Structural and functional analysis of the *HSP90AA1* gene: distribution of polymorphisms among sheep with different responses to scrapie

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Abstract Scrapie is a transmissible spongiform encephalopathy in sheep and goats. Susceptibility to this neurodegenerative disease is mainly controlled by point mutations at the *PRNP* locus. Other genes, apart from *PRNP*, have been reported to modulate resistance/susceptibility to scrapie. On the basis of several studies in Alzheimer and different transmissible spongiform encephalopathy models,

HSP90AA1 was chosen as a putative positional and functional candidate gene that might be involved in the polygenic variance mentioned above. In the present work, the ovine *HSP90AA1* gene including the promoter and other regulatory regions has been isolated and characterized. Several sequence polymorphisms have also been identified. FISH-mapping localized the *HSP90AA1* gene on ovine chromosome OAR19q24dist, which was confirmed by linkage analysis. This chromosome region has been shown to include a quantitative trait loci (QTL) for scrapie incubation period in sheep. Expression analyses were carried out in spleen and cerebellum samples. No differences in the expression of the *HSP90AA1* gene were found in any of these tissues ($p > 0.05$) between control and infected animal samples. Nevertheless, association analyses revealed that several polymorphisms in the 5' and 3' regions of the *HSP90AA1* gene were differentially distributed among animals with different responses to scrapie infection. Thus, results presented here support the hypothesis that *HSP90AA1* could be a positional and functional candidate gene modulating the response to scrapie in sheep.

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Introduction

Scrapie (SC) is a transmissible spongiform encephalopathy (TSE) of sheep and goats. TSEs are neurodegenerative diseases caused by transmissible proteinaceous particles (prions) devoid of nucleic acid that affect man and various animals. The key event in the pathogenesis of prion diseases is the conversion of the host encoded and naturally

expressed cellular prion protein (PrP^C) into its aberrant counterpart PrP^{Sc}. During the conversion process, PrP^{Sc} acquires new biophysical and biochemical characteristics, which lead to its accumulation in the form of amyloid plaques in nervous and lymphoid tissues (Prusiner 1998). However, the mechanism by which PrP^{Sc} causes neurodegeneration remains unclear. Like SC, most of the neurodegenerative diseases are associated with events of protein misfolding. The accumulation of misfolded protein aggregates can overwhelm the ubiquitin-proteasome system inducing apoptosis and increasing neuronal vulnerability to subsequent insults (Kang et al. 2004).

Susceptibility to SC is associated with polymorphisms in the amino acid sequence of the PrP protein. Twenty-five polymorphic codons and 40 haplotypes have been described to date in the open reading frame of the gene encoding the PrP (*PRNP*; Goldmann et al. 2005). In a slightly simplified assessment of genetic susceptibility for a sheep to develop SC, polymorphisms are classified attending to amino acid changes at positions V136A, R154H, and QH171R (Baylis et al. 2004). However, significant differences in the incubation period for this neurodegenerative disease have been reported in mice and sheep with the same *PRNP* genotype (Carlson et al. 1988; Diaz et al. 2005; Dickinson 1975; Westaway et al. 1987). Additionally, evidence for other genomic regions containing genes that influence the incubation period for SC in mice (Lloyd et al. 2001; Lloyd et al. 2002; Manolakou et al. 2001; Moreno et al. 2003a,b; Stephenson et al. 2000) and sheep (Moreno et al. 2003a) have been reported. Thus, a reasonable hypothesis is that a major gene (*PRNP*) controlling resistance/susceptibility to SC coexists with a number of other genes that modulate its effect.

Chaperones are essential proteins involved in the formation and maintenance of the proper conformation of other proteins and promoting cell survival after a large variety of environmental stresses. They protect other proteins against aggregation, solubilize initial, loose protein aggregates, assist in the folding of nascent proteins or in the refolding of damaged proteins, target severely damaged proteins to degradation and in case of excessive damage, sequester damaged proteins to larger aggregates (Soti et al. 2005).

A QTL interval modulating SC incubation period in sheep has been described on ovine chromosome OAR18 (Moreno et al. 2003a). By comparing this ovine region with the human and murine genomes, herein we have inferred that it should include the Hsp90 α (cytosolic) class A member 1 gene (*HSP90AA1*).

The chaperone protein known as 90 kDa heat shock protein, Hsp90, is one of the most abundant proteins in eukaryotic cells, comprising 1–2% of cellular proteins under nonstress conditions. There are two major cytoplasmic isoforms of Hsp90, which have arisen by gene

duplication, Hsp90 α (inducible form) and Hsp90 β (constitutive form). It is also known that additional Hsp90 paralogs are localized in the endoplasmic reticulum and mitochondria, denoted Grp94/gp96 and Hsp75/TRAP1, respectively. Since separating biochemically the Hsp90 α and Hsp90 β isoforms is rather difficult because they share 85% of sequence identity in higher eukaryotes, most studies have been carried out with a mixture of both isoforms (Sreedhar et al. 2004).

Cytosolic Hsp90's contribution to various cellular processes including signal transduction, protein folding, protein degradation, cell survival, and morphological evolution has been extensively studied.

It has been shown that Hsp90 specifically influences aggregation at “early” stages in Alzheimer disease avoiding the production of toxic oligomers (Evans et al. 2006). Furthermore, Kakimura et al. (2002) reported that Hsp90 induced microglial activation participating in compensatory neuroprotection through the production of cytokines, enhancement of phagocytosis and clearance of A β in this human disease.

Although the exact role of this chaperone in SC pathology is unknown, it could participate in several aspects of the disease. Thus, Hsp90 α acts as a repressor of HSF1, which is an activator of heat shock genes. Under stress conditions, nonnative proteins accumulate and compete with HSF1 for binding to Hsp90. As a result, HSF1 is released from this complex and becomes active (Zou et al. 1998). In addition, it is well known that Hsp90 maintains the functional integrity of the 26S proteasome and participates in its assembly. It is also responsible for refolding stress-damaged proteins and thereby might be sequestered into such damaged proteins after severe insults. In this way, the disassembly of the 26S proteasome could be a consequence of changes in the physiological state of Hsp90 due to stress conditions (Imai et al. 2003). It has been demonstrated that wild-type PrP is subjected to ubiquitination and degradation by the proteasome and that a significant fraction of PrP enriched in unglycosylated forms accumulates in the cytoplasm when the activity of the proteasome is compromised (Ma and Lindquist 2001; Yedidia et al. 2001). Furthermore, proteasomal activity decreases in mouse SC brains (Kang et al. 2004). Taking into account these reports and that both unglycosylated PrP forms and cytoplasmic environment favor the formation of PrP^{Sc}-like forms, it can be hypothesized that after SC infection, PrP^{Sc} starts to accumulate because its degradation is impaired. Hsp90 could contribute to the correct folding of PrP^{Sc} instead of participating in other functions and thus result in an increased expression of other heat shock proteins and in a minor proteasomal function. Additionally, as a consequence of the reduced proteasomal activity, the PrP^C, which in normal circumstances is efficiently degraded

by this machinery, would accumulate in the cytoplasm, increasing the amount of substrate susceptible of being transformed to the PrP^{Sc} isoform. These arguments led us to study *HSP90AA1* as a possible positional and functional candidate gene contributing to ovine SC resistance/susceptibility modulation.

The first objective of this work was to isolate and characterize the gene, and determine the cytogenetic position of *HSP90AA1* on the ovine genome to verify if its localization was in concordance with previous reports describing QTLs associated with resistance/susceptibility and incubation period for different TSEs in ovine (Moreno et al. 2003a) and murine models (Lloyd et al. 2001; Lloyd et al. 2002; Manolakou et al. 2001; Moreno et al. 2003b; Stephenson et al. 2000). The next step was to verify if there was any difference in the expression of this gene between SC infected and uninfected sheep presenting the same genotype for *PRNP* (*ARQ/ARQ*). Since, as it has been described above, there is no need of Hsp90 α overproduction to trigger a stress response, the aim of this point was to gain insight into the possible role of Hsp90 α in SC and in the way it participates in this neurodegenerative disease. This comparison was performed using real-time RT-PCR on samples of spleen and cerebellum tissues. These are important organs in the development of SC and other TSEs since, after oral infection, prions replicate in the spleen and subsequently migrate to the central nervous system causing a profound neurodegeneration. Finally, the *HSP90AA1* polymorphism was studied to perform further association analyses to identify putative mutations in the ovine *HSP90AA1* locus that could explain, at least in part, the polygenic response to SC in sheep.

Materials and methods

Isolation of sheep specific DNA fragments

Genomic DNA was extracted from ovine lymphocytes according to the salting-out procedure described by Miller et al. (1988). BAC DNA was purified by a Maxiprep kit with the Nucleobond PC 100 Kit (Macherey-Nagel) according to the manufacturer's instructions.

To isolate and sequence *HSP90AA1* fragments, 12 heterologous primers from human (GenBank NT_026437), murine (GenBank NT_039551) and bovine sequences (GenBank NW_930044.1, NW_270059 and NM_001012670.1), and thirteen specific primers based on the sequence of the ovine fragments previously isolated with the primers described above, were synthesized (ESM Appendix 1). Bovine *HSPCAB* mRNA sequence (AB072369) was also taken into account to avoid unspecific amplifications due to the high homology level between *HSP90AA1* and *HSPCAB* (gene

encoding Hsp90 β protein) coding regions. *HSP90AA1* regulatory regions were inferred by sequencing both the BAC DNA insert and PCR amplified fragments from genomic and BAC DNA. Genomic (60–100 ng) and BAC DNA (100 ng) were amplified in a final volume of 25 μ l containing 0.5 μ M of each primer, 200 μ M of dNTPs, 1.5–2 mM MgCl₂, 2.5 μ l of 10 $\times$ buffer MgCl₂ free (Biotools) and 1 U Taq polymerase (Biotools). In some cases, it was necessary to add DMSO to the PCR mix (see ESM Appendix 1). The following PCR conditions were used: denaturation at 94°C for 5 min, 30 amplification cycles of denaturation at 94°C for 45 s, annealing at 52°C to 59°C for 30 s, and extension at 72°C for 30 s to 1 min 30 s followed by a final 5–10 min extension at 72°C. Primer pairs and their amplification conditions are shown in ESM Appendix 1. The resulting PCR fragments were purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) and bidirectionally sequenced with the PCR primers. The identity of the fragments was confirmed by BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>). As indicated in ESM Appendix 1, the fragment generated with primers that hybridized with regions at –570 and intron 2 had to be sequenced with different primers from those used to perform the PCR reaction due to the secondary structures that they formed. Additional primers also had to be used to sequence the region amplified between –700 and +400 positions because of the presence of insertion/deletion polymorphisms.

Chromosomal location

Ovine *HSP90AA1* gene was localized in the ovine genome by two approaches: cytogenetic and genetic mapping.

Cytogenetic mapping: Primers located in *HSP90AA1* intron 10 and exon 11 (see ESM Appendix 1) were used to screen an ovine BAC library (Vaiman et al. 1999). DNA from the identified BAC clone was labelled by nick-translation in the presence of biotin-14-dATP (BioNick TM 18247-015 labelling system) and used as probe for in situ hybridisation on RBP-banded ovine chromosome preparations following the protocol described by Hayes et al. (1991).

Genetic mapping: Genomic DNA from five domestic sheep breeds [Latxa ($n=2$), Manchega ($n=5$), Awassi ($n=1$), Assaf ($n=1$) and Rasa Aragonesa ($n=1$)] was amplified and sequenced as previously described and analyzed using CHROMAS 1.43 and ClustalW (<http://www.ebi.ac.uk/clustalw/>) software to detect polymorphisms along the isolated sequences of *HSP90AA1*, without taking into account either their *PRNP* genotype or their pattern of SC resistance/susceptibility. Animals were chosen from different breeds to avoid any problem due to allele fixation because of the selective status of these breeds in our country (Spain), and the identification of a representative polymorphism at the species level.

To follow the distribution of the gene within the pedigrees of the AgResearch International Mapping Flock (IMF), the SNP (A/G) located at position 84 in the *HSP90AA1* intron 10 was analyzed by PCR-RFLP using primers that hybridized *HSP90AA1* exons 10 and 11 (see ESM Appendix 1). The reaction product (8 μ l) was digested with 0.2 U of *Tsp509I* at 65°C over 19 h in a final volume of 18 μ l and then electrophoresed on a 3% agarose gel for visualization.

Thus, cytogenetic localization was confirmed by linkage mapping in nine sheep families. *HSP90AA1* gene was mapped against markers on the sheep framework map (Maddox et al. 2001). Multipoint linkage analysis of the IMF pedigrees (Crawford et al. 1995) was performed using CRI-MAP (Lander and Green 1987).

Gene expression

A total of 13 Rasa Aragonesa female sheep (aged 3–5 years) with the same *PRNP* genotype (*ARQ/ARQ*) were included in this study. Eight of them exhibited clinical signs of SC in a terminal state. *PRNP* genotypes were determined according to Acin et al. (2004). The diagnosis was confirmed using a rapid test and immunohistochemistry to detect PrP^{Sc} (Bolea et al. 2005). These animals belong to a flock of sheep, conserved for research purposes by the Prion Research Centre of the University of Zaragoza and where several SC cases have appeared in the last 4 years. Control animals ($n=5$) were selected from a different flock belonging to the same breed, where no SC had been reported to date.

The aim of this sample design was to determine if SC disease might cause any change in the expression pattern of *HSP90AA1*. Thus, small fragments of spleen and cerebellum were included in RNeasy (Ambion). Total mRNA was purified from spleen and cerebellum with the RNeasy Mini Kit and the RNeasy Lipid Tissue Mini Kit (Qiagen), respectively. Complementary DNA (cDNA) was synthesized from 1 μ g of each RNA preparation using random hexamer primers with the SuperScript First-Stranded Synthesis System for RT-PCR (Invitrogen). Gene expression levels were subsequently determined by real-time RT-PCR. All RT-PCR reactions were run in duplicate. Two tissue-specific housekeeping genes were used to normalize each set of results. Data from the spleen were normalized for succinate dehydrogenase complex, subunit A (*SDHA*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. Hexose-6-phosphate dehydrogenase (*G6PDH*) and *18S rRNA* mRNA were used in the case of cerebellum. Primers and probes used for gene expression analysis, their concentrations and amplicon sizes are shown in ESM Appendix 2. Amplification was carried out in a final volume of 25 μ l containing SYBRGreen PCR Master Mix

(Applied Biosystems) or TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), depending on the gene analyzed (see ESM Appendix 2). After preheating the mix at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 30 s were carried out.

It was necessary to sequence a fragment of the cDNA containing part of exon 10 through intron 11 to synthesize a pair of primers adequate to carry out the real-time RT-PCR. These primers were F: 5'- TTGGTGACATCCCCAT GCTG -3' and R: 5'- CAACAGTCATCTGTTTAGG -3', which hybridized with *HSP90AA1* exons 10 and 11, respectively. Amplification conditions were those described in the first section of Materials and methods. The annealing temperature was 56°C, the extension time 30 s and MgCl₂ concentration 2 mM. All the resulting PCR fragments were purified with ExoSAP-IT (USB) and bidirectionally sequenced using the PCR primers and the BigDye Terminator V 3.1 Cycle Sequencing kit (Applied Biosystems). The identity of genes was confirmed by BLAST comparison with the GenBank database.

The normalization factor (NF; Vandesompele et al. 2002) was calculated as the geometric mean of the quantity (Q) of the two housekeeping genes of each tissue (*SDHA* and *GAPDH* for spleen and *G6PDH* and *18S* mRNA for cerebellum). The normalized expression level of *HSP90AA1* mRNA (n *HSP90AA1*) was calculated as the ratio between the Q values of *HSP90AA1* and the NF calculated for each sample. Later, this ratio (Q *HSP90AA1*/NF) was transformed according to the expression arc sin $\sqrt{(n$ *HSP90AA1* $\times 100^{-1})$ as recommended for parametric tests on relative data. Finally, a t test was run to compare the means of the two samples. The test was constructed to determine whether the differences between the means of expression data from naturally infected and control animals were equal to zero.

Polymorphism detection

The cDNA from the 13 Rasa Aragonesa breed sheep with the same *PRNP* genotype (*ARQ/ARQ*) used in the expression analysis was used in an attempt to identify polymorphism in the coding region of *HSP90AA1* gene that might cause an amino acid change. In addition, genomic DNA from 80 *ARQ/ARQ*, Rasa Aragonesa sheep (see association analysis section and Tables 1, 2, 3) was examined and used to perform association analysis. Thirty-one of them (infected) exhibited clinical and immunohistochemical signs of SC while the remaining forty-nine (healthy) did not. All these animals belong to six flocks of sheep, conserved for research purposes by the Prion Research Centre of the University of Zaragoza and where several SC cases have appeared in the last 4 years. *PRNP* genotypes and SC diagnosis were assessed as explained before (Acin

Table 1 Genotypic frequencies and confidence interval for the SNPs in the 5' flanking region of the *HSPCAAI* gene in animals of several sheep breeds

Position	Genotype	Whole (n=67)	Rasa Aragonesa frequency (%) Healthy (n=40)	Disease (n=27)	Manchega and Assaf frequency (%; n=38)
-660	CC	59.4 (47.3–71.4)	76.3 (62.8–89.8)	34.6 (16.3–52.9)	50.0 (34.1–65.9)
	CG	35.9 (24.2–47.7)	21.1 (8.1–34.0)	57.7 (38.7–76.7)	34.2 (19.1–49.3)
	GG	4.7*	2.6*	7.7*	15.8 (4.2–27.4)
-528	AA	59.4 (47.3–71.4)	76.3 (62.8–89.8)	34.6 (16.3–52.9)	60.5 (45.0–76.0)
	AG	35.9 (24.2–47.7)	21.1 (8.1–34.0)	57.7 (38.7–76.7)	31.6 (16.8–46.3)
	GG	4.7*	2.6*	7.7*	7.9*
-524	TT	75.0 (64.4–85.6)	71.0 (56.6–85.4)	80.8 (65.6–95.9)	68.4 (53.6–83.2)
	GT	25.0 (14.4–35.6)	29.0 (14.5–43.3)	19.2 (4.1–34.4)	26.3 (12.3–40.3)
	GG	0.0	0.0	0.0	5.3*

Confidence interval at 95% in brackets

*Frequency estimates no different from zero.

et al. 2004; Bolea et al. 2005). To determine if the SNP's genotype distribution found in this group of animals was representative of other breeds or exclusive of the Rasa Aragonesa group, an additional genotyping of 38 *ARQ/ARQ* sheep belonging to the Manchega and Assaf breeds, which had no contact with SC, was carried out.

Association analysis

It is well established that there are significant differences among breeds in which *PRNP* genotypes are attacked by SC. For instance, the *ARQ/ARQ* genotype shows different resistance to SC infection depending on the breed studied (Baylis et al. 2004). Nevertheless, there is no evidence of any correlation between the occurrence of a specific *ARQ* haplotype, considering all the SNPs between codons 112 and 241 of the *PRNP*, and the SC disease status of a flock (Goldmann et al. 2005).

HSP90AA1 polymorphism was studied on a total of 80 Rasa Aragonesa breed sheep displaying the same *PRNP* genotype (*ARQ/ARQ*).

Since one of the major sources of variability in SC resistance/susceptibility and/or incubation period associated with Hsp90 α might be explained by differences of expression of this chaperone, we decided to look for polymorphism in the 5' and 3' flanking regions of *HSP90AA1* gene. There were 67 sheep (27 infected and 40 healthy) sequenced to study the 5' flanking region (13 sheep from the 80 initial sample were not available in this case). To analyze the 3' flanking region, 80 animals (31 infected and 49 healthy) were studied. Thus, genomic DNA from these sheep was amplified, sequenced, and analyzed as previously described. The primer pairs used hybridized with regions located at -700 and +400 bp from the transcription start site and in exons 10 and 11.

Finally, association analysis was performed with the CATMOD procedure of the SAS statistical package (SAS 1989), which is a procedure for categorical data modeling. In this case, the standard response function (generalized logits) was used because there were only two response levels (infected vs healthy). The Maximum Likelihood was the estimation method used. Analysis took into account response

Table 2 Genotypes detected for the SNPs at positions -660, -528 and -524 in the 5' flanking region of the *HSP90AA1* gene

Genotype LYF SIF SP1 ^a	Number of animals	Number of healthy animals	Number of infected animals	Probability of healthy animals	Probability of infected animals
CC AA GG ^b	2	2	0	0.050	0.000
CC AA GT	11	10	1	0.250	0.037
CC AA TT	27	19	8	0.475	0.296
CG AA TT ^b	1	0	1	0.000	0.037
CG AG GT ^b	5	1	4	0.025	0.149
CG AG TT	18	7	11	0.175	0.407
GG GG TT ^b	3	1	2	0.025	0.074
	67	40	27		

The number of healthy and infected Rasa Aragonesa breed sheep and the probability of developing the disease based on the genotype are shown. $\chi^2 = 15.9452$ for the contingency table and p value = 0.014 ($p < 0.05$)

^a Putative regulatory motif that could be affected by the SNP

^b The estimated probabilities for these genotypes should be cautiously considered due to the small number of animals found in the analyzed sample.

Table 3 Genotypes detected for the SNPs at positions 40, 165, 178, 205, 220, and 239 of the ovine *HSP90AA1* intron 10

Genotype	<i>n</i> ^o animals	<i>n</i> ^o healthy animals	<i>n</i> ^o infected animals	Probability of healthy	Probability of infected
GG CC GG CC TT TT	75	49	26	1.000	0.839
AG CT CG CT CT CT	5	0	5	0.000	0.161
	80	49	31		

The number of healthy and infected Rasa Aragonesa breed sheep and the probability of developing the disease based on the genotype are shown. $\chi^2=8.4301$ for the contingency table and p value=0.004 ($p<0.005$)

to SC (presence/absence of clinical signs and positive immunohistochemistry for PrP^{Sc}) as the dependent variable and the genotypes for the SNPs tested as the independent factor.

Results

Isolation of the ovine *HSP90AA1* gene and polymorphism detection

Two cDNA fragments corresponding to exons 2 to 5 (921 bp) and exons 7 to 11 (1,123 bp) (Genbank accession number EF091713), and a sequence of 5,917 bp containing the complete coding sequence, interrupted by 10 introns, and 1,000 bp of the 5' flanking region of *HSP90AA1* gene DQ983231 were isolated.

Exons were identified by comparison with the ovine and bovine mRNA sequences (EF091713 and AB072368.1, respectively). Each *HSP90AA1* intron showed the GT/AG consensus splice junction described by Breathnach and Chambon 1981. Thus, the whole *HSP90AA1* ovine gene comprises 11 exons and 10 introns. The precise length and full sequence of each one were determined (GenBank accession no DQ983231).

In addition to the coding region of the *HSP90AA1* gene, a sequence of more than 1,000 bp corresponding to the 5' flanking region of the gene was analyzed. Several consensus sequences typical of promoter regions including a putative TATA box and several SP1 consensus sites (Dale et al. 1996) were identified in this 5' flanking region. More interesting is the presence of several heat shock elements (HSEs), which are binding sites for the heat shock factor (HSF-1) involved in the inducible gene expression of hsp90 α . The human *HSP90AA1* core promoter described by Zhang et al. (1999) is also conserved in sheep. Additional *cis*-regulatory elements were identified with the informatics programs TFSEARCH and Signal Scan (<http://www.cbrc.jp/research/db/TFSEARCH.html> and <http://bimas.dcrct.nih.gov/molbio/signal/>, respectively), which predicted several putative binding sites for various *trans*-acting factors, the most important ones being Lyf-1, CdxA, SIF, and of course, SP-1, TATA binding protein and HSF-1.

There were 34 polymorphisms identified within the isolated fragments: 12 in the coding region (exons 4, 7, 8, 9, 10 and 11), 14 in the 5' flanking region, and 8 in the *HSP90AA1* intron 10 (see Fig. 1 and DQ983231). None of the SNPs located in the *HSP90AA1* coding region produced any change in the amino acid sequence. The SNP (A/G) located at position 84 in the ovine *HSP90AA1* intron 10 was further analyzed within the IMF families for linkage mapping.

Chromosomal localization of ovine *HSP90AA1* gene

The ovine *HSP90AA1* gene was mapped to OAR18q24dist by FISH using the ovine BAC library (Vaiman et al. 1999) at INRA-CRJ (Jouy-en-Josas, France). These results were confirmed by linkage mapping using the IMF pedigrees. The best supported linkage map of the relevant region of chromosome 18 including the *HSP90AA1* gene is shown in Fig. 2 and was obtained by CRI-MAP multipoint analysis, after performing the two-point and flips options. *HSP90AA1* was located at the end of ovine chromosome 18, next to VNTR16.

Gene expression analyses

The expression of Hsp90 α was studied by real-time RT-PCR in spleen and cerebellum tissues. Standard curves for the genes studied present the appropriate slopes and correlation values (slope>-4, $r^2=0.99$). No significant differences in the expression of *HSP90AA1* between

Fig. 1 Polymorphisms and *cis*-acting elements localized within ovine *HSP90AA1* 5' (a and b) and intron 10 (c and d) flanking regions. **a** Sequence amplified with -700 and +400 primers. Here are represented the polymorphisms found within this region and putative binding sites for Lyf-1 (-667 to -657), and CdxA (-450 to -444) factors predicted with the TFSEARCH program. *Cis*-acting elements previously described for the *HSP90AA1* murine gene (Dale et al. 1996) are also indicated: SP-1 sites (-525 to -520 and -256 to -251), HSE (-82 to -62) and the TATA box (-29 to -23). The binding sites for ZBP-89 (-667 to -660) and SIF (-531 to -526) factors described in the promoters of other genes are also shown (Wagner et al. 1990; Zhang et al. 2003). Exon 1 is shown in **bold capital letters**. **b** Summary of the most interesting polymorphism found within the *HSP90AA1* 5' flanking region. Their location, the *trans*-acting factor that binds to them and the sequence of the *cis*-element are shown. **c** Intron 10 is represented. **d** Summary of the most interesting polymorphism found within *HSP90AA1* intron 10

a

-719 accgctgccctggcaaagagatgcgccctagaccctctaatacgctgcccgga (c) (c) cccccc/gaaaccctaacc
 -647 atggacctgagcctgcgatcaatgcccgaagcatataacctgtgacaa/cgggactggaccctcaccggtttgatcc
 -569 cctaagcgcggcctcaaggctcgaaacctagccccaggccca/gtagg/tta/gggggg (g) tcccgatgctgggggat
 -498 cgggagcgcacactatctggggacacgcttg/taaccaccgagagtttacttttcta/ggacttcccagagctgctggca
 -422 cgggagcgcacactatctggggacacgcttg/taaccaccgagagtttacttttcta/ggacttcccagagctgctggca
 -342 ggccagcgggtgtgaggcggcgggggcgacagaggtttgggtcgggggagcgtaggcccgcgcgccaagcggggttggcg
 -268 gaaggcgcgagacccgc/gcggaaacgctccggatagcgcaggcgcactgcggcgggcgcgggcacgcttggcacgcaggc
 -190 gccctgaggcgtgggagcgcgcggcgggccccgaggagggggcgggcggtccagtgcgcaggcgcaggcgcgcggcc
 -110 gctccggcggttggggaggggttcttcggaaggttcgggagggcttctggaaaaggcgcgcgcgctgggcgggcccgct
 -30 ctatataaggcgcgcgggggcgggcgcgcc**CAGTTGCCTCTGCGTCCCGGTGCTGCGTTCGGGAGCCCGTGCTAGTC**
 +51 **CTTCAGCCAAG**gtgaggggagggcggttgtagggggacgcggggatccacgggcgggtgggcggcgggcgagg

b

Position	Polymorphism	Cis-Factor	Sequence
-667	(C) and/or (CC)	Lyf-1 or	gga(c)(c)cccccc/gaaa
-660	C/G	ZBP-89	
-528	A/G	SIF	ccca/gta
-524	G/T	SP-1	gg/tca/ggg
-522	A/G		
-444	A/G	CdxA	ttttcta/g
-252	C/G	SP-1	ccgc/gc

c

1 gtaagccttgtgcaagtaccatgtgtaataccattgggga/ga/gaaacctaacagatgactgttgaaaaggggtgaact
 77 ttcagcaa/gtttcaacaaagttgggcactcttgtttttccatttgcaaaacactgaagtacatgagttaaaaggggt
 155 aaataagccc/ttttcgtcatgtc/gtttctaattcgctcagccttgagaagc/ggggtatgaggaacc/tgtagt
 227 ttggacc/taattac/tgcggcttgaagaaacttaatccgtgttttttatttccttcaag

d

	Position	Polymorphism
Intron 10	40	A/G
	165	C/T
	178	C/G
	205	C/G
	220	C/T
	239	C/T

Fig. 2 Cytogenetic (left) and genetic (right) maps showing *HSP90AA1* gene localization on sheep chromosome 18

affected and healthy animals were found either in spleen or in cerebellum ($p > 0.05$).

Polymorphism detection and association analysis

Considering the many pathways in which this chaperone is involved, it is possible that a polymorphism affecting *HSP90AA1* expression could explain part of the inter-individual response to SC that is not related with the *PRNP* genotype.

The polymorphism at 5' flanking region and intron 10 of *HSP90AA1* was studied on animals belonging to Rasa Aragonesa ($n=80$) and Manchega and Assaf ($n=38$) breeds sheep, displaying the same *PRNP* genotype (*ARQ/ARQ*).

5' flanking region Fourteen polymorphisms were detected in the 5' flanking region. As previously mentioned, part of these polymorphisms were located within possible *trans*-acting factors binding sites. For instance, the C/G SNP at -660 is in a putative Lyf-1 site, the -528 and -524 A/G and G/T substitutions are present in SIF and SP-1 consensus sites, respectively, and an additional A/G polymorphism was found at -444 affecting a CdxA site. These represent a small sample of the putative *cis*-regulatory elements identified within this region. The 14 polymorphisms identified in the *HSP90AA1* 5' flanking region are represented in Fig. 1a and b. After discarding the SNPs presenting extreme allelic frequencies, only the genotype of the three SNPs located at positions -660, -528, and -524 were analyzed. Table 1 showed genotypic frequencies and its confidence intervals (95%) for the SNPs

identified at positions -660, -528 and -524 in Rasa Aragonesa healthy and disease animals and in animals of Manchega and Assaf breeds. Since samples had enough size ($n > 30$), we can assume that binomial proportions trend to normal distributions, and therefore, confidence intervals for the frequencies can be calculated. In those cases in which proportions were no different from zero, confidence intervals could not be calculated. Similar genotypic frequencies in Rasa Aragonesa, Manchega, and Assaf breeds were observed for the three SNPs studied (Table 1). However, between healthy and disease animals of Rasa Aragonesa breed clear differences between genotypic frequencies at positions -660 and -528 were detected. Thus, the CCAA linked genotype at these positions, was more frequent in the healthy than in the disease group. Conversely, the heterozygous genotype CGAG, for these SNPs, was predominant in the disease sample. This event was independent from the genotype obtained for the SNP at -524 position. The grouped genotypes for the three SNPs at positions -660, -528, and -524 of the *HSP90AA1* 5' flanking region, in Rasa Aragonesa healthy and disease animals, are shown in Table 2. Although it is not possible to know the ancestral allele, taking into account the frequencies of genotypes found in the analyzed sample (see Table 2), the existence of the CAT, CAG, GGT, and GAT alleles could be inferred. It is worthy to mention that despite the GAT allele appeared just one within the Rasa Aragonesa sample, within the Manchega and Assaf group it appears three times, confirming the real existence of such allele. Distribution of SC cases among genotypes indicate that the SNP located at -524 has not influence in the probability to develop the disease (see Tables 1 and 2). For this reason, only the two first SNPs at the 5' flanking region (-660 and -528) were used to carry out the association analyses. In this way, maximum likelihood variance analyses revealed that predicted haplotypes for these two positions were statistically associated (p value < 0.0002) with the presence/absence of the disease. Thus, the CA allele was overrepresented in healthy sheep, whereas the GG was more common in infected animals. The GA allele was not considered due to its reduced representation in this case.

3' flanking region After discarding the SNPs presenting extreme allelic frequencies, six SNPs located at positions 40, 165, 178, 205, 220, and 239 of *HSP90AA1* intron 10, which showed a complete linkage, were analyzed. Frequencies for the homozygous genotype (GG CC GG CC TT TT) were 93.8% of Rasa Aragonesa and 94.8% of the Manchega and Assaf sheep breeds. The remaining 6.2% of Rasa Aragonesa and 5.2% of the Manchega and Assaf sheep breeds, were heterozygous with a AG CT CG CT CT CT genotype. No animal with the AA TT CC TT CC CC genotype was identified.

Maximum likelihood variance analyses of the genotypes for this six SNPs showed statistical significance (p value = 0.0022) with the presence/absence of the disease. Within Rasa Aragonesa breed, the 100% of the heterozygous sheep were infected, while only the 35% of the homozygous animals catch the disease.

Discussion

We have isolated and characterized the ovine *HSP90AA1* gene, which consists of 11 exons ranging in size from 60 to 367 bp interrupted by 10 introns ranging in size from 92 to 456 bp. This gene organization is conserved in the human (at least in the region isolated in the present work, see below), murine, and bovine homologues (Dale et al. 1996; NW_930044.1, NW_270059, and NM001012670.1). The relative position of each intron is also conserved including the position of the first intron exactly at the boundary between exon 1 and the initiation of translation site (ATG). This unusual positioning of the first intron is a feature shared by other members of the *HSP90* gene family (Dale et al. 1996). Additionally, various regulatory regions, also present in man, mouse and cattle, have been determined.

The coding region is very similar among these three species (>90% sequence homology), except that the human sequence has three nucleotides less than the ruminant counterpart. Thus, the protein in cattle and sheep contains 733 amino acids, whereas the human homolog lacks a glutamic acid at position 266. Amino acid sequences in cattle and sheep are 100% identical. At this point, it is interesting to mention that five different transcript variants have been described for this gene in man, the larger form consisting of 854 amino acids whereas the shortest has only 413 amino acids (Chen et al. 2005). Although we could not identify the coding region for all these variants neither in cattle nor in sheep, the sequence isolated in the present work contains the variant that is expressed most ubiquitously in man (Chen et al. 2005).

The localization of the ovine *HSP90AA1* gene on OAR18q24dist, next to the marker OY5 (Fig. 2), is consistent with comparative mapping information since this gene maps to human chromosome 14 and mouse chromosome 12. Ovine chromosome 18 has already been reported as containing a QTL region involved in incubation period to SC (Moreno et al. 2003a) and it is interesting to note that the *HSP90AA1* gene is located within the proposed interval. This result supports the hypothesis that *HSP90AA1* may be a positional candidate gene modulating the response to SC infection in sheep.

Expression results revealed no differences in *HSP90AA1* mRNA concentration between the two groups of sheep (infected and uninfected) analyzed. This suggests that SC

infection is not the cause of any change at the *HSP90AA1* mRNA level. However, there are several things that should be taken into account to avoid inferring erroneous conclusions: (1) the number of tissues analyzed (spleen and cerebellum) was limited. It is possible that *HSP90AA1* mRNA varies in other regions not studied in the present work. (2) the existence of transcript variants that could not be detected with the primers used (they hybridise with exons 10 and 11), (3) Jacquier-Sarlin et al. (1995) have suggested that under conditions associated with sustained Hsps expression such as inflammation or ischemia, Hsp synthesis results from a posttranscriptional regulatory mechanism. Thus, the regulation of *HSP90AA1* during SC could occur at the translational level, and the increase in Hsp90 α synthesis might be via the activation of transcription of stored mRNA. This is conceivable since the *HSP90AA1* mRNA level decreases by 20 and 10%, in spleen and cerebellum, respectively, in SC infected sheep as compared with controls (data not shown).

Although it is real that Hsp90 α does not change during SC infection, we suggest that any cause leading to an up or down regulation of cytosolic Hsp90 could have important consequences along the SC neurodegeneration process. Genetic polymorphism is an acknowledged source of phenotypic variation. Considering that Hsp90 α is the inducible form of cytoplasmic Hsp90s, any polymorphism at the *HSP90AA1* locus modifying the activity of Hsp90 α could affect the neurodegeneration process in SC infected animals. This is why we decided to study the polymorphism in the regulatory regions of these genes among sheep with the same *PRNP* genotype (*ARQ/ARQ*), which, although exposed to SC infection, present different responses to it (infected vs healthy).

Among the 34 polymorphisms identified within the isolated fragments, 3 SNPs in the 5' flanking region (positions: -660, -528, -524) and 6 SNPs in the 3' end (positions: 40, 165, 178, 205, 220, 239 of intron 10) were included in the association analyses. These analyses showed significant results for the C/G and A/G substitutions at -660 and -528 ($p < 0.001$) and for the A/G C/T C/G C/G C/T C/T linked SNPs in the *HSP90AA1* intron 10 ($p < 0.01$).

A survey of the literature and the use of the programs TFSEARCH and Signal Scan to determine if all these changes affect any regulatory element showed that the region upstream of the *HSP90AA1* minimal promoter contains apparently multiple elements controlling *HSP90AA1* gene transcription (Fig. 1a). Thus, the (C)(C) CCCCCC/gA sequence, located between -667 and -659 positions, is very similar to the consensus motif for IKAROS/LYF-1 family members described in the human B29 promoter (CCTCCCCCA) by Thompson et al. (1996). In addition, this ovine sequence is identical with a ZBP-89 binding site described in the 5' region of the vimentin gene

(GGACCCCCCCC) by Zhang et al. (2003). ZBP-89 has been reported to bind regulatory regions of various genes, and it appears to act both as transcriptional activator and repressor (Yamada et al. 2001). In addition, ZBP-89 is known to interact with SP elements and the SNPs (G/T and A/G) at locations -524 and -522 in the *HSP90AA1* 5' flanking region are both affecting an SP1 binding site described by Dale et al. (1996) in the *HSP90AA1* murine promoter. On the other hand, the SNP (A/G) at -528 affects a putative *Sis*-Inducing Factor (SIF) binding site (CCCG/aTM). The sequence of this putative regulatory element is very similar to the core sequence of the SIF binding site described by Wagner et al. (1990) in the *c-fos* promoter (CCCGTC). SIF complexes are dimerized forms of signal transducers and activators of transcription (SAS Institute Inc.) factors which, after being activated by different ligands, translocate into the nucleus to direct the transcription of specific target genes (Wang et al. 2006). Thus, mutations at these sites might yield different rates of ovine *HSP90AA1* transcription due to positive or negative interactions between different transcriptional regulatory elements. These different transcription levels might have important effects on SC incubation period.

Sequence analysis of intron 10 and exon 11 revealed eleven other polymorphisms. In this case, results from the association analysis were not as clear as those reported above because only five infected individuals presented the genotype (AG CT CG CT CT CT) for the six linked SNPs in *HSP90AA1* intron 10. Nevertheless, since it has been shown that efficiency of RNA 3'-end formation is correlated with the efficiency of the final intron removal, and that the influence of the 3'-terminal intron on 3'-end formation can be attributable to the determinants of splicing efficiency (Nesic and Maquat 1994), these SNPs are not less interesting than those described in the 5' flanking region. Thus, some of the mutations found within the ovine *HSP90AA1* intron 10 may interfere with these post-transcriptional regulatory steps affecting the level of *HSP90AA1* expression. To sum up, individuals capable of producing more Hsp90 α under different conditions would have a minor proteasomal activity inhibition when they are submitted to SC infection. As a consequence, in these individuals, cytoplasmic PrP^C accumulation would be lower, decreasing the conversion and aggregation process and extending the incubation period.

Although additional experiments should be performed to determine the exact implication of these polymorphisms in SC development, it can be concluded that *HSP90AA1* is a good positional and functional candidate gene modulating SC incubation period in sheep. Thus, the GG and ATCTCC alleles, for positions described at 5' flanking region and intron 10 are associated with higher susceptibility to SC. In a new project, which we have just started with the aim of

studying in depth the *HSP90AA1* polymorphism, we have found the third genotype for the intron 10 region (AA TT CC TT CC CC). Additionally, it seems that some of the polymorphisms are differentially distributed within different breeds. Nevertheless, further analyses should be performed to see if there are any differences in Hsp90 α expression that could be explained by the polymorphisms described in the present work, and to test if combinations of the alleles described for the *HSP90AA1* 5' flanking region and intron 10 can interact to modulate the response to SC in sheep.

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