Research article

Genomic organization and the tissue distribution of alternatively spliced isoforms of the mouse Spatial gene

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Abstract

Background: The stromal component of the thymic microenvironment is critical for T lymphocyte generation. Thymocyte differentiation involves a cascade of coordinated stromal genes controlling thymocyte survival, lineage commitment and selection. The "Stromal Protein Associated with Thymus And Lymph-node" (Spatial) gene encodes a putative transcription factor which may be involved in T-cell development. In the testis, the Spatial gene is also expressed by round spermatids during spermatogenesis.

Results: The Spatial gene maps to the B3-B4 region of murine chromosome 10 corresponding to the human syntenic region 10q22.1. The mouse Spatial genomic DNA is organised into 10 exons and is alternatively spliced to generate two short isoforms (Spatial-α and -γ) and two other long isoforms (Spatial-δ and -ε) comprising 5 additional exons on the 3’ site. Here, we report the cloning of a new short isoform, Spatial-β, which differs from other isoforms by an additional alternative exon of 69 bases. This new exon encodes an interesting proline-rich signature that could confer to the 34 kDa Spatial-β protein a particular function. By quantitative TaqMan RT-PCR, we have shown that the short isoforms are highly expressed in the thymus while the long isoforms are highly expressed in the testis. We further examined the inter-species conservation of Spatial between several mammals and identified that the protein which is rich in proline and positive amino acids, is highly conserved.

Conclusions: The Spatial gene generates at least five alternative spliced variants: three short isoforms (Spatial-α, -β and -γ) highly expressed in the thymus and two long isoforms (Spatial-δ and -ε) highly expressed in the testis. These alternative spliced variants could have a tissue specific function.

Background

The immune system is composed of many interdependent cell types that collectively protect the body. The hematopoietic precursors leave the bone marrow and migrate into the thymus, primary site of T cells development, providing a unique microenvironment that efficiently
generates functional T lymphocytes. This maturation requires the interaction of immature thymocytes with the thymic stroma. The lympho-epithelial interaction delivers signals necessary for survival, proliferation, differentiation and selection of developing thymocytes [1]. After a remarkable maturation process, mature T cells are then released into the bloodstream and migrate to secondary lymphoid organs, such as the spleen and lymph nodes.

Although the T cell differentiation process is well documented, little is known about the underlying stromal molecules involved in the lympho-epithelial interaction. In order to identify new stromal genes potentially involved in these mechanisms, we developed a systematic approach of gene expression profiling to evaluate, in comparison to the wild-type littermate, the gene expression of several thymus deficient mice showing a blockage at different stages of T cell development [2-4]. Among the identified stromal genes, we selected a gene, dependent on the three-dimensional organization of the thymus, named Spatial for “Stromal Protein Associated with Thymic And Lymph-node” [5]. RT-PCR analysis on 48 distinct mouse tissues showed that Spatial is highly expressed in the thymus and testis. In the testis, in situ hybridization experiments showed that Spatial expression is tightly regulated and restricted to step 2–10 in haploid round spermatids during spermiogenesis [6].

Here, we describe the genomic organization of the Spatial gene and subsequently identify a new alternatively spliced isoform expressed in the thymus. In addition, we precisely evaluate the tissue distribution of the alternative spliced variants of Spatial gene by quantitative TaqMan RT-PCR.

Results and discussion
Chromosomal localization
We determined the chromosomal localization of Spatial gene by fluorescence in situ hybridization using a 40 kb cosmid isolated from the rzpd genomic library. 98% of 30 analysed metaphase cells showed specific fluorescent spots on the B3-B4 region of murine chromosome 10 (Fig. 1A). This single signal strongly suggests that Spatial does not belong to a gene family. This region contains the Sim1 [7], Zfa [8], Hsf2 [9] and Edar [10] genes in the B3 band and Eif4ebp2 [11], Sgpl1 [12], Ddx21 [13] and Gp49b [14] genes in the B4 band (Fig. 1B). Furthermore, the human synthetic B3-B4 region is divided into two regions located on chromosome 6 and 10 where SIM1 and HSF2 genes are located on chromosome 6 and the SGPL1, DDX21 and EIF4EBP2 genes were mapped on chromosome 10. This observation suggests that these genes are respectively located above and below the mouse translocation points T(2; 10) and T(10; 18). In parallel, by BLAST analysis, we identified a chromosome 10q22.1 human contig which displays similarities to Spatial gene (Genbank Accession No NT_008583) where no pathologies were described in this region until now. All together, these data show that Spatial is located on the mouse chromosome 10-B4 band between Ddx21 and Eif4ebp2 (Fig. 1C).

Identification of a new alternative spliced variant of Spatial
In order to study the stromal molecular mechanisms involved in T cell development, we isolated new genes, by quantitative differential screening using array technology, between wild-type and several knock-out mice models displaying a stromal disorganization. We screened from an adult mouse thymus library (Mouse Thymus Adult, MTA) three Spatial cDNA clones: MTA.H09.096, MTA.C03.094 and MTA.F04.072 (GenBank Accession No. W91576, W91584, AY243458 respectively). In addition, by screening a 15 days embryonic thymus mouse library (Mouse Embryonic Thymus, MFT, library accessible in the RZPD resource center), another Spatial cDNA clone was isolated: MFT.G17.012 (GenBank Accession No. AY243459).

The sequencing of these clones showed that MTA.C03.094 is similar to MTA.F04.072 while MTA.H09.096 and MTA.F04.072 differ by a 102 bases additional exon, corresponding to two adult variants already described in the thymus (GenBank Accession No. AF257502 and AF257503) [5]. In addition, sequence analysis of the MFT.G17.012 showed that it contains an additional exon of 69 bases. We confirmed the presence of this new exon by performing RT-PCR analysis on total RNA extract from adult mouse thymus, using specific primers for this new additional exon (Fig. 2A). Thereafter, we cloned and fully sequenced this third isoform reporting the expression of this new Spatial splicing variant in the thymus (GenBank Accession No. AY243457). According to the size of alternative spliced variants, the longest and the shortest isoforms were respectively named Spatial-α (1035 bp) and Spatial-γ (933 bp) and the third new mid-sized isoform Spatial-β (1002 bp) (Fig. 2A). In addition, we already described two other isoforms in the testis differing by the same 102 bases alternative exon mentioned above: Spatial-ε (1454 bp) for the longest (GenBank Accession No. AF521592) and Spatial-δ (1353 bp) for the shortest (GenBank Accession No. AF521591) [6]. In order to study the protein product of Spatial, we have developed a polyclonal antibody to be able to recognize all isoforms. As shown in figure 2B, this antibody reacts with the three protein products of thymic isoforms on the nuclear extract. The apparent molecular masses of the upper and the lowest bands correspond to Spatial-α and Spatial-γ at 38 and 32 kDa respectively [5]. In the cytosolic fraction, we can detect Spatial-α which is probably not yet translocated into the nucleus. On the basis of the size of Spatial isoforms, we have deduced that the third band at 34 kDa probably corresponds to the newly described Spatial-β.
suggesting that this isoform would generate a protein in the thymus.

**Organization of Spatial alternative spliced variants**

We determined by analyzing the mouse genomic contig (Mus musculus, Genbank Accession No, NT_039495) that Spatial is composed of 10 exons (Fig. 3A). Four exons of Spatial are present in Spatial-α, -β, -γ isoforms (exons 2, 3, 4 and 5) and 9 exons in Spatial-δ, -ε isoforms (exons 1, 3, 4, 5, 6, 7, 8, 9 and 10) (Fig. 3B). Spatial-α and Spatial-ε contain an alternatively spliced internal region of 102 bases located in exon 4 and the new described Spatial-β contains an alternatively spliced internal region of 69 bases in exon 5. The exon organization of Spatial-δ and -ε shows that 5 exons are added at the 3' end compared to Spatial-α, -β, -γ. In addition, the start and the stop codons are respectively localized in exon 2 and exon 5 for Spatial-α, -β, -γ while they are localized in exon 1 and exon 10 for Spatial-δ, -ε. We classified Spatial-α, -β, -γ as short isoforms and Spatial-δ, -ε as long isoforms. The exon length and the exact position of the start and the stop of different Spatial isoforms were determined by detailed analysis of mouse chromosome 10 (Table 1A,1B) [15]. Furthermore, short isoforms contain in the 3' end of exon 5 a nuclear localization signal (NLS) allowing the transport of the protein from the cytosol to the nucleus. This observation strongly

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**Figure 1**

**Chromosomal localization.** (A) Localization of the Spatial probe on WMP murine metaphase: R-banded chromosomes are red-stained with propidium iodide. The FITC fluorescent signals (green), corresponding to Spatial gene, are located on chromosome 10 and belong to the robertsonian translocation Rb(10; 17). (B) On the left, the map of mouse chromosome 10 zooming on B3-B4 bands where are localized 2 translocation points, and on the right, 2 human syntenic regions located on Ch6 and Ch10. (C) The repartition of genes within B3 to B5.3 bands on mouse chromosome 10.
suggests that long and short isoforms could have different subcellular localizations which potentially leads to different functions.

**Tissue distribution of mouse Spatial isoforms**

Previous RT-PCR analysis on a large panel of mouse tissues showed that *Spatial* is highly expressed in the thymus and testis, but also to a lesser extent in brain cortex, cerebellum and hippocampus [6]. In order to precisely decipher the tissue distribution of short and long *Spatial* isoforms, quantitative TaqMan RT-PCR was performed on the thymus, testis as well as the kidney as a negative control. The expression of *Spatial* isoforms in different adult tissues was measured relatively to the expression of the housekeeping 18S-rRNA gene.

This analysis shows a residual expression of $1.36 \times 10^{-4}$ RQ value in the kidney for the two isoform types confirming a null expression, while short and long *Spatial* isoforms were differentially expressed in the thymus and the testis. Short isoforms are highly expressed in the thymus whereas long isoforms are highly expressed in the testis. Short isoforms are expressed in the thymus 85-fold higher than the testis and an expression of $1.18 \times 10^{-2}$ RQ value is observed in testis signing a basal expression (Fig. 4A). Long isoforms are expressed in the testis 25-fold higher than the thymus and in this case the basal expression in the thymus is $3.9 \times 10^{-2}$ RQ value (Fig. 4B).

This study reveals a mirror image of short and long isoforms expression in these organs. Actually, the basal expression detected by RT-PCR experiments which is very sensitive and known to amplify very low expression in classical conditions (30 cycles) can lead to an overestimation of the expression level analysis. Indeed, in the thymus only the short isoforms are translated and give rise to three corresponding proteins (see above). In addition, previous analyses on thymus sections have shown that *Spatial* gene is expressed by subcapsular stromal cells which are resident cells known to be involved in thymocytes development [16]. In testis, the *Spatial* gene is expressed in round spermatids which are migrating cells representing a cell stage prior to spermatozoa maturation [17]. In conclusion, short isoforms are highly expressed in the thymus and at a basal-rate in testis whereas long isoforms are highly expressed in the testis with a basal-rate in thymus. Major isoforms type are expressed in specific cell types suggesting a tissue specific function.

**Comparison of the mouse Spatial ortholog protein sequences**

We further examined the inter-species homologies by performing a BLAST search of the Expressed Sequence Tag (EST) database with *Spatial* mouse cDNA and identified several mammalian ESTs of pig (*Sus scrofa*, GenBank Accession No. BM484264) and bovine (*Bos taurus*, GenBank Accession No. BF077282) in addition to rat (GenBank Accession No. XM_228291) and human (GenBank Accession No. AK057382) [18,19]. The analysis of the rat EST showed that it corresponds to the mouse *Spatial*-ε isoform. In addition, bioinformatic studies of the rat contig (*Rattus norvegicus*, Genbank Accession No NW_043448) showed that the corresponding EST, in
comparison to the mouse Spatial-ε isoform, contains an additional exon in the 5’ end separated from the second exon by an intron of about 8 kb. Therefore, we performed an RT-PCR analysis with primers specific for the first predicted exon in the rat and deduced that this exon does not exist (data not shown). This result indicates that the Spatial-ε mRNA contains 9 exons in rat as in mouse, while it is composed of 11 exons in human. The alignment of the deduced amino acid sequences is shown in figure 5 [see Additional file 1]. As a reference, we have chosen the mouse Spatial-α protein, that shows 90% identity with other mouse Spatial isoforms and 55% of inter-species homology. However, we did not find any EST from widely sequenced non-mammalian eukaryotes such as Drosophila melanogaster, Caenorhabditis elegans and Saccharomyces cerevisae.
Bioinformatic analyses have predicted that the molecular weights of Spatial-α, -β and -γ are 25.8, 24.6 and 22.2 kDa respectively and present an isoelectric point of 10. The apparent mobility of the in vivo proteins (38, 34 and 32 kDa) is greater than the predicted mass based on the cDNA sequence (Fig 2B), this could be explained by a post-translational modifications of these proteins. In addition short isoforms where the NLS sequence is conserved, are detected in the nuclear fraction of the thymus and present proline-rich domains (10%) as there are two other positive amino acid rich domains (arginine, lysine, histidine) which seem to exhibit homology with zinc-finger proteins known to be involved in protein-DNA interaction [20], but the C2H2 consensus motif is not conserved in Spatial proteins. In addition, the 23 amino acid alternative exon (residues 190–212), only found in Spatial-β, presents an interesting proline-rich signature (residues 196–208) that could confer to Spatial-β a particular function. Moreover, these short isoforms possess a secondary structure with 72% of random coil, suggesting an unstable structure. So, these nuclear proteins may have to interact with DNA and other cofactors in order to stabilise their conformations. Concerning long isoforms, Spatial-δ and -ε are encoded with a 40.2 and 43.8 kDa prediction molecular mass and present an isoelectric point of 6.5. In contrast to short isoforms, they lost the NLS sequence but gained an additional rich leucine sequence (15.5%) in the 3' end, with a high percentage of alpha-helix (44.5%) suggesting a more stable structure. Taking in consideration all these data, Spatial is a relatively well conserved protein in mammals. Long isoforms observed in human, mouse and rat display alpha-helix in the C-terminal region suggesting a stable structure of the protein. Short isoforms lack any similarity to other known domains apart the putative zinc-finger homology. However, conserving the NLS sequence, short isoforms encode nuclear factors which could play a role in transcriptional regulation cell process. Interestingly, we describe here a new short isoform Spatial-β, which has an additional

Table 1: Exon-intron organization of Spatial gene in the mouse chromosome 10. (A) The exact size as the position of the start and the end of each exon are precisely calculated for different Spatial isoforms. The exon 4 contains two alternatively spliced internal regions (4 a, b) while the exon 5 contains three alternatively spliced internal regions (5 a, b, c). (B) The exact position of the start, the stop and the polyA tail is calculated for short and long isoforms. Feature positions in bp are calculated relatively to position 63050000 of the mouse chromosome 10 (Ensembl build 32). Introns can not be determined because the genomic sequence contains stretches of nucleotides.

### A

| Exon | Start | End   | Length | Spatial-α | Spatial-β | Spatial-γ | Spatial-δ | Spatial-ε |
|------|-------|-------|--------|-----------|-----------|-----------|-----------|-----------|
| 1    | 3873  | 4027  | 155    | 4834...4975| 5030...5550| 5030...5550| 5030...5550| 5030...5550|
| 2    | 4834  | 4975  | 142    | 4834...4975| 5030...5550| 5030...5550| 5030...5550| 5030...5550|
| 3    | 5303  | 5550  | 248    | 8090...8350| 8192...8350| 8192...8350| 8192...8350| 8192...8350|
| 4 ab | 8090  | 8350  | 261    | 8090...8350| 8192...8350| 8192...8350| 8192...8350| 8192...8350|
| 4 b  | 8192  | 8350  | 159    | 8090...8350| 8192...8350| 8192...8350| 8192...8350| 8192...8350|
| 5 abc| 9093  | 9543  | 451    | 9093...9543| 9160...9543| 9160...9543| 9160...9543| 9160...9543|
| 5 b  | 9160  | 9543  | 384    | 9160...9543| 9160...9543| 9160...9543| 9160...9543| 9160...9543|
| 5 b  | 9160  | 9239  | 80     | 9160...9239| 9160...9239| 9160...9239| 9160...9239| 9160...9239|
| 6    | 12958 | 13149 | 192    | 12958...13149| 12958...13149| 12958...13149| 12958...13149| 12958...13149|
| 7    | 14960 | 15041 | 82     | 14960...15041| 14960...15041| 14960...15041| 14960...15041| 14960...15041|
| 8    | 15696 | 15837 | 142    | 15696...15837| 15696...15837| 15696...15837| 15696...15837| 15696...15837|
| 9    | 17083 | 17135 | 53     | 17083...17135| 17083...17135| 17083...17135| 17083...17135| 17083...17135|
| 10   | 18002 | 18242 | 241    | 18002...18242| 18002...18242| 18002...18242| 18002...18242| 18002...18242|

### B

|                      | Short isoforms | Long isoforms |
|----------------------|----------------|--------------|
| Start                | 4917           | 3984         |
| Stop                 | 9285           | 18079        |
| PolyA Signal         | 9499           | 18201        |
Conclusions

We report the genomic characterization of the Spatial gene, which presents three short and two long splice variants. Here, a new Spatial isoform is characterized: it contains a sequence encoding for NLS protein signal and an internal splice site producing a 69 bases exon 5 encoding a proline-rich domain. Short isoforms are highly expressed in the thymus by stromal cells which are involved in T cell development. Long isoforms are highly expressed by round spermatids in testis, and appear to be involved in a specific stage of spermatozoa differentiation. This unique gene, localized in B4 band of mouse chromosome 10, is well conserved in mammals and displays 5 different messengers generated by alternative splicing which is one of the main process for differential expression in mammals [21-23]. Future work is now required to understand Spatial specific tissues function and link alternatively spliced isoforms to specific promoter regulatory elements.

Methods

Animals

Mice C57BL/6 were maintained under specific-pathogen-free conditions and used between 4 to 8 weeks of age. All experimental and surgical procedures involving animals were approved by the veterinary office of the Ministry of agriculture, France.

RT-PCR

Total RNA was purified using TRIzol reagent (Gibco-BRL, Life Technologies). Single-strand cDNA was synthesized by reverse transcription on 0.3–5 µg of total RNA using oligo(dT)25 and SuperScript II (Gibco-BRL) in a final volume of 20 µl. PCR reaction was performed in a PTC 200 Peltier Thermal Cycler (MJ research) using 2 µl of the RT reaction product in a final volume of 50 µl, using the following conditions: 94°C for 3 min (1 cycle), 30 cycles of 94°C for 30 s, 62°C for 40 s, 72°C for 1 min, and 72°C for 10 min (1 cycle). Of the amplification product, 10 µl were resolved on a 1% agarose gel. The sequence primers used to detect specifically Spatial-β are: forward primer: 5'-CTGAAGACAGGGAGGACA-3' and reverse primer 5'-CGCTGTCACCTTCGAGGTTA-3' while Spatial-α, -γ were amplified with forward primer: 5'-AGTCCAAAGGCGACGCCACA-GAGCCCA-3' reverse primer 5'-CGCTGTCACCTTCGAGGTTA-3' and the housekeeping gene GAPDH with forward primer: 5'-AACGACCCCTTACGAC-3' and reverse primer 5'-TCCAGCAGCACTTCAGCAC-3'.
produced by immunizing two rabbits against the mouse recombinant 6 × His Spatial-α protein isoform and further purified on a protein G column (Eurogentec). Rabbit polyclonal anti-actin was used as a control charge (1:600; Santa cruz biotechnology). Proteins were visualized using horseradish peroxidase-conjugated secondary antibody (1:1000; Amersham Pharmacia Biotech) and the enhanced chemiluminescence (ECL) detection system (Pierce).

**Quantitative TaqMan RT-PCR**

Quantitative RT-PCR was performed on the thymus, testis and kidney chosen as a negative control to evaluate the distribution of the short and long Spatial isoforms by using the ABI PRISM 7000 Sequence Detection System. Random hexamers and the TaqMan reverse transcription reagents from the RT reaction mix (Applied Biosystems) were used to reverse transcribe total RNA. After, the PCR step was performed with TaqMan universal PCR master Mix and assays-on-demand gene expression probes (Applied Biosystems). Primers and the TaqMan probe used to detect specifically short isoforms were: forward primer: 5'-TTGGAACACGCCCCGTGTIT-3', reverse primer 5'-GTTCCTCGCITCCTCITC-3' and FAM 5'-CCTTGACACTGCTCCTCAT-3' NFQ, respectively. Primers and the TaqMan probe used to detect specifically long isoforms were: forward primer: 5'-GCTTCAAGAGCCTCAAGACA-3', reverse primer 5'-GGTGGTGACCTAGTCTCTTCAG-3' and FAM 5'-ACTGTAGGCTGCCTCTTG-3' NFQ, respectively. The 18S-rRNA was amplified from all samples on each plate as a housekeeping gene to normalize expression between different samples and to monitor assay reproducibility. A non-template control was included for each target analysed. Relative quantification of all targets was calculated by using the comparative cycle threshold method [24].

**Fluorescence in situ hybridization**

Metaphase spreads were prepared from a WMP female mouse, in which all the autosomes except chromosome 19 were in the form of metacentric Robertsonian translocations [25] Concanavalin A-stimulated lymphocytes were cultured at 37°C for 72 h with 5-bromodeoxyuridine added for the final 6 h of culture (60 µg/ml of medium) to ensure a chromosomal R-banding of good quality. The Spatial clone was biotinylated by random priming with biotin-14-dUTP, as outlined by the Gibco-BRL protocol (Life Technologies). Hybridization to chromosome spreads was performed with standard protocol. The biotin-labelled DNA was mixed with hybridization solution at a final concentration of 10 µg/ml and used 80 ng per slide. Before hybridization, the labelled probe was annealed with a 250-fold excess amount of murine Cot-1 DNA (Gibco-BRL) (for 45 minutes at 37°C) in order to compete the aspecific repetitive sequences. The hybridized probe was detected by means of fluorescence isothiocyanate-conjugated avidin (Vector laboratories). Chromosomes were counterstained and R-banded with propidium iodide diluted in antifade solution pH 11.0.

**Sequence analysis**

For the chromosomal localization, the genomic cosmid was isolated from rzpd genomic library http://www.rzpd.de. The human-mouse homology map was analysed with NCBI synten database http://www.ncbi.nlm.nih.gov/Homology/. The Spatial corresponding contig was downloaded from the Ensembl Genome Browser http://www.ensembl.org. BLAST searches were conducted using the BLAST server http://www.ncbi.nlm.nih.gov/BLAST. The exact size and precise position of the start and the end of each exon for different Spatial isoforms are calculated using the AAT server http://genome.cs.mtu.edu/aat/aat.html. The sequence translation, exon organization, the multiple alignment and protein signature sequences have been analysed with Biology workbench http://workbench.sdsc.edu.

**Abbreviations**

NCBI: National Centre of Biotechnology Information. RT-PCR: Reverse Transcription Polymerase Chain Reaction. BLAST: Basic Local Alignment Search Tool

**Authors’ contributions**

MI contributed to the conception, design, and coordination; conducted the expression experiments, cloning, sequencing analysis, and. wrote parts of the manuscript.

DP participated in helpful discussion concerning the conception, and coordination of this study.

SG participated in helpful discussion concerning the revision and the sequence analysis.

FL participated to the analysis of mouse ortholog of Spatial gene.

GV participated to technical advises and maintenance of the laboratory.

MGM conducted the experiment of fluorescent in situ hybridization.

CN participated in the conception, designed, and coordination of the study and edited the manuscript.

All authors read and approved the final manuscript.
Additional material

Additional File 1

Figure 5: Word: document, sequence comparison of Spatial orthologs with the mouse protein. Sequence comparison of Spatial proteins in different species: Sus scrofa, Ss; Bos Taurus, Bt; Homo sapiens, Hs; Mus musculus, Mm and Rattus norvegicus, Rn. Residues conserved with the mouse protein are shown by (*), strongly conserved residues by (•) and weakly conserved residues by (○). Residues are colour coded: basic, DE, blue; acidic, KR, pink; polar, C/V/Q/N/Y, green; and hydrophobic, AFILMPVW, red. The start and the stop of the translation for the different protein isoforms, are indicated by a red M and (•), respectively. A proline rich domain, specific of Spatial-ß isoform, is highlighted in gray. Two positive amino acid rich domains are underlined. A putative nuclear localization signal is indicated, NLS and highlighted in yellow. Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-2164-5-41-S1.doc](http://www.biomedcentral.com/content/supplementary/1471-2164-5-41-S1.doc)

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