of macaque testis cDNA, free of genomic DNA, did not reveal a Sp17-2 transcript (Figure 4.3AB).

4.4 Northern blot hybridization

Northern blots of total RNA extracted from multiple cynomologous macaque tissues were hybridized with a human Sp17 coding region specific ^{32}P-labelled nick-translated DNA probe. A strong Sp17 hybridization to a single band at 950bp was detected in the total mRNA extracted from adult testes. However, Sp17 hybridization was not observed in RNA extracts from non-testes cynomologous macaque tissues (Figure 4.4).

4.5 Western blot analysis

Western blots of total protein extracted from multiple cynomologous macaque tissues and rhesus adult testes tissue were probed with rabbit anti-recombinant Sp17 polyclonal sera. A strong anti-Sp17 reactivity to a single band of ~ 28kDa was detected in the total protein from rhesus testes tissue. Similarly, a ~35kDa was detected in the total protein extracted from cynomologous macaque adult testes, suggesting a Sp17 multimer (Figure 4.5). In addition, a strong anti-Sp17 reactivity was observed against a single 18.8kDa band corresponding to the Sp17 recombinant protein. Anti-Sp17 reactivity was not observed to the total protein extracts from any other cynomologous macaque tissues including the adrenal gland, brain, colon, esophagus, heart, kidney, liver, lung, lymph node, pancreas, prostate, skin, skeletal muscle, small intestine, spleen, synovium, thyroid, uterus, synovium, juvenile testes and adult testes. In addition, western blots probed with rabbit preimmune sera were negative for immune reactivity.

5. Discussion

The Sp17-1 and Sp17-2 nucleotide sequences were amplified from normal adult cynomologous and rhesus macaque testes tissue. Both Sp17 nucleotide sequences exhibited 100% identity to the human Sp17-1 and Sp17-2 nucleotide sequences (Chapter 1, Chapter 2). Thus, it is likely that the Sp17 gene is highly conserved among primates.

In addition to adult testes tissue, the Sp17-1 transcript was also detected in cynomologous macaque esophagus, kidney, pancreas, small intestine, spinal column, spleen, thyroid and adult testes. However, a second round of PCR was needed to visualize DNA products, suggesting that the Sp17-1 transcript in these tissues is in low abundance.

Furthermore, RT-PCR analysis of Sp17-1 alternative transcriptional start sites in multiple cynomologous macaque tissues detected a single Sp17 transcriptional start site, Sp17-1a. Thus, only Sp17-1a appears to be transcribed in the cynomologous macaque. Conversely, both the Sp17-1a and Sp17-1b transcriptional start regions were detected in human testes (Chapter 1). However, the human Sp17 mRNA pool, the cynomologous macaque, appears to be dominated by a single mRNA isoform, Sp17-1a (11, Chapter 1).

Similar to the human, the Sp17-2 gene variant was detected in the genomic DNA of the cynomologous and rhesus macaques. The detection of the Sp17-2 gene in both macaques suggests the likelihood that the Sp17-2 gene variant will be observed among other primates. However, the Sp17-2 gene was not found in the rat (Chapter 2). Thus, these data support the hypothesis that the retroposition of Sp17-1 to create Sp17-2 may have occurred recently on the evolutionary timeline.

Sp17 mRNA expression and the Sp17 protein were only detected in normal adult cynomologous testes. However, it is particularly interesting to note that the Sp17 mRNA and Sp17 protein were identified in adult testes, but were not detected in juvenile testes. These findings suggest that Sp17 may be a developmental gene whose transcription, mRNA expression and translation may be age dependent and possibly hormonally regulated. Thus, examining the regulation of the Sp17 transcript may provide insight into male infertility and male contraception.

Although the Sp17-1 transcript is expressed, in low abundance, in multiple non-testes tissues and is conserved among mammals, its role in non-testes tissues is not yet known. However, the Sp17 gene has been identified in highly proliferating diseased tissues, such as RA and metastatic neoplasias (3, 5, 6). Therefore, Sp17 may have an alternative role in highly proliferating tissues, possibly in the mediation of signal transduction, protein synthesis and/or unregulated growth (12). In addition, Sp17 has been implicated as a cancer-testis antigen (6).

Although the role of Sp17 in non-testes tissues is unknown, altered Sp17 gene expression may contribute to changes in pathologic behavior, including pathways involved in signal transduction, cell growth and death, cell recognition and adhesion, angiogenesis, and host immunity. Thus, the characterization of the Sp17 gene in diseased tissues may provide further insight into the regulation and function of Sp17.

6. Conclusion

A multiple tissue, primate model of Sp17 detected the Sp17-1 transcript and Sp17-2 gene variant. In addition, the Sp17-1a transcript was detected in normal non-testes tissues, although in low levels. However, Sp17 mRNA expression and translation in non-testes tissues was not detected. Moreover, the role of the Sp17 transcript in normal, non-testes tissues is not known. Thus, an investigation of the alternative, possibly pathogenic, role of the Sp17 transcript in non-testes tissues merits attention.

7. Tables and Figures

Table 4.1. Sp17 specific sense and anti-sense primers used in RT-PCR to detect the Sp17-1 transcript, differentiate between alternative transcriptional start sites and detect the Sp17-2 gene variant.

CODING REGION			
Sense (Shu)	5'	CGCGGATCCATGTCGATTCCATTCTCC	3'
Anti-Sense (Ahu)	5'	CGGGGTACCAACCAGTGTCCTCACTTG	3'
GENE 1			
Sense (Sg1a)	5'	CGCCCCTTCTCGGCCGCCGTAG	3’
Sense (Ssp1/2)	5'	CCCAGCAACTAGAAAAACAACCGGAA	3’
Anti-Sense (Ag1)	5'	GATAATAGTTAGAACACATAACCTC	3'
GENE 2			
Sense (Sgr2)	5'	GAGGTCAGAGGAGGAAGTAAGCTG	3'
Sense (Sg2)	5'	CCCTAGAACATGAGGTCAGAGGAG	3'
Sense (Shu)	5'	CGCGGATCCATGTCGATTCCATTCTCC	3'
Sense (Sg2full)	5'	CCAATGACTGATGGACACAGACTG	3'
Anti-Sense (aCDS)	5'	GAGTCTAAGATGGTGACTGATG	3'
Anti-Sense (Ag2)	5'	CCATGAGCCAAAATGGTGGTAGTC	3'
Anti-Sense (Ag2full)	5'	GAGATGTGATAGCTGGCACTCATG	3'

Table 4.2. Sp17 sense and anti-sense primers used to develop northern blot DNA probe.

Sense (sspe3)	5'	AAAACCAACTTTGATCCAGCAGAATG	3'
Anti-Sense (Ag1)	5'	GATAATAGTTAGAACACATAACCTC	3'

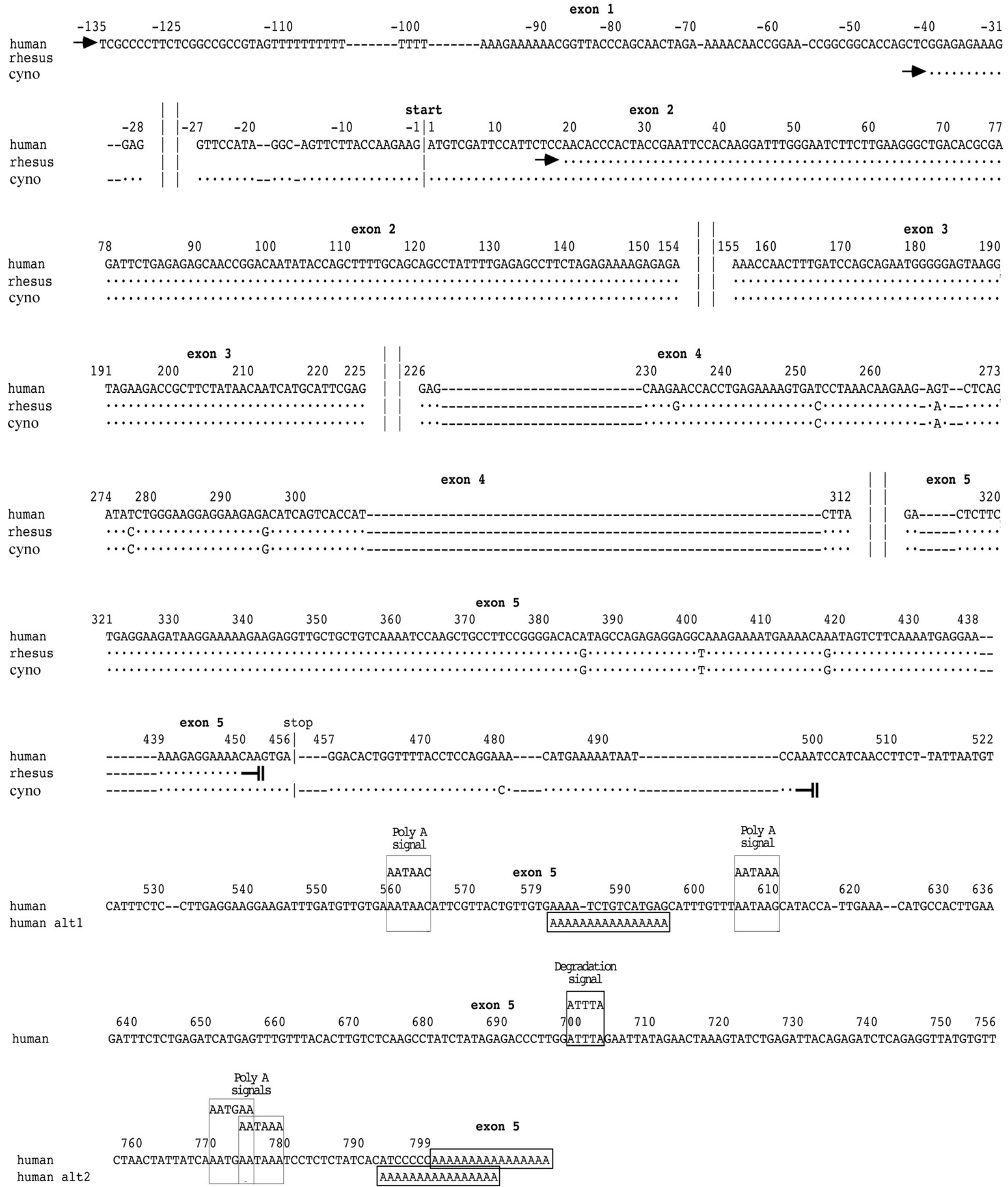
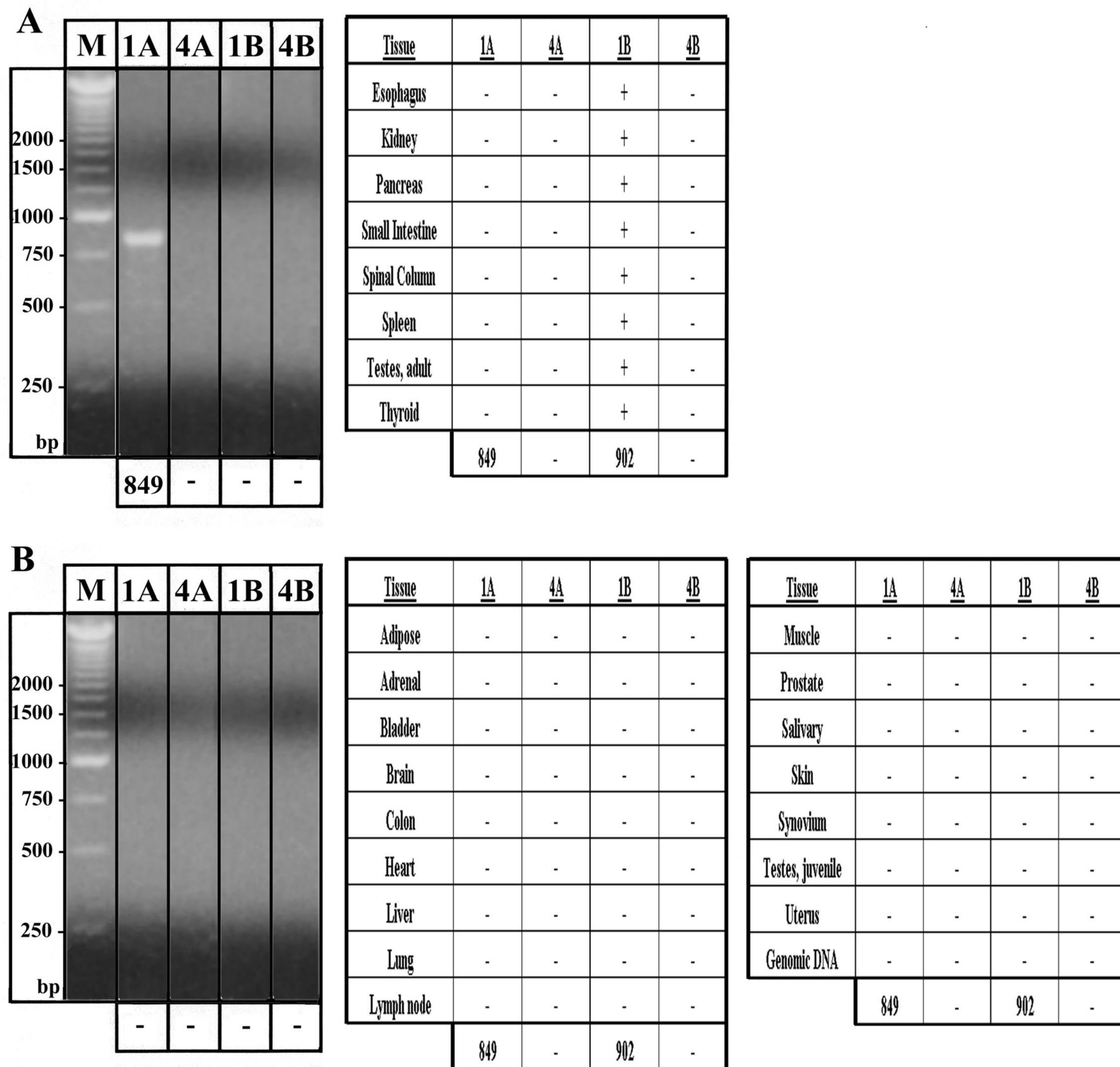


Figure 4.1. Alignment of the Sp17 nucleotide sequence from the human (AF334735), cynomologous macaque (cyno, AF005551) and rhesus macaque (AF334809). The Sp17 nucleotide sequences were aligned by Clustal algorithm with respect to the human Sp17 sequence. Sequence homology is represented by dots. Dashes were used to maintain alignment. The sequence start (arrows), sequence end (double-bars), start and stop codons (vertical line) and exons (double vertical line) are indicated.



Tissue	1A	4A	1B	4B
Esophagus	-	-	+	-
Kidney	-	-	+	-
Pancreas	-	-	+	-
Small Intestine	-	-	+	-
Spinal Column	-	-	+	-
Spleen	-	-	+	-
Testes, adult	-	-	+	-
Thyroid	-	-	+	-
	849	-	902	-

Tissue	1A	4A	1B	4B
Adipose	-	-	-	-
Adrenal	-	-	-	-
Bladder	-	-	-	-
Brain	-	-	-	-
Colon	-	-	-	-
Heart	-	-	-	-
Liver	-	-	-	-
Lung	-	-	-	-
Lymph node	-	-	-	-
	849	-	902	-

Tissue	1A	4A	1B	4B
Muscle	-	-	-	-
Prostate	-	-	-	-
Salivary	-	-	-	-
Skin	-	-	-	-
Synovium	-	-	-	-
Testes, juvenile	-	-	-	-
Uterus	-	-	-	-
Genomic DNA	-	-	-	-
	849	-	902	-

Figure 4.2. RT-PCR analysis of the Sp17-1 transcript in multiple cynomologous macaque tissues. Sp17 specific primers were used to differentiate between alternative transcriptional start sites, Sp17-1a (1A; 849bp) and Sp17-1b (1B; 902bp). The DNA fragments were measured by a DNA marker (M) in bp. The PCR cocktails were prepared with the following template 1: cDNA; 4: none (Chapter 1, Table 1.1). **A.** RT-PCR analysis revealed DNA amplification of the Sp17-1a transcript in the tissues listed on the right. **B.** The Sp17-1b transcript was not detected in the tissues listed on the right. All controls (4A, 4B) were negative for genomic DNA.

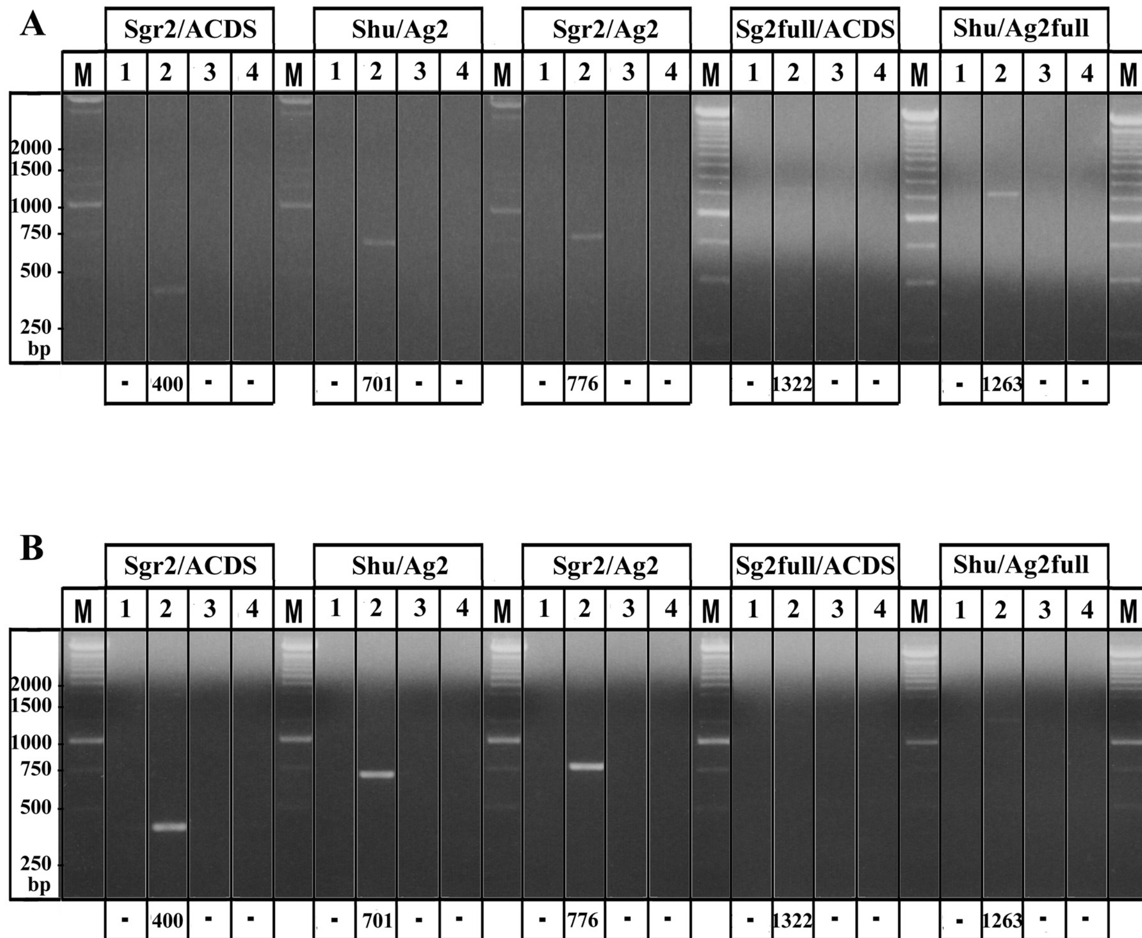


Figure 4.3. RT-PCR analysis of the Sp17-2 gene in normal cynomologous macaque (**A**) and rhesus macaque (**B**) testis tissue. Sp17-2 specific primers were used to elucidate Sp17-2 in the cDNA versus the genomic DNA (Chapter 2, Figure 2.2). The PCR cocktails were prepared with the following templates 1: cDNA; 2: gDNA; 3: RNA; 4: none (Chapter 1, Table 1.1). The PCR products were measured in bp adjacent to a DNA marker (M). All controls (3 and 4) were negative for genomic DNA.

Figure 4.4. Northern blot analysis of total RNA extracted from multiple normal cynomologous macaque tissues. The blot was hybridized with a ^{32}P-labeled nick-translated Sp17 DNA probe. Strong hybridization to adult testes was detected at 950bp as measured by a RNA marker (bp). However, hybridization was not observed to any other RNA tested.

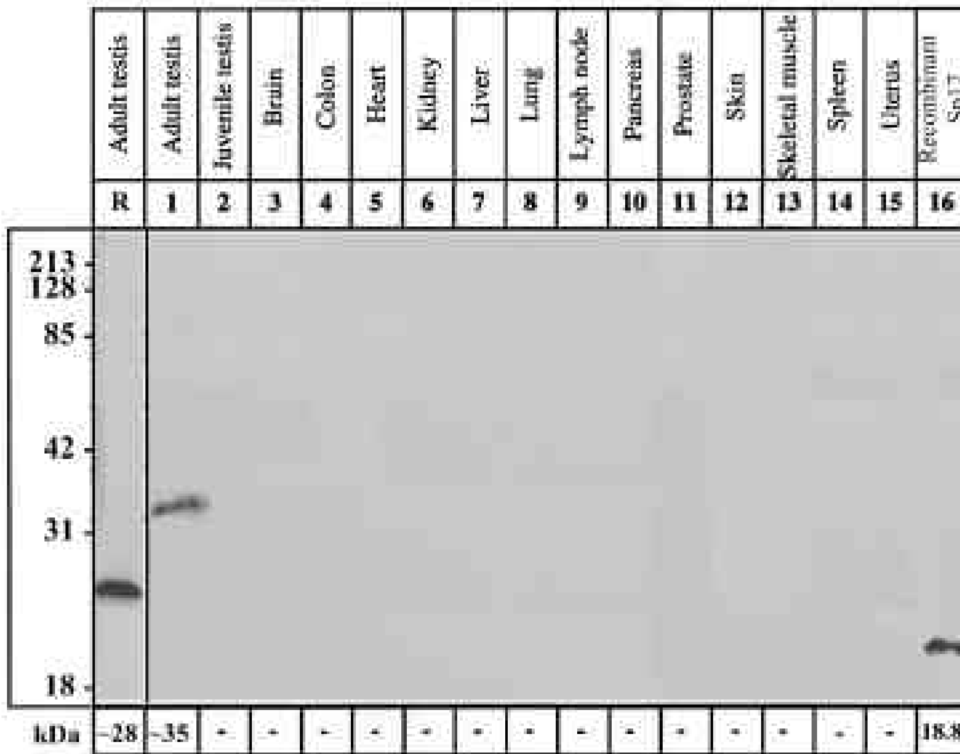


Figure 4.5. Western blot analysis of total protein extracted from multiple normal cynomologous macaque tissues and rhesus macaque (R) testis tissue. The blot was probed with rabbit anti-recombinant Sp17 anti-sera. Strong hybridization to cynomologous adult testes (~35kDa), rhesus adult testes (~28) and native recombinant Sp17 protein (18.8kDa) was observed. However, anti-Sp17 immune reactivity was not detected to any other cynomologous tissue examined.

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