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Full Length Article

Single Nucleotide Polymorphism in Prolactin (PRL) Gene of Yak (*Bos grunniens*) Population of Gilgit-Baltistan, Pakistan

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Abstract

Prolactin secreted by the pituitary gland is a polypeptide hormone plays vital role in the mammary gland's development, milk secretion and reproduction in mammals. The PRL gene was sequenced to see whether polymorphisms existed in the PRL gene (PRL) in 10 domestic Yaks of Gilgit-Baltistan. SNPs in exon 2 of PRL gene were detected by MEGA6 software. A total of 5 SNPs were identified at positions 30, 52, 55, 58 and 75 among which 3 were transversions and two were transitional substitutions. Effect of these mutations at protein level showed that three mutations were non-synonymous at amino acids positions 18, 19 & 20 whereas the other two mutations were synonymous (at positions 10 and 25). Multiple sequence alignment was performed by using ClustalW software and NCBI BLAST was used to study the homology of Yak with other bovine and caprine species. It was concluded that the PRL gene at exon 2 in Domestic Yak of Pakistan closely resembled other bovine species such as *Bos mutus, B. indicus and B. taurus, Bubalus bubalis* and other bovine species depicting the conservation of PRL gene among different bovine and mammalian species. The future prospect of this study is to find the association of prolactin gene SNPs in exon 2 region with the quantitative characters especially milk production of the yak.

Keywords: Prolactin; Single Nucleotide Polymorphism (SNP); Domestic Yak; Gilgit-Baltistan; Pakistan

Introduction

Prolactin, secreted from pituitary gland is a polypeptide hormone which regulates lactation in mammals and involves in lactose, protein and lipid synthesis in milk (Le Provost *et al.* 1994; Horseman *et al.* 1997). It is not only involved in milk production and mammary gland development but also plays role in growth and development, reproduction, metabolism and immunity (Bole-Feysot *et al.* 1998; Gregerson 2006). The PRL gene was found on chromosome 23 with 5 exons and 4 introns in bovine (Camper *et al.* 1984; Hallerman *et al.* 1988). 199 amino acids reside in a PRL gene which codes for Prolactin hormone (Cao *et al.* 2002). Many studies have been conducted on dairy cattle to find polymorphism within the gene of PRL and it is observed that there is a considerable link between mutations in PRL gene and production of milk (Lü *et al.* 2011). In past, many single nucleotide polymorphisms (SNP) have been reported in PRL genes in dairy cattle and more than twenty SNPs have been detected so far (Halabian *et al.* 2008; Uddin *et al.* 2013).

Among domestic cattle, domestic Yak is a beneficial source of milk and meat in mountainous regions all over the world (Hussain *et al.* 2021). Its milk is used to extract dairy products like cheese, butter and yoghurt (Jianlin *et al.* 2002). In Pakistan, the herds of yak are restricted and only found in the higher elevated areas of Chitral and Gilgit-Baltistan. So far, limited works have been conducted to find out the genetic polymorphism in PRL gene affecting its milk production and reproduction.

The present study was aimed to determine the polymorphic sites in PRL gene at exon 2 in domestic yak

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(*Bos grunniens*) of Gilgit-Baltistan, Pakistan. The phylogenetic relation of yak through prolactin gene exon 2 with other yak population was also evaluated.

Materials and Methods

Blood samples of ten domestic yak were collected from Gilgit-Baltistan Pakistan. Wet lab work was conducted at Molecular Biology lab, Virtual University of Pakistan, Davis Road Campus (DRC), Lahore.

Blood samples and DNA extraction

Blood samples were collected in EDTA-containing vacutainers and stored at -20°C. Genomic DNA was extracted using standard organic DNA extraction method by using the phenol-chloroform technique (Sambrook and Russell 2006).

Primer designing, synthesis and optimization

Primer3 online software tool was used for primer designing and the specificity of primers was checked through in silico PCR. After synthesis, primers were optimized at different conditions (primer concentration and volume, buffer, DNA concentration, thermocycling profiles) and the conditions at which best amplification was achieved were recorded and used for the amplification of the exon 2 region of yak's PRL gene.

Polymerase Chain Reaction (PCR)

PCR was performed to amplify fragment of the expected size of 182 bp. The PCR was performed using initial denaturation at 95ºC for 5 min, followed by 35 cycles using 95ºC for 40 sec, 53ºC for 40 sec and 72ºC for 40 sec followed by a final extension of 10 min at 72ºC. The amplified PCR fragments were resolved on 1.5% Agarose gel and observed under UV trans illuminator or gel documentation system and compared with DNA ladder to confirm the size of amplified PCR product (Table 2). Previously, used primers F-AGGGAAGGGCAGAAAGATAG and R-ATGGCAGACTGTTGAGGATC (Rajan *et al.* 2011) were used for the PCR amplification of the prolactin gene exon 2 (Table 1).

Precipitation of PCR products and sequencing

PCR products were precipitated through ethanol precipitation method and sent for Sanger DNA sequencing using the sequencing PCR through ABI genetic Analyzer.

Bioinformatics analysis

The sequences obtained from sequencing were edited and aligned using BioEdit software. Basic Local Alignment Tool (NCBI BLAST) was used to align the prolactin gene sequences already reported in GenBank for further sequence analysis. MEGA v6.0 and DnaSP v5.0 software were used for detection of DNA polymorphism and phylogenetic analysis among studied and already reported gene sequences from NCBI GenBank.

Results

Sequence analysis and multiple sequence alignment

Sequencing results were analyzed by using BLASTN of NCBI. The Clustal Omega was used for multiple sequence alignment of exon 2 of PRL gene with reference sequences and nucleotide coding sequences were compared with NCBI database (Hall 1999). MEGA 6.06 software was utilized for phylogenetic analysis as well as to detect the single nucleotide polymorphism (SNPs). Multiple sequence alignment was performed by using ClustalW software. Obtained sequences were compared with the reference nucleotide and amino acid sequences retrieved from the NCBI database and mutations are highlighted (Fig. 1 and 2).

Mutational analysis of PRL gene Exon 2 of domestic yak

Mutational analysis of Exon 2 of PRL gene in yak was done by using the MEGA 6.0 software. This analysis showed a total of 5 SNPs at positions 30, 52, 55, 58 and 75. 60% of detected SNPs were found to be transversions (pyrimidine were replaced by purines or vice versa) that included position 30→ T>A, 52→ G>C, 75→ C>A and 40% (55→ T>C and 58→ T>C) were transitional mutations (purine to replace purines and pyrimidine replace pyrimidine) (Table 4). These nucleotide sequences were also translated into amino acid sequences to check the effect of SNPs at protein level. Results showed that 60% (n=3) mutations were non-synonymous and 40% (n=2) were synonymous substitutions. In non-synonymous condition, mutation at nucleotide level causes changes at amino acid level which may affect the structure and function of protein whereas synonymous substitutions are those where mutation and nucleotide level does not cause change at an amino acid level when compared with reference sequence retrieved from NCBI (Fig. 3–7).

Similarity with other bovine species and their phylogenetic analysis

Homology of the studied samples of domestic yak PRL Exon 2 with other bovine and caprine species was also analyzed using NCBI BLAST (Basic local alignment Search Tool) that showed the highest similarity with *B. mutus, B. indicus and B. taurus* (99.45%) followed by *Bubalus bubalis* (98.90%), *Capra hircus* (97.25%), *Bison bison* (96.48%), *Cervus elaphus* (96.70%), *Ovis aries* (96.15%), *Orcinus orca* (87.91%), *Globicephala melas* (87.91%), *Tursiops truncates* (87.91%), *Canis lupus* (85.71%), *Camelus ferus, C. dromedariu, Sus scrofa and C. bactrianus* (84.62%), *Equus caballus* (85.63%), *Macaca mulatta* (81.76%) and *Homo sapiens* (81.18%) (Table 3). These results showed the higher

Table 1: Primers Sequence used for amplification of exon-2 of Yak PRL gene

Primers Name	Primers sequence (5'→3')	Amplicon size (bp)	Annealing temperature (°C)
PRL-F	AGGGAAGGGCAGAAAGATAG		
PRL-R	ATGGCAGACTGTTGAGGATC	182	50–53

Table 2: PCR reaction mixture for amplification of exon-2 of Yak PRL gene

Reagents	Volume used
Genomic DNA (25 ng/µL)	2 µL
10XPCR buffer	2.0 µL
Prolactin gene-Forward primer	1 µL
Prolactin gene-Reverse primer	1 µL
$MgCl_2$	2.5 µL
25 mM dNTPs	2.5 µL
Taq polymerase	0.5 µL
Nuclease free water	14 µL
Total volume	20 µL

Table 3: Percentage similarity of Domestic Yak PRL Exon2 with other Mammalian species

Species	Common Name	% Identity	Accession Number
Bos mutus	Wild Yak	99.45%	XM005894272.2
B. indicus	Zebu Cattle	99.45%	KX685939.1
B. taurus	Cattle	99.45%	KX602711.1
Bubalus bubalis	Water Buffalo	98.90%	NM_001290885.1
Capra hircus	Domestic goat	97.25%	NM_001285547.1
Bison bison	American Buffalo	96.84%	XM_010845567.1
Cervus elaphus	Red deer	96.70%	AY373035.1
Ovis aries	Sheep	96.15%	KC764410.1
Orcinus orca	Killer whale	87.91%	XM_012534348.2
Globicephala melas	Long-finned pilot whale	87.91%	XM_030881186.1
Tursiops truncates	Common bottlenose dolphin	87.91%	XM_019944976.1
Canis lupus	Wolf	85.71%	NM_001008275.2
Camelus ferus	Bactrian camel	84.62%	XM_006182220.3
C. dromedaries	Arabian camel	84.62%	XM_010980119.2
Sus scrofa	Wild Pig	84.62%	XM_005665624.3
Camelus bactrianus	Bactrian camel	84.62%	XM_010974249.1
Equus caballus	Horse	85.63%	XM_014734200.1
Macaca mulatta	Rhesus monkey	81.76%	NM_001047128.3
Homo sapiens	Human	81.18%	NM_001163558.3

Table 4: SNPs (Single Nucleotide Polymorphisms) Distribution in Exon 2 of Yak Prolactin gene

Nucleotide Position	Ref seq# *B. taurus*	Y-1	Y-2	Y-3	Y-4	Y-5	Y-6	Y-7	Y-8	Y-9	Y-10	Transition/ Transversion	Synonymous/ Non-synonymous	AA Position	AA Change
30	T	T	T	T	T	T	T	T	T	A	T	Transv	Synonymous	10	C>C
52	G	G	G	G	G	G	G	G	G	G	C	Transv	Non-synonymous	18	V>L
55	T	T	T	T	T	T	T	T	T	T	C	Trans	Non-synonymous	19	W/R
58	T	T	T	T	T	T	T	T	T	T	C	Trans	Non-synonymous	20	S/P
75	C	C	A	C	C	C	C	C	C	C	A	Transv	Synonymous	25	V/V

similarity and conservation of PRL gene among different bovine and mammalian species. Phylogenetic analysis was done using MEGA 6.0 software. BLAST was run using the obtained sequences results of exon 2 PRL gene and orthologs were found. Already available sequences of PRL gene of different mammalian and bovine species were retrieved and downloaded from NCBI. To infer the evolutionary history of yak prolactin gene a Neighbor-Joining tree was build using evolutionary distances computed through Maximum Composite Likelihood method. Evolutionary analysis showed close relatedness among all mammalian species including *B. mutus*, *B. indicus*, *B. taurus* as the nearest neighbors and they are shown in the same clade with our studied yak sequences. Other highly resembling species were *B. bubalis*, *B. bison*, *O. aries* and *C. hircus* having same ancestor. However, *M. mullata* and *H. sapiens* formed separate group evolving much faster than others as they were shown as farthest species. This clade was followed by an adjacent clade of *C. lupus* and *Equus caballus*. The phylogenetic tree showed a separate clade for whale and dolphin [*Orcinus orca* (Killer whale)], *G. melas* (Long-finned pilot whale) and *T. truncates* (Common bottlenose dolphin)). Contrarily, *C. lupus* (Wolf), *C. ferus* (Bactrian camel), *C. dromedaries* (Arabian camel), *Sus scrofa* (Wild Pig) and *C. bactrianus* (Bactrian camel) were shown in a single separate clade exhibiting resemblance among then as shown in the tree (Fig. 8 and 9).

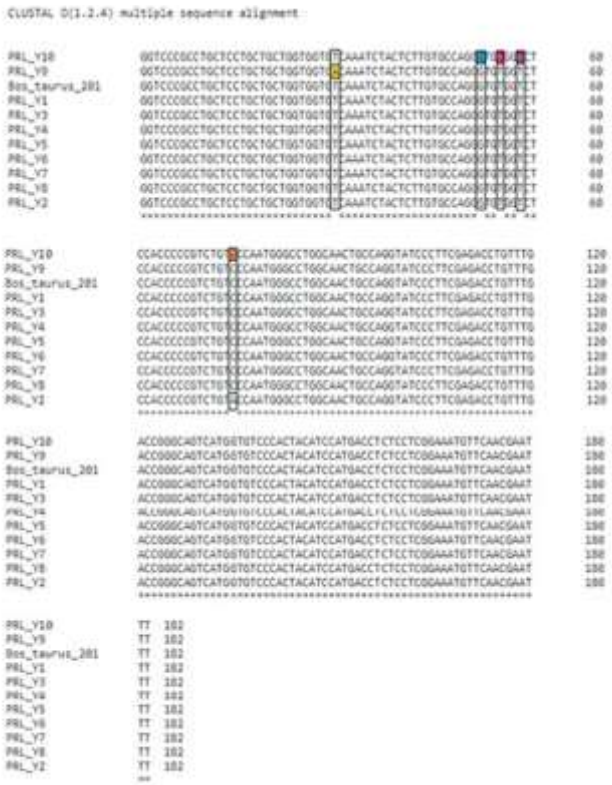

Fig. 1: Comparison and Multiple alignments with Reference nucleotide sequence of Bos Taurus

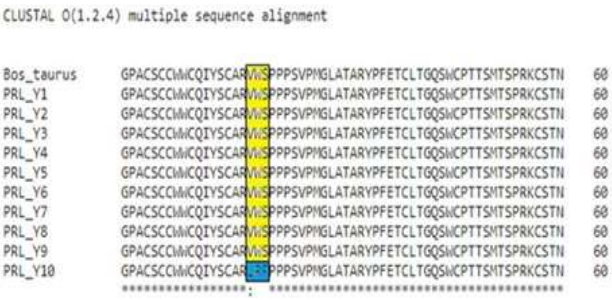

Fig. 2: Comparison and Multiple alignments with Reference amino acid sequence of Bos taurus

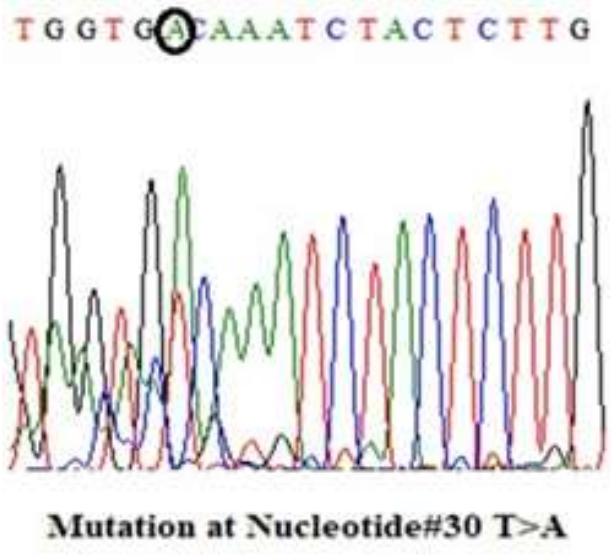

Fig. 3: Mutation at position 30 T>A (Thymine replaced by Adenine in Y-9)

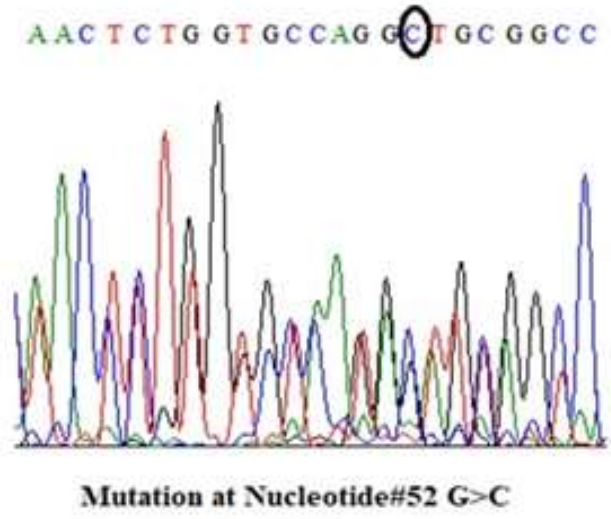

Fig. 4: Mutation at position 52 G>C (Guanine replaced by Cytosine in Y-10) caused addition of Leucine (L) instead of Valine (V) at position 18

Discussion

A similar study was conducted for the polymorphic evaluation of the bovine PRL gene in Pakistani cattle and a total of five mutations in the exonic region and eleven in the intronic regions were found (Uddin *et al.* 2013). In another study, a total of three SNPs was detected in buffaloes, two of them were in the promoter region while one was found in the exon2 region in buffaloes. The SNP in the exon2 region was found to be associated with an amino acid change of Arginine to Cysteine in the signalling domain (Kumar *et al.* 2017).

A study on Chinese Holstein cows reveals that PRL gene has SNPs in exon 10 using PCR and sequence analysis. Two newly discovered single nucleotide polymorphisms in PRL gene cause a change in amino acid (Lü *et al.* 2011).

In a study, the phylogenetic analysis of PRL gene family has been screened in the mouse, rat, and cow where the mouse and rat show similarity in the organization of PRL gene. The presence of PRL gene in cow is assured however its resemblance with mouse and rat PRL is not found similar. PRL gene in mice and rat consist of a unique group of 6-exons that are PRL related. Human and dogs share a similarity concerning locus of gene. Both have only one gene locus. PRL in human also encodes for growth hormone (Alam *et al.* 2006).

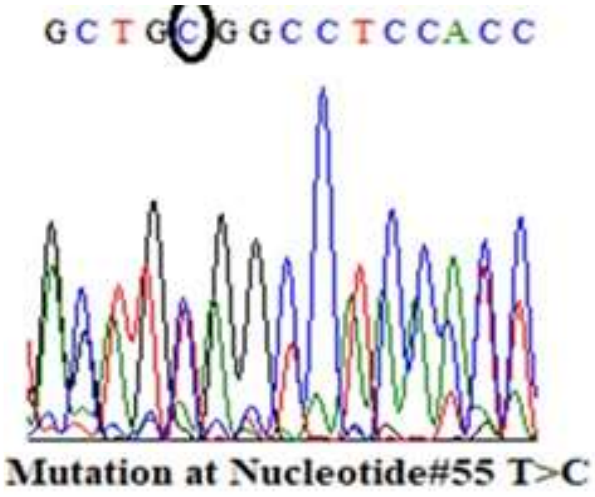

Fig. 5: Mutation at position 55 T>C (Thymine replaced by Cytosine in Y-10) caused addition of Arginine (R) instead of Tryptophan (W) at position 19

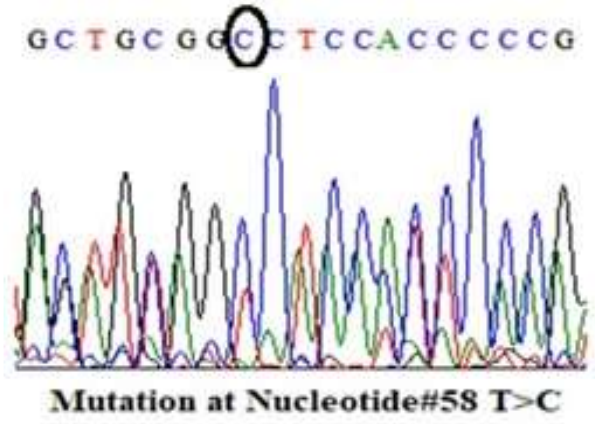

Fig. 6: Mutation at position 58 T>C (Thymine replaced by Cytosine in Y-10) caused addition of Proline (P) instead of Serine (S) at position 20

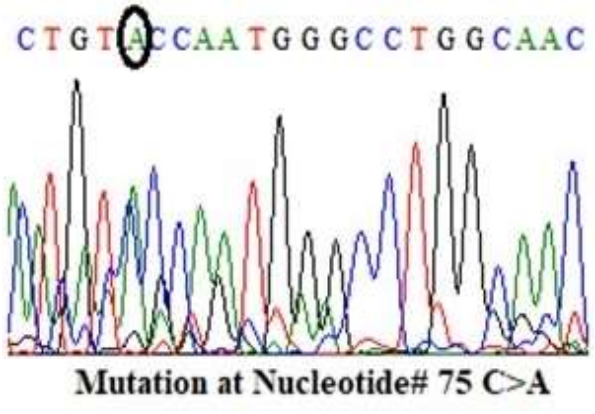

Fig. 7: Mutation at position 75 C>A (Cytosine replaced by Adenine in Y-2 & Y10)

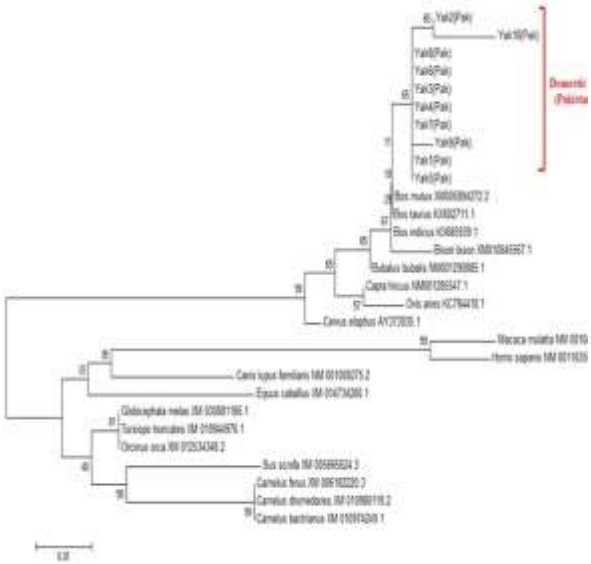

Fig. 8: Phylogenetic analysis of PRL gene Exon 2 in Domestic Yak of Pakistan

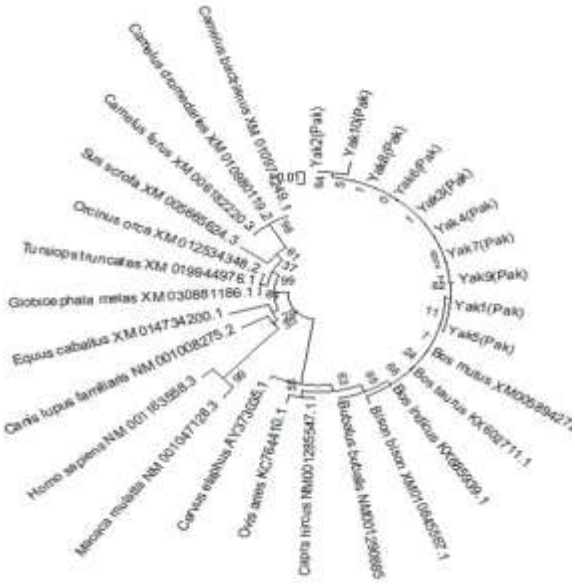

Fig. 9: Phylogenetic analysis of PRL gene Exon 2 in Domestic Yak of Pakistan

Conclusion

In conclusion, the change in nucleotide position has led to changes at the protein level. The sequence analysis has shown that among the yak population, yak is physically different but genetically resembles with the other bovine animals. The phylogenetic analysis illustrates that there is the highest resemblance clade of yak with other mammals. Taking into consideration the importance of yak milk, it is the need of the hour to conduct more research on PRL in domestic yak. The present study would provide insight into the association of

the SNPs found at exon 2 of PRL gene in milk production trait and reproduction.

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Author Contributions

TH, MEB conceived the idea,TH, AW, TA, AA, SMR collected field samples, SN, QA conducted lab work, TH, AW, BB analysed the data and wrote the manuscript, TH, MEB reviewed the manuscript

Conflicts of Interest

No conflict of interest among authors

Data Availability

Data is avaiabe and can be shared on demand

Ethics Approval

The experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Virtual University of Pakistan

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