# The conserved role of Smu1 in splicing is characterized in its mammalian temperature-sensitive mutant

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## Summary

**Temperature-sensitive CHO-K1 mutant cell line tsTM18 exhibits chromosomal instability and cell-cycle arrest at S and G2 phases with decreased DNA synthesis at the nonpermissive temperature, 39°C. We previously identified an amino acid substitution in Smu1 that underlies the temperature-sensitive phenotypes of tsTM18 cells. In the present study, we confirmed that *Smu1* is associated with the temperature-sensitive defect of tsTM18 by RNA interference. We also found an early temperature effect in DNA synthesis. Because genetic studies of nematodes revealed that *smu-1* is involved in splicing of the *unc52/perlecan* pre-mRNA, we analysed the *perlecan* transcript in tsTM18 cells by reverse transcription-polymerase chain reaction (RT-PCR). The *perlecan* PCR product amplified from RNA of tsTM18 cells cultured at 39°C appeared to be a mixture of variants. Sequence analysis identified at least six variants that result from alternative splicing and intron retention. Comparison of the results of *perlecan* RT-PCR analysis with those of analysis of four other genes suggested that the splicing defect in the *perlecan* gene is unique and that it is conserved through evolution.**

Key words: Alternative splicing, DNA synthesis, Intron retention, RNA interference, Temperature-sensitive mutation

## Introduction

To identify genes responsible for the maintenance of chromosome integrity, we previously isolated 25 temperature-sensitive (ts) CHO-K1 cell mutants exhibiting chromosome instability (Tsuji et al., 1990). One ts mutant, tsTM18, carries a mutation that interferes with maintenance of chromosome integrity and progression through the cell cycle. At the nonpermissive temperature, mutant cells exhibit accumulation of single-stranded DNA in the nucleus, abnormal chromosome configuration with incomplete assembly of the spindle, failure of proper activation of cdc2 kinase during G2, and cell-cycle arrest at S and G2 phases with decreased DNA synthesis and chromosome instability (Tsuji et al., 1990; Sugaya et al., 2005).

As part of our effort to identify the gene(s) responsible for the ts defects of tsTM18 cells, we found that the ts phenotype of tsTM18 cells was caused by a G-to-A transition at nucleotide (nt) 1465 of *Smu1* that results in a G-to-A substitution at amino acid 489 in the WD-repeat region of Smu1 (Sugaya et al., 2005). The *smu-1* gene of *C. elegans* encodes a nuclear protein that contains five WD motifs and that is expressed ubiquitously (Spike et al., 2001). Genetic studies in *C. elegans* have suggested that *smu-1* is involved in alternative splicing of *unc-52*, which is a homolog of mammalian *perlecan* (Spike et al., 2001).

Perlecan is a major heparan sulfate proteoglycan in basement membranes and connective tissues (reviewed by Iozzo et al., 1994; Jiang and Couchman, 2003). The core protein of perlecan is divided into five domains on the basis of sequence homology to other known proteins. Domain IV of perlecan is the largest domain and contains a long series of immunoglobulin (Ig)-like repeats. Complex patterns of alternative splicing of the Ig repeat region of *perlecan/unc-52* have been observed in nematodes. Alternative splicing of exons 16, 17 and 18 is regulated by the *mec-8* (Lundquist et al., 1996; Spike et al., 2002) and *smu-1* genes (Spike et al., 2001). A presumptive null mutation in *smu-1* (*smu* for suppressor of *mec-8* and *unc-52*) suppresses nonsense mutations in exon 17 but not in exon 18, indicating that SMU-1 regulates the splicing of exon 17, which is enhanced in the absence of functional SMU-1 (Spike et al., 2001).

Although Spartz and colleagues reported (Spartz et al., 2004) that human SMU1 is a component of the spliceosome fSAP57 (Zhou et al., 2002), the function of *Smu1*, especially in mammalian species, is not well understood. It is possible that the mutation in *Smu1* affects spliceosome function, resulting in generation of improper splice variants, which could explain the phenotypes of tsTM18 cells. To clarify the relation between mutation of *Smu1* and the phenotypes of tsTM18 cells, we analysed splicing of several genes in tsTM18 cells by reverse transcription-polymerase chain reaction (RT-PCR). In this study, we describe a defect in splicing of the *perlecan* transcript in tsTM18 cells and discuss the conserved role of mammalian Smu1 in spliceosomes.

We also wanted to study the dynamics of Smu1 in living cells. Our strategy was to construct a hybrid gene encoding a fluorescently tagged form of Smu1 and to use this gene to correct the genetic defect in tsTM18 cells. Previously, we

developed cells expressing functional RNA polymerase II tagged with green fluorescent protein (GFP) and used the same strategy against tsTM4 cells, which have a ts mutation in polymerase (Sugaya et al., 2000). With the use of this cell line, the dynamics of RNA polymerase II, especially the transcription cycle of polymerase in living cells, was revealed (Kimura et al., 2002). We hoped that introduction and expression of a hybrid Smu1 would enable tsTM18 cells to grow at 39°C. However, we failed several times using plasmids encoding a variety of fusion proteins. Therefore, we performed RNA interference (RNAi) knockdown experiments to confirm that the mutation in *Smu1* underlies the ts defects in tsTM18 cells.

## Results

### RNA interference knockdown experiment for Smu1

On the basis of the nucleotide (nt) sequence similarities between human *SMU1* and hamster *Smu1*, we designed three plasmid vectors expressing short interfering RNA (siRNA) for *Smu1*. Sequence 'S1' is common between human and hamster, and sequences 'S2' and 'S3' are identical to the human *SMU1* sequence but not to the hamster sequence. The locations of target sequences are shown in Fig. 1A. Previously, during experiments to isolate hamster *Smu1* as the gene underlying the defects of tsTM18 cells, we isolated transformed tsTM18 cells (e.g. 18H-1c30 and 18K-1c1 in Fig. 1) by complementation with human genomic DNA fragments (Sugaya et al., 2005). These cells can grow at 39°C because they express wild-type human SMU1. After transfection of cells with plasmid DNAs encoding siRNAs, cells were incubated at 34°C or 39°C with 5 μg ml$^{-1}$ puromycin for the appropriate number of days. Colonies on dishes were stained with methylene blue, and the mean number of colonies from triplicate dishes was calculated (Fig. 1B,C). Most cells formed colonies after transfection with a plasmid encoding siRNA against GFP (control). Cells survived at 34°C after transfection of plasmids encoding siRNAs S2 or S3, confirming that hamster *Smu1*, which includes the ts mutation, is functional at the permissive temperature. However, few cells grew at 39°C and 34°C after transfection of siRNA S1 (Fig. 1B). These results are consistent with the sequence similarities between target molecules (i.e. human *SMU1* and hamster *Smu1*) and siRNAs (Fig. 1C). These RNAi knockdown experiments confirmed that the mutation in *Smu1* underlies the phenotype of tsTM18 cells.

### Early temperature effect in DNA synthesis in tsTM18 cells

Previously, we observed a decrease in DNA synthesis activity of tsTM18 cells after at least 10 hours of incubation at 39°C (Tsuji et al., 1990). The tsTM18 cells also display chromosome instability, including sister chromatid exchange

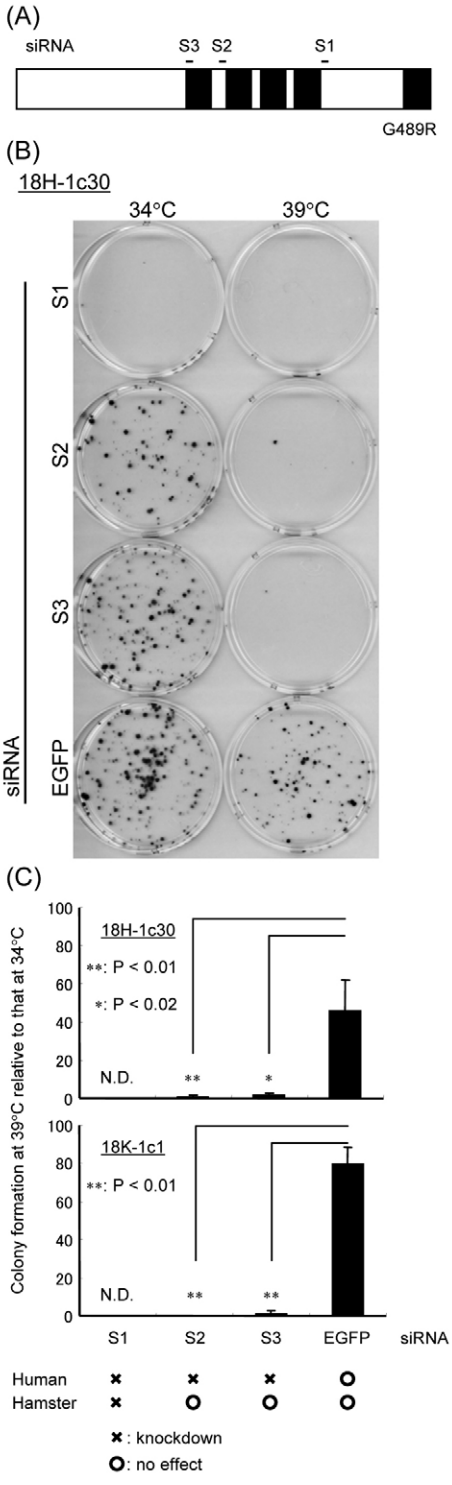


**Fig. 1.** Effect of expression of siRNA against *SMU1/Smu1* on colony formation. (A) Diagram of the structures of mammalian Smu1. Black boxes, WD-repeat motifs. Locations of the sequence corresponding to siRNAs are indicated as S1, S2 and S3 above the diagram. An amino acid substitution found in the last WD-repeat of Smu1 of tsTM18 is indicated as G498R. (B) A photograph of colonies of 18H-1c30 cells after transfection of plasmid DNAs encoding siRNAs. Colonies on dishes after 12 days of incubation at 34°C or 39°C with 5 μg ml$^{-1}$ puromycin were stained with methylene blue. Cells survived at 34°C after transfection of S2 or S3; however, few cells grew at 39°C or at 34°C after transfection of S1. Most cells formed colonies after transfection of an EGFP plasmid (control). (C) Colony formation at 39°C relative to that at 34°C by 18H-1c30 (upper) and 18K-1c1 (lower) cells. The mean and s.d. of six independent experiments are indicated as a solid bar and line, respectively. *P* values were calculated by Student's *t*-test. Significant differences in colony formation were found between EGFP and S2 and S3.

and chromosomal aberrations, in which most aberrations are chromatid-type (Tsuji et al., 1990). These findings suggest that the mutant phenotype is associated with DNA replication. After 24 hours of incubation at 39ºC, accumulation of single-stranded DNA was observed in nuclei of mutant cells (Sugaya et al., 2005), suggesting that the *Smu1* mutation may be related to generation of single-stranded DNA. It is possible that the mutation in *Smu1* causes a defect in DNA replication that in turn leads to generation of single-stranded DNA, resulting in chromosome instability and cell-cycle arrest.

Time course analyses of DNA synthesis showed that [$^3$H]thymidine incorporation in both wild-type (CHO-K1) and mutant (tsTM18) cells increased gradually from 20 minutes to 60 minutes (Fig. 2A,B). The rate of DNA synthesis in CHO-K1 cells at 39°C was 1.34 times faster than that at 34°C (Fig. 2A). However, the rate of DNA synthesis in tsTM18 cells after 4 hours of incubation at 39°C was 77% lower than that at 34°C, although the DNA synthesis activity at 34°C was similar to that of CHO-K1 cells (Fig. 2B). These results suggest that an early effect of the temperature shift is on DNA synthesis in tsTM18 cells. Incubation times of less than 4 hours at 39°C were not sufficient to detect the effect of temperature on DNA synthesis (data not shown).

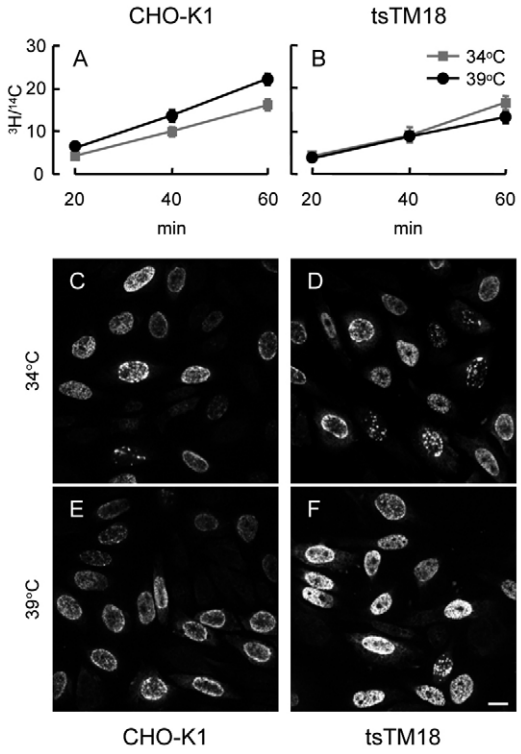


**Fig. 2.** Effects of incubation for 4 hours at 39°C on DNA synthesis. (A,B) DNA synthesis activity of CHO-K1 and tsTM18 cells were measured by [$^3$H]thymidine incorporation as described in the Materials and methods. The average and standard deviation of at least three independent experiments are indicated. Incorporation rate at 39°C relative to that at 34°C of replication was 1.34 for CHO-K1 and 0.77 for tsTM18 cells, respectively. The temperature shift appears to reduce DNA synthesis in tsTM18 cells. (C-F) Images of replication were obtained as described in the Materials and methods. Single equatorial sections were collected with a confocal microscope. Scale bar, 10 μm.

We next examined the distributions of nascent DNA at 34°C and 39°C. Cells were incubated with bromodeoxyuridine (BrdU) to label nascent DNA, fixed, and the resulting Br-DNA was indirectly immunolabeled with Alexa Fluor 488. Br-DNA staining yielded different patterns, suggesting that cells containing nascent Br-DNAs were in different stages of S phase (Fig. 2C-F). Patterns of indirect labeling for Br-DNA were similar at 34°C and 39°C in both wild-type and mutant cells. These results are consistent with our findings that DNA synthesis in tsTM18 cells was decreased but still active after 4 hours of incubation at the nonpermissive temperature.

### RT-PCR analysis for splice variants of the hamster *perlecan* transcript

The Ig-like repeats six, seven and eight in domain IV of *C. elegans unc-52* are encoded by exons 16, 17 and 18 (Rogalski et al., 1993) (Fig. 3A). Alternative splicing of exons 16, 17 and 18 is regulated by the *mec-8* (Lundquist et al., 1996; Spike et al., 2002) and *smu-1* genes (Spike et al., 2001). We analysed expression of the *perlecan* transcript in tsTM18 cells by RT-PCR. Because the hamster *perlecan* sequence has not been reported in a public database, we used multiple sequence alignment analysis of the human perlecan, mouse perlecan and *C. elegans* UNC-52 proteins to design primers to amplify the hamster sequence that corresponds to the fifth to eleventh Ig-like repeats in domain IV of *C. elegans unc-52* (f2, f3, f4, f6, r2, r3, r4 in Fig. 3C).

Four hours of incubation at 39°C was selected because an early, significant effect on DNA synthesis was observed in tsTM18 cells (Fig. 2B). RT-PCR analysis of RNAs from CHO-K1, tsTM4 and tsTM18 cells was performed with the f2/r2 primer pair as described in the Materials and Methods. A 1661-base pair (bp) product was present in RNA from wild-type CHO-K1 cells (Fig. 3B). Although the 1661-bp product was also amplified from RNA isolated from tsTM18 cells incubated at 34ºC, RT-PCR products from tsTM18 cells cultured at 39°C were a mixture of several size variants (Fig. 3B). Another ts mutant cell line, tsTM4, has a mutation in the largest subunit of RNA polymerase II and shows similar phenotypes to tsTM18, including abnormal induction of sister chromatid exchange and cell-cycle arrest at S phase with decreased DNA synthesis (Tsuji et al., 1990; Sugaya et al., 1997). There was no significant change in the amount of *perlecan* transcript after 4 hours of incubation at 39°C in tsTM4 cells, suggesting that the defect in splicing of *perlecan* pre-mRNA is unique to tsTM18 cells (Fig. 3B).

To analyse the *perlecan* size variants in tsTM18 cells, we extracted, cloned and sequenced the RT-PCR products with the primers listed in Fig. 3C. Sequence analysis of these RT-PCR products revealed that there is one full-length form of the transcript and at least six variants resulting from alternative splicing and retention of intron sequences (Fig. 3C). The 1661-nt partial sequence of the hamster *perlecan* transcript was deposited under Accession No. AB259765 in the DDBJ, GenBank and EMBL databases. An amino acid sequence deduced from this 1661-nt hamster *perlecan* sequence showed significant homology both to the region between the fourth and tenth Ig-like repeats in domain IV of mouse *perlecan* and the region between the eleventh and seventeenth Ig-like repeats in

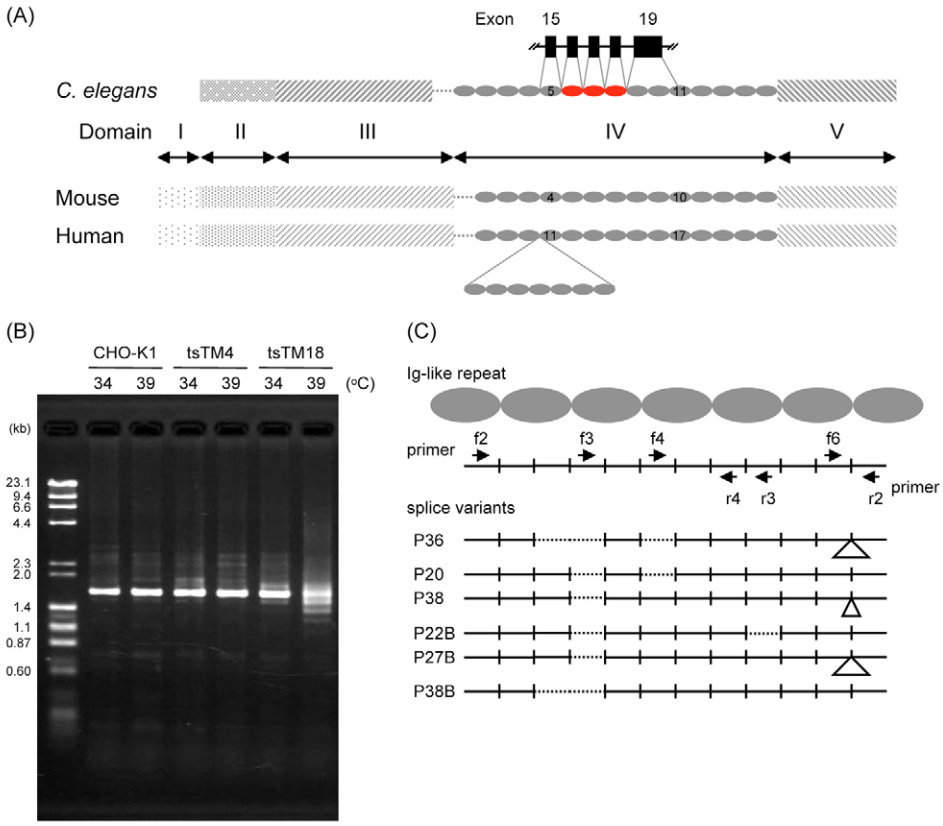


**Fig. 3.** Schematic diagram of the structures of the *perlecan* gene and its splice variants in a hamster *smu1* mutant cell line. (A) Schematic structure of the *C. elegans*, mouse and human homologs of perlecan. The five major domains are indicated by four boxes and ellipses with Roman numerals. The alternatively spliced region of the *C. elegans* counterpart of *perlecan*, *unc-52*, in *smu-1* mutant is indicated above the diagram. Exons (15-19) that correspond to Ig-like repeats are represented by boxes with the introns indicated by horizontal lines. The number of Ig repeats in domain IV varies between species. Mouse perlecan has 14 repeats, whereas human perlecan has 21 repeats resulting from the probable insertion of seven Ig-like repeats. (B) RT-PCR analysis of splice variants of the hamster *perlecan* transcripts. RT-PCR analysis for CHO-K1, tsTM4 and tsTM18 cells was carried out as described in the Materials and Methods. A 1661-bp product is present in RNAs from CHO-K1 and tsTM4 cells. Although 1661-bp product was also amplified from RNA isolated from tsTM18 cells incubated at 34°C, RT-PCR products from tsTM18 cells cultured at 39°C were a mixture of several size variants. Similar results were observed in two additional experiments (Fig. 4B and Fig. 5). (C) Positions of primers and structures of splice variants. Lines indicate exon sequences, dashed lines indicate sequences that are spliced out, and vertical lines indicate exon/intron junctions. Retained intron sequences are indicated by a triangle under the transcript.

domain IV of human *perlecan* with 86.1% and 84.6% identities, respectively. The number of Ig repeats in domain IV varies among species, and sequence analysis revealed the probable insertion of seven Ig repeats after the third Ig repeat of human perlecan (Fig. 3A). We also found the conserved structure of mammalian *perlecan* genes. Exon/intron junctions were conserved in both the mouse and human *perlecan* genes; the sites within the corresponding sequences to the present 1661-nt hamster *perlecan* were identical between mouse and human. Therefore, on the basis of the structures of the mouse and human *perlecan* genes, we predicted the exon/intron junctions in the 1661-nt hamster *perlecan* sequence. Structures of the size variants are shown in Fig. 3C. All six variants identified in this study lacked exon 4, and four of the six lacked multiple exons (variants P36, P20, P22B and P38B in Fig. 3C). Moreover, three of the six contained at least part of intron 11; P36 and P27B contained full-length intron 11, and P38 contained a partial fragment of intron 11 (Fig. 3C). The exon/intron junctions identified through sequence analysis were consistent with those predicted from the comparison between mouse and human sequences described above. The complicated pattern of splice variants suggests that Smu1 participates in at least two steps of splicing, splice-site selection and accuracy of splicing. Interestingly, the splicing abnormality in the *perlecan/unc-52* transcript caused by the *Smu1/smu-1* mutation was identified again by genetics in a mammalian cell line.

## Retention of introns in some splice variants

To confirm that genomic DNA sequences were retained in the *perlecan* transcript, total RNAs were treated with DNase following RT-PCR. A 97-bp fragment was amplified with the f6/r2 primer pair from RNAs isolated from both CHO-K1 and tsTM18 cells cultured at 34°C or 39°C (Fig. 4A). In addition to a 97-bp product, a 300-bp fragment was present in RT-PCR products amplified from RNAs isolated from tsTM18 cells incubated for 4 hours at 39°C (Fig. 4A). The presence of splice variants in tsTM18 cells incubated at 39°C was confirmed by RT-PCR with primer pair f2/r2 (Fig. 4B). The 300-bp product was also observed in PCRs with f6/r2 primer pair and genomic DNAs without DNase treatment (Fig. 4C). Sequence analysis of this 300-bp fragment revealed that it contained the 97-bp sequence and a 203-bp intron. The sequence of the 203-nt intron of the hamster *perlecan* gene was deposited under Accession No. AB259766 in the DDBJ, GenBank and EMBL databases.

## Accumulation of splice variants in tsTM18 cells after culture at 39°C

To clarify the effect of temperature on splicing, we analysed accumulation of splice variants of the *perlecan* gene in tsTM18 cells by RT-PCR. Total RNAs prepared from CHO-K1 and tsTM18 cells after various incubation times at 39°C were analysed by RT-PCR with the f2/r2 primer pair. The 1661-bp full-length *perlecan* transcript was detected in CHO-K1 and

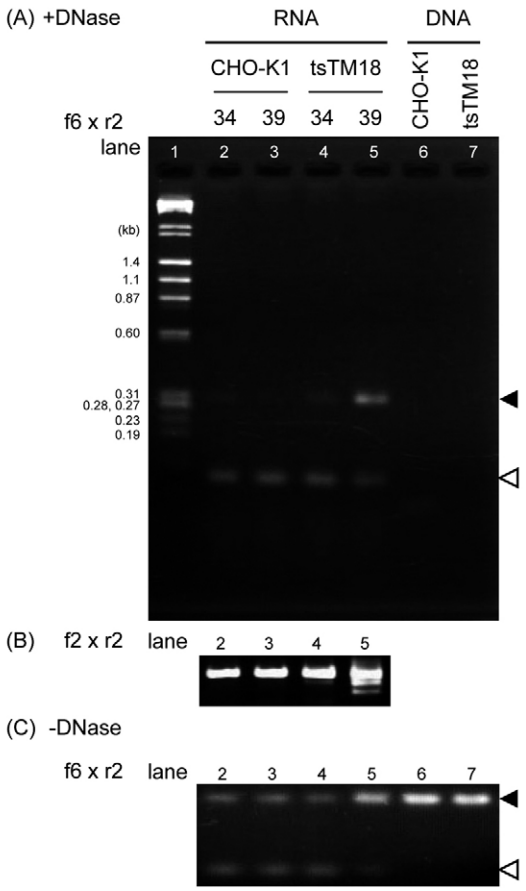


**Fig. 4.** Retention of intron sequences in the *perlecan* transcript in hamster tsTM18 cells. Total RNA and genomic DNA of CHO-K1 and tsTM18 cells were treated with or without DNase and then analysed with primer pair f6/r2 by RT-PCR for RNA or by PCR for DNA. (A) After treatment with DNase, a 97-bp product appears in RNA fraction, and a 300-bp product is present in RNA from tsTM18 cells cultured at 39°C. No PCR products were obtained with DNA as template. The closed triangle indicates a transcript with retained intron sequences, and the open triangle indicates the transcript predicted from the sequence. (B) Result of RT-PCR with primer pair f2/r2. Lane numbers correspond to those in panel A. Splice variants are seen in the amplification products of tsTM18 cells incubated at 39°C (lane 5). (C) Results of RT-PCR and PCR without DNase pre-treatment. Triangles indicate the product predicted from the sequence. A 300-bp PCR product was amplified from DNA.

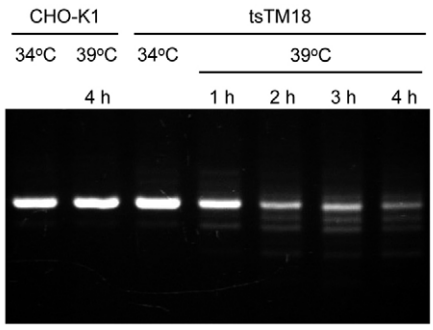


**Fig. 5.** Accumulation of splice variants of *perlecan* in tsTM18 cells during culture at 39°C. Incubation times at 39°C are indicated above the gel. A 1661-bp RT-PCR product was predicted on the basis of the sequence. This product is present in CHO-K1 cells and in tsTM18 cells incubated at 34°C. However, during culture of tsTM18 at 39°C, the amount of the 1661-bp product decreases gradually, and a number of different splice variants appear.

tsTM18 cells incubated at 34°C (Fig. 5). However, when cells were cultured at 39°C, the amount of the 1661-bp product decreased gradually, and a number of splice variants appeared in tsTM18 cells. The defect in splicing of the *perlecan* pre-mRNA appears slightly earlier than the defect in DNA synthesis.

## RT-PCR analysis of other transcripts

To clarify whether the splicing defect in the *perlecan* pre-mRNA in tsTM18 cells at the nonpermissive temperature is specific to the *perlecan* gene, we examined transcripts of four other genes, *Aptx*, *Dnaja1*, *Tnc* and *Smu1*, by RT-PCR. *Aptx* encodes aprataxin, which is a member of the histidine triad superfamily, some members of which have nt-binding and diadenosine polyphosphate hydrolase activities. It was reported that aprataxin may play a role in single-stranded DNA repair; mutations in this gene appear to be associated with ataxia-ocular apraxia, and multiple splice variants encoding distinct isoforms have been identified (Date et al., 2001; Moreira et al., 2001). RT-PCR analysis of the coding sequence of *Aptx* revealed that at least three variants exist, and the shortest variant became the most prominent in both CHO-K1 and tsTM18 cells during culture at 39°C (Fig. 6A). *Dnaja1* shows significant homology to the bacterial heat shock protein DnaJ (Hsp40) and may function in protein folding and transport (Chellaiah et al., 1993). DnaJ was originally isolated as one of the genes involved in DNA replication in bacteria. A single RT-PCR product was obtained from both CHO-K1 and tsTM18 cells incubated at 34°C and 39°C (Fig. 6B). Expression of *Smu1* was uniform and confirmed to be lower than expression of the *Aptx*, *Dnaja1*, *Tnc* and *perlecan* genes (Fig. 6C). *Tnc* encodes extracellular matrix glycoprotein tenascin-C, is expressed in a variety of tissues and is thought to play a role in mesenchymal-epidermal interactions not only during organ development but also under conditions of tissue remodeling, such as tumorgenesis and wound healing (Erickson and Bourdon, 1989; Chiquet-Ehrismann et al., 1995). Tnc was chosen as an example of an extracellular matrix molecule that contains repeated sequences similar to perlecan. Mouse tenascin-C contains six alternatively spliced sites between its fifth and sixth type-III fibronectin domains, which generate a large number of Tnc isoforms in mammalian development and in cancer progression (Joester and Faissner, 1999). When the region between the fifth and sixth type-III fibronectin domains was amplified by RT-PCR, single products were obtained from all templates, even tsTM18 cells grown at 39°C (Fig. 6D). When the results of RT-PCR analysis of the *perlecan* gene were compared with those of the other four genes, the splicing defect in the *perlecan* gene of *Smu1* mutant cells appeared to be unique. However, the present results are not sufficient to conclude that the splicing defect is specific to the *perlecan* gene in tsTM18 cells. Therefore, analyses of additional genes involved in replication and in maintenance of genomic integrity are needed. Development

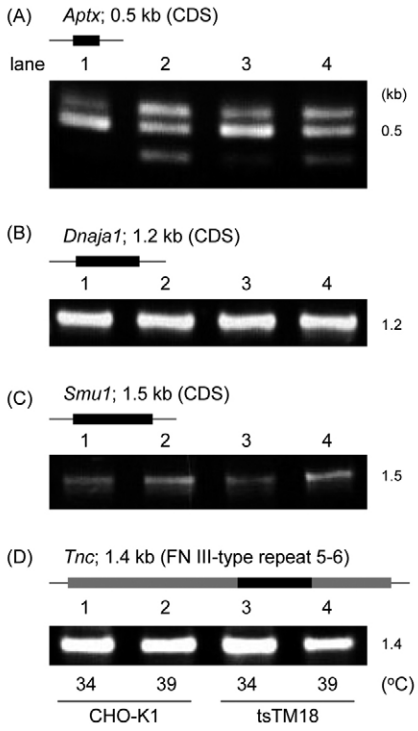


**Fig. 6.** RT-PCR analysis of the *Aptx*, *Dnaja1*, *Smu1* and *Tnc* transcripts in CHO-K1 and tsTM18 cells. Amplified regions are indicated by black boxes above the photograph. The coding sequences of *Aptx*, *Dnaja1* and *Smu1* were amplified. The FN III-type repeat 5-6 region of *Tnc*, which is alternatively spliced in mammalian development and in cancer progression (Joester and Faissner, 1999), was analysed. There were no changes in expression of *Dnaja1*, *Smu1* and *Tnc* in CHO-K1 and tsTM18 cells incubated at 39°C. There were at least three size variants of *Aptx*, and the shortest one appeared in both CHO-K1 and tsTM18 cells during culture at 39°C.

of a comprehensive procedure to assess alternative splicing of all genes in the genome would be helpful for such analyses.

## Discussion

### Alternative splicing and intron retention in the *perlecan* pre-mRNA in tsTM18 cells

Because genetic analyses of nematodes revealed that *smu-1* affects splicing of the *unc-52/perlecan* pre-mRNA, we analysed expression of *perlecan* in *Smu1* mutant cells by RT-PCR and found that *Smu1* affects splicing of the *perlecan* pre-mRNA. These findings indicate that the role of Smu1 in splicing is highly conserved among multicellular eukaryotes and that the abnormal splicing of the *perlecan/unc-52* transcript caused by *Smu1/smu-1* mutation is also conserved. Sequence analysis of the splice variants revealed that they resulted from alternative splicing and intron retention. These results suggest that Smu1 participates in at least two steps of splicing: splice-site choice and control of accuracy (intron retention).

Regulated alternative splicing of pre-mRNA most often results in the synthesis of multiple (functional) proteins from the same pre-mRNA, but alternative splicing can also function as an on-off switch for gene function (Baker, 1989). Sequence analyses of amino acid sequences deduced from six *perlecan* splice variants revealed that five of the six encoded truncated forms of the protein due to generation of stop codons by alternative splicing (variants P36, P20, P38, P22B and P27B in Fig. 3C). Interestingly, one variant did not contain any nonsense codons and encoded a truncated form that lacked a single Ig-repeat (P38B in Fig. 3C).

Some important regulatory events are controlled by intron retention. For the *Drosophila* Msl2 transcript, an intron is retained in the final mRNA in female flies (Gebauer et al., 1998). Retention of this intron requires binding of Sxl protein to both the 5′ and 3′ splice sites. Another well-characterized system of intron retention is the P element transcript in *Drosophila* (Rio, 1991). In our RT-PCR and sequence analyses of the variant transcripts, it remains unclear whether the introns retained in *perlecan* due to *Smu1* mutation have any physiologic significance.

### Role of Smu1 in spliceosomes

RT-PCR analysis of transcripts of four other genes revealed that the splicing defect is restricted to the *perlecan* gene, suggesting that functional Smu1 is not required for basic splicing activity in mutant cells. Comprehensive proteomic analysis of human spliceosomes revealed that the human homologue of Smu1 is fSAP57 (Zhou et al., 2002). However, as Spike and colleagues suggested (Spike et al., 2001), there is no homolog of *Smu1* in a *Saccharomyces cerevisiae*, a basic model organism, further supporting that *Smu1* does not encode a core component of the spliceosome. On the basis of the facts described above, we believe that Smu1 is an essential RNA splicing factor for one or more specific genes. However, the role of Smu1 in the spliceosome may be very important because regulation of editing of the *perlecan/unc-52* pre-mRNA is evolutionarily conserved.

### Enzymes involved in RNA metabolism and their ts mutants

Both ts mutant cell lines, tsTM4 and tsTM18, have mutations in enzymes involved in RNA metabolism (the largest subunit of RNA polymerase II in tsTM4, transcription; Smu1 in tsTM18, splicing) and exhibit similar phenotypes including abnormal induction of sister chromatid exchange and cell-cycle arrest at S phase with decreased DNA synthesis (Tsuji et al., 1990; Sugaya et al., 1997; Sugaya et al., 2005). Taken together with the results of previous and present analyses of two hamster ts mutants, enzymes involved in RNA synthesis and editing may have some connection with replication and the maintenance of chromosome integrity. A recent study established a link between splicing, transcription and the maintenance of genomic stability (Li and Manley, 2005). They showed that a significant function of SF2/ASF is to prevent the formation of R loop structures between nascent transcripts and template DNAs. SF2/ASF is a member of the SR splicing factors (Ge and Manley, 1990; Krainer et al., 1990), which are proteins necessary for an early step in spliceosome assembly and that can influence the selection of alternative splice sites (Fu, 1995; Manley and Tacke, 1996; Graveley, 2000). Members of this family have characteristic RNA-binding domains and Ser/Arg-rich (SR) motifs. In tsTM18 cells cultured at the nonpermissive temperature, the ts defect of Smu1 appears to alter localization of SF2/ASF, suggesting that there is a link between Smu1 and SF2/ASF (our unpublished

results). Our ts-mutant cell lines should prove useful in the analysis of the function of enzymes involved in RNA metabolism in connection with replication and the maintenance of chromosome integrity.

We conclude that one of the earliest observable effects of temperature shift in tsTM18 cells occurs in DNA synthesis. We also found that the defect in splicing of the *perlecan* pre-mRNA appears slightly earlier than the defect in DNA synthesis because 4 hours of incubation at 39°C is needed to observe the effect of temperature on DNA synthesis. However, we have no evidence to explain the connection between perlecan and replication. In tsTM4 cells, there was no significant change in *perlecan* expression after 4 hours of incubation at 39°C (Fig. 3B). This result suggests that both the alterations (defects) in replication and in splicing of *perlecan* pre-mRNA may be caused independently by the *Smu1* mutation. Furthermore, the relation between the defects in replication (DNA synthesis) and splicing (RNA editing) in tsTM18 cells at the nonpermissive temperature remains unclear. Further studies to clarify the functions of Smu1 in replication and splicing are needed.

## Materials and Methods

### Cells

The Chinese hamster cell line, CHO-K1, and its temperature-sensitive (ts) mutant cells, tsTM4 and tsTM18, were grown in Ham's F-12 medium (Sigma-Aldrich, St Louis, MO) containing 10% fetal calf serum, 2 mM L-glutamine and antibiotics (Gibco/Invitrogen, Carlsbad, CA). 18H-1c30 and 18K-1c1 cells were isolated previously by introduction of the human *SMU1* gene to complement the mutant phenotype of tsTM18 cells (Sugaya et al., 2005).

### Plasmids and transfection

Plasmid vectors expressing short interfering RNAs (siRNAs) were generated with piGENE PUR hU6 vector by iGENE Therapeutics (Tsukuba, Japan). Sequences were S1, 5′-ACCACAGAATGTTCAAATA-3′; S2, 5′-GGCCCAAGATAACTTT-ATG-3′; S3, 5′-GTCAGAAATCACATGTGGA-3′ and EGFP, 5′-TACGGCAAG-CTGACCCTGAAGTTCATCTGC-3′. Plasmids were introduced into 18H-1c30 and 18K-1c1 cells with FuGENE6 transfection reagent (Roche Diagnostics, Mannheim, Germany). After selection with 5 μg $ml^{-1}$ puromycin (Sigma-Aldrich), colonies in dishes were stained with methylene blue and counted, and the average colony number for triplicate dishes was calculated.

### Radiolabeling

Cells were grown on glass coverslips at 34°C overnight with 1.85 kBq $ml^{-1}$ methyl-[$^{14}$C]thymidine (American Radiolabeled Chemicals, St Louis, MO, USA) to label DNA uniformly. Cells were then grown at 34°C or 39°C for 4 hours and incubated with 1.11 MBq $ml^{-1}$ methyl-[$^{3}$H]thymidine (American Radiolabeled Chemicals) to label nascent DNA for the periods indicated in Fig. 2A,B. Incorporation was stopped by adding an equal volume of 0.5% sodium azide. Cells on coverslips were washed with phosphate-buffered saline (PBS), fixed with methanol-acetic acid (3:1) and air dried. After cells were washed with 5% trichloroacetic acid (TCA), radioactivity was measured by scintillation counting. $^{3}$H counts were normalized to $^{14}$C counts.

### Indirect immunolabeling and microscopy

Cells were grown on glass coverslips at 34°C or 39°C for 4 hours, incubated with 50 μM bromodeoxyuridine (BrdU) for 20 minutes, washed with PBS and then fixed (20 minutes at 4°C) with 4% paraformaldehyde (PFA) in 250 mM HEPES. After acid denaturation (1 hour) with 2 N HCl, cells were incubated (20 minutes) with 0.2% Triton X-100 in PBS and blocked with PBS containing 40 mM glycine, 1% bovine serum albumin and 0.2% gelatin. Br-DNA was indirectly immunolabeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR). The primary antibody was mouse anti-BrdU (clone MD5110; 1:800; Caltag, Burlingame, CA). Images were collected with a Yokogawa CSU10 confocal microscope and 'contrast-stretched' with Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

### RT-PCR

Total RNA was isolated from CHO-K1 and tsTM18 cells with TRIzol reagent (Invitrogen). At least three independent samples of RNAs were prepared from wild-type and mutant cells. For analysis of retention of genomic sequences in the *perlecan* transcript, total RNA and genomic DNA were treated with DNase (RQ1 RNase-free DNase, Promega, Madison, WI).

According to the manufacturer's protocol (Takara Bio, Otsu, Shiga, Japan), 1 μg of total RNA was reverse transcribed in a 10 μl volume containing dNTPs, RNase Inhibitor, oligo(dT) primer and BcaBEST polymerase in the supplied buffer and incubated at 65°C for 1 minute, 40°C for 5 minutes, 65°C for 30 minutes and 98°C for 5 minutes. The following primers were used for subsequent amplification of the hamster *perlecan* transcript: f2, 5′-GGCGAGTATGTGTGCCGRGCC-3′; f3, 5′-ATCGAGACCTCTTCCTCCCGA-3′; f4, 5′-GCCCAGCCTATCTATATAGAG-3′; f6, 5′-AAGAAGCTGAGCTGACCTTGG-3′; r2, 5′-CGCAGCTTGGACCAGTG-GATG-3′; r3, 5′-TGAGATTGACGACACTCTGGG-3′ and r4, 5′-GAAACTGG-CATCTTGGCCCTG-3′. After initial denaturation for 2 minutes at 94°C, 35 cycles of PCR were performed. Each cycle consisted of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 63°C and extension for 3 minutes for the f2/r2 and 1 minute for the f6/r2 primer pairs at 72°C. After the last cycle, all samples were incubated for an additional for 3 minutes for the f2/r2 and 1 minute for the f6/r2 primer pairs at 72°C. PCR products were separated by 1.5% agarose (f2/r2 products) or 3% NuSieve 3:1 agarose (f6/r2 products) gel electrophoresis. Sequence confirmation of bands was performed by gel extraction, cloning into the pUC118 vector with a Mighty Cloning Kit (Takara Bio) and sequencing of vector inserts with the primers listed above.

RT-PCR of the hamster *Aptx*, *Dnaja1*, *Smu1* and *Tnc* transcripts was carried out with the following primer pairs: *Aptx*, 5′-ATGMARGACCCCAAAATGCAGGTT-3′ and 5′-CYSKSYCCAGTGCTTCCTGAGRTG-3′; *Dnaja1*, 5′-ATGGTGAAA-GAAACMACTTACTAC-3′ and 5′-AGAGGTCTGACACTGAACRCCACC-3′; *Smu1*, 5′-GGGGTACCATGTCGATCGAAATC-3′ and 5′-GCGGATCCGCGWG-GTTTCCAGAG-3′ and *Tnc*, 5′-GCACGTGTGAAGGCATCCAC-3′ and 5′-CCC-ATGGCTGTTGTTGCTAT-3′. After initial denaturation for 2 minutes at 94°C, 30 cycles of PCR were performed. Each cycle consisted of a denaturing period (30 seconds at 94°C), an annealing phase (30 seconds at 50°C for *Aptx* and *Smu1*, 30 seconds at 55°C for *Dnaja1*, 30 seconds at 65°C for *Tnc*), and an extension period (1 minute for *Aptx*, 2 minutes for *Dnaja1* and *Tnc*, 3 minutes for *Smu1* at 72°C). After the last cycle, all samples were incubated at 72°C for an additional extension period shown above. Amplified products were separated by 1.5% agarose gel electrophoresis.

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