

Full Length Research Paper

Polymorphism in *TNP-1* gene of Murrah buffalo bulls

S. K. Panigrahi* and B. R. Yadav

Livestock Genome Analysis Laboratory, Dairy Cattle Breeding Division, National Dairy Research Institute, Karnal - 132 001, Haryana, India.

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Transition nuclear proteins (TPs), the major proteins found in chromatin of condensing spermatids, have been reported to be important for histone displacement and chromatin condensation during mammalian spermatogenesis. In the present study, transition nuclear protein-1 (*TNP-1*) gene was analyzed using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) technique- to detect polymorphism in Murrah bulls. Analysis of *TNP-1* gene sequence of Murrah buffalo revealed 3 single nucleotide polymorphisms (SNPs) at 205, 340 and 346 bp positions of intronic region. The effect of this polymorphism was explored on individual motility, mass activity and maturation of spermatozoa. Analysis of variance indicates that two variants C and D of Murrah buffalo had significant effect on spermatozoal maturation. However, their effects on individual motility and mass activity was non significant.

Key words: TNP-1 gene, polymorphism, infertility, sub-fertility, sperm maturation, SNP.

INTRODUCTION

In animal breeding, a major objective in a cow-calf operation is to produce one calf from each cow annually. The degree to which producers meet this goal influences their net income. A key component of efficient calf production is a high fertility level in each breeding animal in the herd. In breeding herds, females also show many different types of anomalies (Yadav et al., 1990), however as an individual bull is used to serve many females, a deficiency in the breeding ability of the bull has a larger impact on the herd productivity than fertility problems of females. In bulls there might be several reasons of infertility and sub-fertility; among these, maturation of spermatozoa is an important one (Foresta et al., 1992). In animal breeding programmes, males with sub-fertility usually cause higher losses as compared to infertility as the later animals can be culled early, whereas the former remains under observation for longer duration.

Spermatogenesis is the biological process of gradual transformation of germ cells into spermatids and finally to spermatozoa over an extended period of time within the boundaries of the seminiferous tubules of the testis through a series of processes. Hess (1999) reviewed the

process of spermatogenesis where he presented that the spermatids begin the differentiation phase by forming round spermatids steps (1 - 7). Round spermatids are slowly transformed into elongated cells (steps 8 -19) and finally into spermatozoa that are released. The spermatozoal transit through the epididymis is associated with significant changes in its chromatin within the nucleus. These specific events are accompanied with spermiogenesis accomplished through specific gene products (Tanaka and Baba, 2005). The sperm nucleus undergoes marked rearrangement, which involves the replacement of histones with various nuclear proteins. Finally, the DNA of spermatozoa is highly condensed in its head with highly positively charged protamines (PRMs) (Tanaka and Baba, 2005). These small, basic, arginine-rich proteins compact the genome into the sperm nucleus. About 85% histones are replaced with protamines and 15% remain as such (Gatewood et al., 1990).

The replacement of histones and the deposition of protamines are supported by different nuclear proteins, including the transition nuclear proteins (TPs) for major remodelling of the chromatin (Meistrich et al., 2003). Almost all of these nuclear basic proteins, including PRMs, are derived from histone H1 and undergo complex processes of modification in mammals (Lewis et al, 2003). The TNP1 gene, which is expressed during spermiogenesis, is located on 2q42-q43 in *Bos taurus*

*Corresponding author. E-mail: pani_vet@yahoo.co.in. Tel.: +911842259111; Fax: +911842250042.

(Kim et al., 1992). The occurrence of TP1 and TP2 has been reported as the predominant TPs found in rodent spermatids (Grimes et al., 1975). TP1 is a 6.2 kDa, highly basic chromosomal protein with evenly distributed residues (Kleene et al., 1988). It has been reported as highly conserved (Kremling et al., 1989) and abundantly expressed in all mammals studied (Heidaran et al., 1989) representing 60% of the basic proteins of mouse step 12 to 13 spermatid nuclei (Yu et al., 2000).

The timing of the appearance of the TPs has been most extensively studied in rat (Alfonso et al., 1993; Kistler et al., 1994). Some studies indicated that both proteins are present at significant levels only in the condensing step 13 to 15 spermatids (Alfonso et al., 1993). However, others have suggested that TP2 first appears at step 10 or 11 during nuclear elongation, whereas TP1 appears slightly later at step 11 or 12 (Kistler et al., 1994).

The degree of sperm nuclear condensation or maturation can be assessed by aniline blue staining, which discriminates between lysine-rich histones on the one hand and arginine and cysteine-rich protamines on the other (Dadoune et al., 1988). The histone-rich nuclei of immature spermatozoa contain abundant lysine and will react positively by taking up the aniline blue stain, whereas the protamine-rich nuclei of mature spermatozoa with abundant arginine and cysteine react negatively and remain unstained. A normal ejaculate should contain at least 75% unstained spermatozoa, which indicates a normal nuclear maturation of ejaculated spermatozoa (Dadoune et al., 1988, Hofmann et al., 1990).

It has been reported that mouse null mutants for either *TNP1* or *TNP2* are sub-fertile (Yu et al., 2000; Zhao et al., 2001) while mice that lack both these *TNPs* are infertile (Zhao et al., 2004). The investigators have shown that these basic proteins are important factors in nuclear formation during spermiogenesis. However, screening of literature did not show any report on *TNPs* in buffalo bulls. In view of this perspective, the present investigation was conducted to detect polymorphism in *TNP-1* and *TNP-2* genes of Murrah bulls and to find the effect of this polymorphism on mass activity, individual motility and maturation of spermatozoa. However, the present report is on *TNP-1* gene.

MATERIALS AND METHODS

Animals

The present investigation was carried out on 60 Murrah buffalo bulls. All the bulls belonged to an organized pedigree herd of the National Dairy Research Institute (NDRI). Mass activity and individual motility data of selected bulls were collected from NDRI semen register. Blood and semen samples of each bull were collected aseptically. Semen samples were collected by artificial vagina (A.V) method; labeling on the collection vials was done for bull number, date of collection and ejaculate number. Blood samples were used to isolate genomic DNA and semen samples for aniline blue test of spermatozoa.

DNA isolation and PCR amplification

DNA was isolated from 10 ml of blood by phenol chloroform extraction method (Clamp et al., 1993) with some modifications (Ganai, 1999). One set of primers (*TNP-1A* and *TNP-1B*) was used to amplify 2 exonic and 1 intronic region of *TNP-1* gene. The primers were designed using online Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www_slow.cgi) with *Bos taurus* cattle sequence (Accession No. - X65041) as template. The forward primer (*TNP-1A*) 5' CTGCAAAGCCCCTCATCTT 3' and reverse primer (*TNP-1B*) 5' CCCTTCTGTTCCGGTTGCT 3' were used to amplify the *TNP-1* gene from 22 to 505 bp. The PCR reaction was performed in a total volume of 25 µl containing 100 ng genomic DNA, 0.2 pmol of each primer, 0.2 mmol of dNTPs and 0.75 U Taq. DNA polymerase (Bangalore Genei, Bangalore, India) and 2.5 µl of 10X PCR buffer (200 mmol Tris-HCl, 100 mmol (NH₄)₂SO₄, 100 mmol KCl, 1% Triton X-100, 20 mmol MgCl₂, pH 8.8). The following PCR conditions were used: initial denaturation at 94°C for 2 min, 37 cycles of denaturation at 94°C for 1 min, annealing at 66°C for 40 s, extension at 72°C for 1 min and final extension at 72°C for 10 min.

Single strand conformation polymorphism and sequence analysis of *TNP-1* gene

Single strand conformation polymorphism (SSCP) was used to screen all the samples before sending them for sequencing. In a PCR tube, 6 µl PCR product and 18 µl loading dye (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA, 10 mM NaOH) were taken. After proper mixing all samples were denatured in a thermocycler at 95°C for 10 min. Denatured PCR product was immediately transferred into ice to prevent renaturation and then loaded onto 10% polyacrylamide gel (49:1 acrylamide to bis-acrylamide) and electrophoresed at 200 V for 8 h at 4°C (Yadav and Kale, 2008).

Various band patterns (due to conformational changes of the single stranded DNA, originating from insertion-deletion or other types of point mutations) of the amplified PCR products were marked and scored. The typical gene variants got sequenced from a commercial agency (Bangalore Genie, India).

Sequence of the variants obtained was edited using Bio Edit Version 7.0.9.0 (Hall, 1999). CLUSTALW (1.83) (<http://www.ebi.ac.uk/Tools/clustalw/ndex.html>) multiple sequence alignment software was used to annotate the consensus sequence and to find out SNPs. Edited sequence was used for nucleotide (BLAST: http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn&MEGABLAST=on&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on) analysis to confirm sequence gene identity. The BLAST analysis was also used to find the percent homology of the sequences that has been obtained in the study and with all other sequences of the other species.

Aniline blue staining of spermatozoa

The collected semen was mixed in the ratio 1:1 with Ham's F10 medium supplemented with 10% egg albumin. The spermatozoa were centrifuged at 300 rpm for 10 min; the pellet was layered with 3 to 4 drops of Ham's F10-medium in Eppendorf tubes and incubated at 37°C in an atmosphere of 5% CO₂.

Subsequent to incubation, 5 µl spermatozoa were spread onto glass slide and allowed to dry. Thus prepared smears were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min. Slides were then stained with 5% aqueous aniline blue mixed with 4% acetic acid (pH 3.5) for 5 min. Per slide 200 sperm cells were evaluated and the percentage of unstained sperm heads

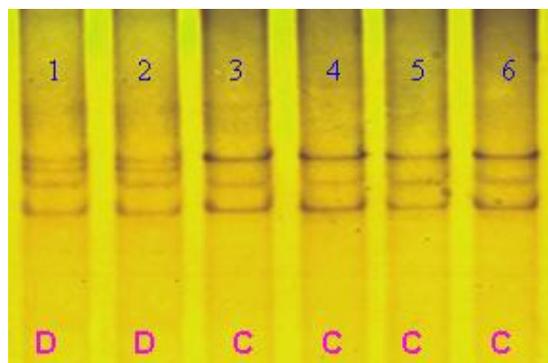


Figure 1. PCR-SSCP band patterns of *TNP-1* gene in Murrah buffalo bulls. Lanes 1 and 2 showing variant-D pattern; Lanes 3 to 6 showing variant-C pattern.

was calculated (Dadoune et al., 1988). Two classes of staining intensities were distinguished: unstained and stained. Stained spermatozoa were immature, whereas unstained spermatozoa were matured.

Statistical analysis

The percentage data generated in acidic aniline blue staining and individual motility were first transformed to arcsine scale. Arcsine transformation of the percentage values was performed using (in Microsoft Excel) the formula:

$$\text{Arcsine value} = \sin [(\text{sqrt}(\text{number}/100))] * 57.3$$

The transformed data were subjected to analysis of variance to study the effect of polymorphism of *TNP-1* gene on aniline blue staining data, individual motility and mass activity of spermatozoa by using SYSTAT software (version-7.0).

RESULTS

SSCP patterns revealed by *TNP-1* primer

The hybridization *TNP-1* primer with genomic DNA covered the first and second exons of the *TNP-1* gene along with the intronic region. The size of the amplified product was so big (484 bp) 10% non-denaturing PAGE was used which gave optimum sharpness and clarity of the bands. Two different band patterns were obtained by SSCP in Murrah bulls (Figure 1).

The observation showed the size of the first exon to be 139 bp, which extended from 834 to 972 bp. The second exon was found from 1193 to 1221 bp, with a size of 29 bp. The intronic region spread from 973 to 1192 bp; size of this region is 220 bp. The size of the amplified product was 484 bp (803 to 1286 bp).

CLUSTAL W multiple sequence alignment of *TNP-1* gene

In the present study the partial sequence of *TNP-1* gene

for the first and second exon and 1 intronic region of Murrah buffalo revealed 3 SNPs between variant-C (NCBI Gene bank accession number- EU-979632; Panigrahi et al., 2008) and variant-D (NCBI Gene bank accession number- EU-979633; Panigrahi et al., 2008). Three SNPs were detected at 205, 341 and 347 bp positions of the intronic region. Variant-C showed guanine at 205 bp position, whereas in variant-D it is substituted with adenine. Substitution of cytosine of variant-C with thymine in variant-D is detected at 341 bp. Substitution of cytosine with adenine is revealed at 347 bp as shown in Figure 2. Chromatogram of variant-C and variant-D of *TNP-1* gene are given in Figures 3 and 4.

CLUSTAL W (1.83) multiple sequence alignment between reference cattle (*B. taurus*) sequence and obtained Murrah buffalo (variant-D and -C) sequence of *TNP-1* gene

CLUSTAL W (1.83) multiple sequence alignment between reference cattle (*B. taurus*) sequence and obtained Murrah buffalo (variant-D) sequence of *TNP-1* gene revealed differences at 913 bp position of the first exonic region; 981, 989, 1003, 1012, 1070, 1135, 1144, 1176 and 1184 bp positions of the intronic region and 1221 bp position of the second exonic region and at 1230 bp position of reference sequence. Whereas, reference cattle (*B. taurus*) sequence and obtained Murrah buffalo (variant-C) sequence of *TNP-1* gene revealed differences at 13 different positions. These differences were at 913 bp position of the first exonic region; 981, 989, 1003, 1008, 1012, 1070, 1135, 1150, 1177 and 1185 bp positions of the intronic region; 1221 bp of second exonic region and at 1230 bp position of reference sequence.

Effect of different SSCP patterns on seminal parameters

The association of different SSCP patterns of *TNP-1* gene with seminal parameters was studied using analysis of variance method. F-ratio obtained from ANOVA indicated that different band patterns revealed by *TNP-1* gene in Murrah buffalo had significant (at 1%) effect on maturation of spermatozoa (Table 1). Least square mean for immature spermatozoa of variant-C and variant-D were 9.7964 ± 0.3061 and 12.1549 ± 0.8837 respectively, which indicated that variant-D pattern had a higher percentage of immature spermatozoa than variant-C. The effect of different SSCP band patterns revealed by *TNP-1* gene on mass activity and individual motility of spermatozoa was not significant (Table 1).

DISCUSSION

During spermiogenesis, major restructuring of the

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VARIANT-C CTGCAAAAGCCCTCATCTTGGCAGAAAGTACAATGTGACCCAGCCGCAAATTAAGAGT C 60
VARIANT-D CTGCAAAAGCCCTCATCTTGGCAGAAAGTACAATGTGACCCAGCCGCAAATTAAGAGT C 60
*****
VARIANT-C AGGGCATGAGGAGGGGCAAGAACAGAACTCCTCACAAGGGAGTCAAAAGAGGTGGCAGCA 120
VARIANT-D AGGGCATGAGGAGGGGCAAGAACAGAACTCCTCACAAGGGAGTCAAAAGAGGTGGCAGCA 120
*****
VARIANT-C AAAGAAAATATCGGAAGAGCAGCCTGAAGAGTAGAAAAAGCTGTGACGATGGTGAGTGAG 180
VARIANT-D AAAGAAAATATCGGAAGAGCAGCCTGAAGAGTAGAAAAAGCTGTGACGATGGTGAGTGAG 180
*****
VARIANT-C GGATGGGTGGGAGAGGAGTCAAACAGGGGACCTTGTGTCTGAGGGCCTTCTTCTTGA 240
VARIANT-D GGATGGGTGGGAGAGGAGTCAAACAGGGGACCTTGTGTCTGAGGGCCTTCTTCTTGA 240
*****
VARIANT-C AGTTAGCTCTCATAGGACCCTGAAACTTCAGCCTGAGAATTTCTGTTCTCAACCAGGTTCC 300
VARIANT-D AGTTAGCTCTCATAGGACCCTGAAACTTCAGCCTGAGAATTTCTGTTCTCAACCAGGTTCC 300
*****
VARIANT-C CATCTGGGCAGGAAGAGGATGAATGGGGTTCGTGAATCTTCAGTGAATATCTAACTGGT 360
VARIANT-D CATCTGGGCAGGAAGAGGATGAATGGGGTTCGTGAATCTTCAGTGAATATCTAACTGGT 360
*****
VARIANT-C GTTCTGTTGCATGCTTGTCTTTCCCTACAGCCAATCGCAATTTGCGCTCCCACTTTTG 420
VARIANT-D GTTCTGTTGCATGCTTGTCTTTCCCTACAGCCAATCGCAATTTGCGCTCCCACTTTTG 420
*****
VARIANT-C ACTCCACCAGCGGGCTCTGCCCTGGTGGAC 450
VARIANT-D ACTCCACCAGCGGGCTCTGCCCTGGTGGAC 450
*****

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Figure 2. CLUSTAL W (1.83) multiple sequence alignment between buffalo TNP1 SSCP patterns C and D.

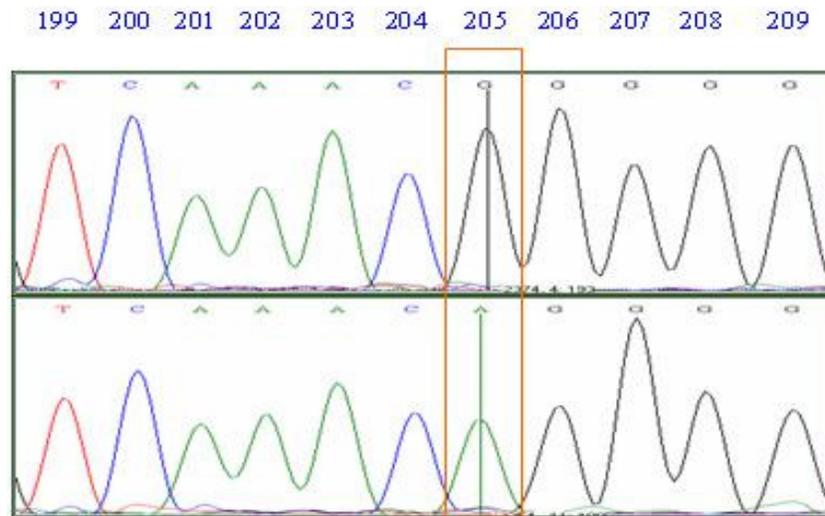


Figure 3. Chromatogram of variant-C and variant-D of TNP-1 gene showing SNP at 205 bp position.

somatic chromatin takes place in which the histones are first replaced by a group of arginine- and lysine-rich proteins called transition proteins (TPs), which are in turn replaced by protamines (Meistrich et al., 1989); this results in the compaction of sperm nucleus. Drastic alteration of chromatin during spermiogenesis is achieved through systematic expression of spermiogenesis-specific nuclear proteins (Tanaka and Baba, 2005).

Because deficiencies in the genes that code these nuclear proteins lead to male infertility in mice, it appears that the essential event in chromatin transformation via the replacement of the somatic-type histones with protamines (PRMs) is the systematic expression of spermiogenesis-specific nuclear proteins (Zhao et al., 2004).

In the present study, assessment was done on the

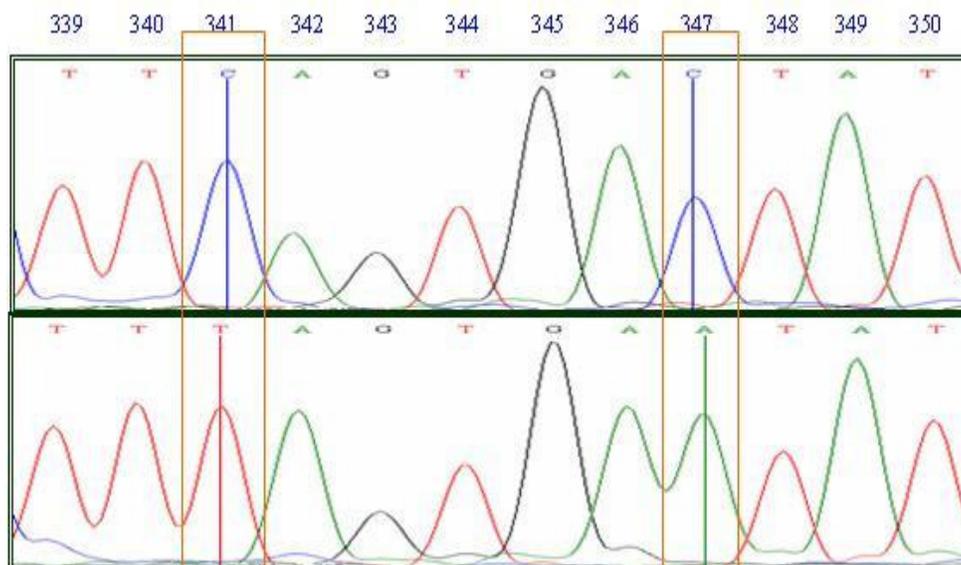


Figure 4. Chromatogram of variant-C and variant-D of TNP-1 gene showing SNPs at 341 and 347 bp positions.

Table 1. Association between different SSCP patterns and seminal parameters.

Seminal parameter	LSM \pm SE		
	Variant-C	Variant-D	F-value
Immature spermatozoa (%)	9.7964 \pm 0.3061	12.1549 \pm 0.8837	6.3593 **
Mass activity	2.040 \pm 0.1571	1.4167 \pm 0.4535	1.6870 ^{NS}
Individual motility (%)	39.2746 \pm 2.0793	31.7540 \pm 6.0025	1.4016 ^{NS}

** indicates significant difference at 1% level of significance; ^{NS} indicates non-significant difference.

polymorphism in *TNP-1* gene of buffalo bulls and their effect on the mass activity, individual motility and aniline blue test result of spermatozoa of the selected animals. wot TPs were identified as abundantly expressed basic nuclear proteins that participate in chromatin condensation by the replacement of somatic-type histones with protamines during spermiogenesis (Meistrich et al., 2003). The TP1 amino acid sequence is highly conserved among various mammals (Kremling et al., 1989). The relative molecular mass (Mr) of TP1 is approximately 6,200, with about 20% arginine and 20% lysine distributed uniformly and without cysteine residues (Kistler et al., 1994). Biochemical analyses have shown that TP1 decreases the melting temperature of DNA (Akama et al., 1998). In contrast, TP2 increases the melting temperature of DNA and compacts the DNA into nucleosomal cores, which indicates that it is a DNA-condensing protein (Baskaran and Rao, 1990). These analyses of TPs show that these proteins play different roles in chromatin remodeling during spermiogenesis.

In general, the loss of the *TNP1* or *TNP2* gene does not lead to infertility in mice, although some *TNP1*-deficient mice are infertile (Yu et al., 2000). The loss of both *TNP1*

and *TNP2* results in reduced litter sizes (Zhao et al., 2004). The idea that these 2 genes compensate each other functionally, evolved from the biochemical characterization and expression kinetics of TP1 and TP2. However, individual *TNP1*-null mice cannot compensate completely for the TP1 deficiency with TP2 (Meistrich et al., 2003), which is probably due to differences in the expression levels of some other proteins. It is clear that TPs, especially TP1, play important roles in mouse spermiogenesis. However, the functions of TPs in buffalo sperm formation are not studied still.

In the present study, *TNP1* gene revealed 3 SNPs in the intronic region between variant-C and variant-D of Murrah buffalo, which is expected to affect significantly the maturation of spermatozoa. Variant-D showed significantly higher percentage of immature spermatozoa (12.1549 \pm 0.8837) than variant-C (9.7964 \pm 0.3061). Mutation in the intronic region has been reported to lead to abnormal splicing which affects the m-RNA and protein synthesis (Cho et al., 2003). Miyagawa et al. (2005) had detected a deletion of the recognition site for the CRE transcription factor in the promoter region of 1 infertile human patient. It might be possible that mutation influen-

ces spermiogenesis by modulating the expression of *TNP1* in somatic cells, such as Sertoli cells. Previously, Schlicker et al. (1994) did not detect any mutation in *PRM1*, *PRM2*, or *TNP1* in 36 infertile men whose spermatozoa showed the presence of histones in the nuclei. Such observations could not be made in the present study and it needs to explore a larger population of proven fertile and infertile animals with defined SNP data to confirm the causal link between *TNP1* gene polymorphisms and male infertility.

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