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Ribosomal proteins expression and phylogeny in alpaca (*Lama pacos*) skin*

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ABSTRACT

Ribosomal proteins (RP) has been reported as a central player in the translation system, and are required for the growth and maintenance of all cell kinds. RP genes form a family of homologous proteins that express in the stable pattern and were used for phylogenetic analysis. Here we constructed a cDNA library of alpaca skin and 13,800 clones were sequenced. In the cDNA library, RP genes from skin cDNA library of alpaca were identified. Then 8 RP genes were selected at random and built the phylogenetic trees from the DNA sequences by using parsimony or maximum likelihood methods for molecular and evolutionary analysis of ribosomal proteins. The results showed that the 42 RP genes of alpaca have been expressed in alpaca skin. They were highly conserved. The phylogeny inferred from all these methods suggested that the evolutionary distances of alpaca RP genes were closer to rat.

Keywords: Ribosomal Protein; Expression; Phylogenetic Tree; Alpaca

1. INTRODUCTION

The ribosome has been reported as a central player in the translation system, which consists of four RNA species and 79 ribosomal proteins (RPs) in mammals. Its function is to decode the nucleotide sequence carried by the mRNA and convert it into an amino acid primary structure by the catalysis of peptide bonds [1]. In eukaryotes, ribosomes consist of two different subunits: a 40S small subunit and a 60S large subunit. In mammals, the 40S subunit contains 33 different proteins and an 18S rRNA, whereas the 60S subunit is composed of 49 unique polypeptides and three rRNAs: a 5S, a 5.8S, and a 28S [2]. Ribosomal proteins are thought to have mainly a scaffolding/chaperone role in facilitating the processing and folding of rRNA during biogenesis and stabilizing the mature particle during protein synthesis [3]. It has been well established that global regulation of protein synthesis in eukaryotes is mainly achieved by posttranslational modification (PTM) of translation factors in response to environmental cues [4].

The family of South American camelids with four members is recognized as two wild species, the guanaco (*Lama guanicoe*) and the vicuna (*Vicugna vicugna*) and two of domestic species, the alpaca (*Lama pacos*) and the llama [5]. Because all potential ancestral forms are extant, South American camelids domestication represents an unusual and useful opportunity to gain insight into the origin and biodiversity of domesticated animals, an issue which is of increasing interest due to the recognized potential economic benefits of indigenous genetic resources and the threats that face marginal and extensive agricultural today (Hall & Bradley, 1995). The molecular evolutionary analysis of the family Camelidae by analyzing the full DNA sequence of the mitochondrial cytochrome b gene was reported. Estimates for the time of divergence of the Old World (Camelini) and New World (Lamini) tribes obtained from sequence data are in agreement with those derived from the fossil record. The DNA sequence data were also used to test current hypotheses concerning the ancestors of the domesticated llama and alpaca. The results showed that hybridization has occurred in the ancestry of both domesticated camelids, obscuring the origin of the domestic species (Helen *et al*., 1994). The evolutionary origins of South America's domestic alpaca and llama remain controversial [5] (Miranda Kadwell *et al*., 2001) due to hybridization, near extirpation during the Spanish conquest and difficulties in archaeological interpretation. At present, although alpaca and llama rearing is a central element of the economy in the high Ands, it is often not profitable due to the poor quality of the animals and their fibre. The reconstruction of fine-fibre breeds and the breeding strategies needed are therefore uniquely dependent upon the contributions of archacozoology and genetic analysis [5].

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The relationship between domestic alpaca and other mammalian species has been reported. The complete mitochondrial (mt) genome of an odontocete and the sperm whale (*Physeter macrocephalus*) were sequenced and included it in phylogenetic analyses together with the previously sequenced complete mtDNAs of two mysticetes (the fin and blue whales) and a number of other mammals, including five artiodactyls (the hippo-potamus, cow, sheep, alpaca, and pig). The most strongly supported cetartiodactyl relationship was: outgroup ((pig, alpaca), ((cow, sheep), (hippopotamus, (sperm whale, (baleen whales). As in previous analyses of complete mtDNAs, the sister-group relationship between the hippopotamus and the whales received strong support, making both Artiodactyla and Suiformes (pigs, peccaries and hippopotamuses) paraphyletic. In addition, the analyses identified a sister-group relationship between Suina (the pig) and Tylopoda (the alpaca), although this relationship was not strongly supported [6]. Ribosomal RNA (rRNA) genes commonly found in eukaryotic and prokaryotes, they are more conservation and have a constant evolution rate variability during the process of evolution. So ribosomal RNA (rRNA) genes is a useful molecular marker for studying organic evolution [7]. Here, we report 8 RP genes (selected randomly) sequences and a phylogenetic analysis of alpaca to find the relationship between alpaca and other animals.

2. MATERIAL AND METHODS

2.1. Animals

The alpacas used in these experiments was maintained at the Shanxi alpaca farm of Shanxi Agricultural University. All animal experiments were performed according to the protocols approved by the institutional committee for use and care of animals.

2.2. cDNA Library Production

The total RNA is extracted by Trizol reagent (Stratagene) and mRNA is isolated by Oligotex (Qiagen). The first strand cDNA is produced at 42˚C in 10 μL reaction with 1 μL PowerScript reverse transcriptase. The second and double strands cDNA(ds cDNA) are produced by LD PCR with 5'PCR primer and CDSIII primer in 100 μL reaction for 24 cycles (95˚C 5 s; 68˚C 6 min). Following the digestion by proteinase K and Sfi, ds cDNA are isolated by CHROMA APIN-400 in the molecular weight order and collected cDNA together with the aimed size. The ds cDNA is ligated with λTriplEx2 vector in the ligation reaction at 16˚C and then the ligation production was packaged in λ-phage (Gigapack III plus packaging extract, Stratagene) at 22˚C.

2.3. Sequencing

The package production was transferred into the XL1-Blue at 37˚C for a night with X-gal and IPTG. 17,400 white clones were picked at random and then were converted from λTriplEx2 to ρTriplEx2 in *E. coli* BM25.8 at 31˚C for circularization of a complete plasmid from the recombinant phage. The clone of circularization production were sequenced in both directions with T7 and 5'special primer.

2.4. Assembly of the Sequences of Targeted Genes

The sequences were assembled by the DNAMAN software and the consensus sequences will be used in the next phylogentic analysis.

2.5. Sequence Analysis

Using alpaca sequences as queries, search for RP sequences was performed in database accessible with the Basic Local Alignment Search Tool (BLAST) on the server of the National Center for Biotechnology Information (NCBI). The obtained nucleotide sequences were loaded into ORF on NCBI, translated into amino-acid sequences and aligned with CLUSTALW in DNAMAN.

2.6. Computer Sequences and Phylogenetic Analysis

The determined nucleotide and amino acid sequences were analyzed using BLAST program search of GenBank for homology with known sequences. The sequence data herein have been submitted to GenBank and the accession numbers assigned in phylogenetic analysis were presented in **Table 1**. Phylogenetic analysis was performed using CLUSTAL X program, the transition/transversion rates were calculated using PUZZLE 4.0.2 program. Bootstrappimg values were calculated using the modules SEQBOOT (random number seed: 13; 100 replicates). PROTDIST (distance estimation maximum likelihood; analysis of 100 data sets). NEIGHBOR (Neighbor joining and UPGMA method; random number seed: 13; analysis of 100 data sets) and CONSENSE from the PHYLIP package version 3.65. TREEVIEW version 1.6.0 was used for visualization of the trees.

3. RESULTS

3.1. Characteristerization of RP Genes

We got 7286 ESTs from 13,800 clones which have been deposited into NCBI (GenBank name: ASCD). In the cDNA library, 42 RP genes from skin cDNA library of alpaca were identified (**Table 1**). Animals possess three classes of acidic ribosomal P proteins: RPLP0, RPLP1

Table 1. Members of RP from the cDNA library of alpaca skin and accession number.

Ribosomal protein names	Abb.	Copies	Accession number
Ribosomal protein L3	RPL3	14	EY413791. *et al*
Ribosomal protein L34	RPL34	4	DQ399523. *et al*
Ribosomal protein L41	RPL41	5	ES609049. *et al*
Ribosomal protein L13	RPL13	6	EY414173. *et al*
Ribosomal protein L21	RPL21	8	EX656717. *et al*
Ribosomal protein L17	RPL17	7	EH219024. *et al*
Ribosomal protein L23	RPL23	6	EY414087. *et al*
Ribosomal protein L18	RPL18	10	EF066341. *et al*
Ribosomal protein L5	RPL5	18	EX656124. *et al*
Ribosomal protein L13a	RPL13a	4	EY414107. *et al*
Ribosomal protein L12	RPL12	11	EX656111. *et al*
Ribosomal protein L26	RPL26	8	ES263593. *et al*
Ribosomal protein L8	RPL8	12	EY414359. *et al*
Ribosomal protein L36	RPL36	2	EY414035. *et al*
Ribosomal protein S5	RPS5	2	ES263622. *et al*
Ribosomal protein S23	RPS23	5	EX656092. *et al*
Ribosomal protein S12	RPS12	14	ES263550. *et al*
Ribosomal protein S19	RPS19	15	ES263588. *et al*
Ribosomal protein S28	RPS28	4	EX161433. *et al*
Ribosomal protein S3	RPS3	14	ES263577. *et al*
Ribosomal protein L19	RPL19	11	DQ646398. *et al*
Ribosomal protein L31	RPL31	2	EX656064. *et al*
Ribosomal protein L11	RPL11	7	ES263543. *et al*
Ribosomal protein L28	RPL28	7	ES263579. *et al*
Ribosomal protein L6	RPL6	7	EX656116. *et al*
Ribosomal protein L27a	RPL27a	5	EY413783. *et al*
Ribosomal protein L14	RPL14	3	EX160914. *et al*
Ribosomal protein L10A	RPL10A	2	EV553863. *et al*
Ribosomal protein S13	RPS13	3	EH219016. *et al*
Ribosomal protein S2	RPS2	15	EY414375. *et al*
Ribosomal protein S17	RPS17	1	EY414252
Ribosomal protein S16	RPS16	9	ES263624. *et al*
Ribosomal protein S14	RPS14	6	EX656055. *et al*
Ribosomal protein L4	RPL4	9	EX656078. *et al*
Ribosomal protein S29	RPS29	4	ES263630. *et al*
Ribosomal protein S4, X chromosome	RPS4, X chromosome	8	EX161341. *et al*
Ribosomal protein S9	RPS9	7	EY414236. *et al*
Ribosomal protein S11	RPS11	10	ES263573. *et al*
Ribosomal protein S8	RPS8	13	ES263554. *et al*
Ribosomal protein S24	RPS24	14	EY414036. *et al*
Laminin receptor 1 (67 kD ribosomal protein SA)	LAMR1	11	ES263560. *et al*
Ribosomal protein Large P2	RPLP2	9	ES263580. *et al*

and RPLP2. Interestingly, RPLP1 and RPLP2 have their own specific characteristics on both expression profiling and amino acid composition by our analyses. In our expression profile, RPLP1 and RPLP2 were highly co-expressed in LND and keratinocytes, forming a sub-cluster. As only RPLP1 and RPLP2 form dimers in the silkworm, they may have gene expression machinery different from those of the other RP genes. This may indicate that RPLP0 is a specific gene not only for P proteins but also for the RP gene family. On the other hand, because all three P protein genes belonged to the main cluster in the study of synonymous codon composition, evolutionarily they might have been affected by selective pressure on codon usage along with other RP genes. From these results, we conclude that RPLP0, RPLP1, and RPLP2 are unique and specific genes compared with the major RP genes, but that these P protein genes are members of the RP gene family. Conserved regions with lengths of over 100 bp were found in regions upstream of the TSS in the following RPgenes: RPS2, RPS4X, RPS7, RPS10, RPS12, RPS14, RPS18, RPS23, RPS27A, RPS30, RPL6, RPL7, RPL10, RPL15, RPL17, RPL18, RPL19, RPL21, RPL22, RPL26, RPL27A, RPL32, RPL35, RPL35A, RPL36A, RPL40, and RPLP1. Most importantly, 14 RP genes were found to have conserved upstream regions of over 100 bp adjacent to the TSS. Conserved intronic regions with lengths of over 100 bp were found in RPS3, RPS6, RPS8, RPS19, RPS27, RPL7, RPL22, RPL23A, and RPL30.

Among those RP genes, there were 15 genes which have the complete ORF and we selected 9 members at random to be the subject for this study. All animals with those genes were searched basing on BLAST (**Table 2**). And we found that some RP genes have the another RP

Table 2. Accession numbers of nucleotide and amino acid sequences of RP for alignment and phylogenetic analysis.

Common name	Species	RPL19	RPL34	RPS5	RPS23
Alpaca	*Lama pacos*	EV554664	DQ407504	EX159979	EX161517
Rat	*Rattus norvegicus*	NM031103	X14401	X58465	NM078617
Mouse	*Mus musculus*	BC083131	BC070208	BC058690	NM024175
Human	*Homo sapiens*	NM000981	BC058118	U14970	BC070221
Pig	*Sus scrofa*	AF435591	CX056619	AU059899	AY461380
Cattle	*Bos taurus*	BC102223	BC103314	BT021032	BC102049
Frog	*Xenopus laevis*	BC041546	BC078541	NM001016992	BC088894
Cat	*Felis catus*	CX535488	-	-	-
Horse	*Equus caballus*	AY246727	XM001490008	XM001495360	AW260956
Sheep	*Ovis aries*	AY158223	DQ399303	-	-
Dog	*Canis familiaris*	AJ388522	XM_848652	XM856770	XM536303
Camel	*Camelus bactrianus*	-	-	-	-
Macaca	*Macaca mulatta*	XM001096265	XM001105069	XM001097103	XM001105094
Common name	Species	RPL41	RPL12	RPL17	RPS3
Alpaca	*Lama pacos*	assembly	assembly	EY413787	ES263577
Rat	*Rattus norvegicus*	NM139083	X53504	BC098644	NM001009239
Mouse	*Mus musculus*	NM_018860	BC090393	BC106165	AK148030
Human	*Homo sapiens*	NM_021104	NM_000976	BC066323	NM001005
Pig	*Sus scrofa*	-	AY550045	AB099057	DQ660373
Cattle	*Bos taurus*	BC141989	BC102693	BC102600	BC102090
Frog	*Xenopus laevis*	NM_001087207	BC041240	AY389972	NM203788
Cat	*Felis catus*	NM_001048157	-	AY738264	-
Horse	*Equus caballus*	AY246729	-	AY246726	-
Sheep	*Ovis aries*	-	-	DQ223555	-
Dog	*Canis familiaris*	XM_548346	XM_548510	XM856283	XM_846323
Camel	*Camelus bactrianus*	-	-	-	-
Macaca	Macaca	XM_001085279	XM001105090	XM_001110180	XM_001089599

genes domain, which is RPL12 with RPL11 domain, RPS11 with RPS17, RPS5 with S7, RPS12 with RPL7, RPL23a with RPS15p, RPL17 (L23) with RP1M, RPS9 with RPS4, RPS2 with RPS5, RPS29 with RPSN, RPS14 with RPSK.

These genes formed 3 main clusters. One cluster contained RPS5, RPL34 and so on. One cluster contained RPL13 and RPS19. The another cluster contained RPL18. In the first cluster, RPP2 and RPLP2 formed a sub-cluster. These genes were named according to their similarity to mammalian RP genes.

3.2. The Homology of RP Genes of Alpaca with Other Animals

Reference gene sequences were obtained from NCBI. Alignments were made with the program DNAMAN. The homology of 9 RP between alpaca and other animals was over 82% at the lever of nucleotide (**Table 3**). Of particular interest was the fact that RPL12 have the conserved RPL11 domain, RPS11 have RPS17 domain, RPS5 have RPS7 domain, RPL17 have RP1M domain, which suggested that RPL12, RPS11, RPS5 and RPL17 may have the similar function to RPL11, RPS17, RPS7 and RP1M, respectively.

3.3. The Phylogenetic Analysis of Alpaca Comparing with Other Animals Collected in GenBank

Phylogenetic analysis were conducted on the basis of amino acid sequences of ribosomal protein genes collected in this study and sequences from the GenBank for several other animals which could be classified into 5 major groups of Rodentia (mouse and rat), Primate (human and macaca), Perissodactyla (horse), Artiodactyla (cattle, sheep, pig, camel) and Carnivora (dog and cat). Additionally, we collected the sequences of frog to be aligned with these sequences. The alignments of these sequences with the homologous regions. The phylogenetic analysis of the sequences was carried out with the program package PHYLIP 3.65. The phylogenetic unrooted trees were constructed with maximum-likelihood (ML) and neighbor-joining (NJ) method. The outgroup special was not set and the multiple dataset was set 100. Because of the limited the species in GenBank, we tried to collect the representatives of every group. The trees of these RP with ML and NJ exhibited the same results which indicated that alpaca clustered together with rat and were more closely to rat on the evolutionary tree, since no RP genes were found in the camel and camelids (**Figure 1**).

4. DISCUSSION

RPS5 is highly conserved in all life forms [8,9]. Of particular interest was the fact that RPL12 have the conserved RPL11 domain, RPS11 have RPS17 domain, RPS5 have RPS7 domain, RPL17 have RP1M domain, which suggested that RPL12, RPS11, RPS5 and RPL17 may have the similar function to RPL11, RPS17, RPS7 and RP1M, respectively. RPL34, RPS5, RPL12, RPL17 and RPS3 of alpaca have the highly conserved regions with that of other counterpart species which may have an equally important translation role in those species and be required for the retention of activity of RP proteins. In past studies, the control mechanisms of gene expression and RP functions were believed to be identical [10]. This highly rate of sequence conservation among difference orders could be due to the highly selective pressure necessitated by the fundamental role of RP in translation function. We

Table 3. Identity of nucleotide and amino acid sequences of RP for alignment and phylogenetic analysis % nucleotide (amino acid) homology.

alpaca	rat	mouse	human	pig	cattle	frog	cat	horse	sheep	dog	camel	macaca
RPL19	99 100	94 100	87 100	89 100	88 100	82 95		89 0	89 100	85 98	-	88 100
RPL34	98 70	90 70	92 70		88 0	82 68					-	
RPS5	99 99	96 100	89 100		87 100					90 100	-	90 100
RPS23	99 0	93 0	92 0	90 0	91 0	82 0					-	
RPS3	100 100	96 100	90 99	88 99	87 99						-	
RPS11	100	93	90	91		83					-	
RPL12	99 98	95 98		90 98	89 98	84 98					-	
RPL17	89 99	89 99	90 100	95 99	95 99	82 96	93 100	92 84		92 100	-	90 100

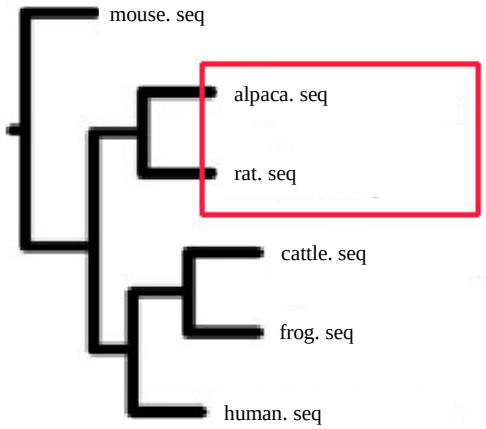

Figure 1. Phylogenetic analysis based on 8 RP amino acid sequences in **Table 3** with NJ and ML produced an evolutionary tree showing the relationship between alpaca and other species. Reference sequences are from GenBank and accession numbers are in **Table 2**.

found that RP genes were the highly conserved and stable characters that decides that RP can be used in the phylogenetic analysis. It has become clear that in *S. cerevisiae* the transcription of ribosomal protein genes, which makes up a major proportion of the total transcription by RNA polymerase II, is controlled by the interaction of three transcription factors, Rap1, Fhl1, and Ifh1 [11]. The RP genes that have characteristically poor TATA boxes [12]. The ATG initiation codon of two thirds genes is present in a C(G)ATG sequence. There also is a marked preference for a C or G before the initiation, while in chloroplasts the preference for a T before the initiation codon. We can speculate they represent an evolutionarily acquirement.

Analysis of agnathans will be necessary to determine the timing of emergence of RP relative to. The differences to those trees obtained with ribonuclease protein sequences can be explained by the influence of convergence of pancreatic RNases from hippopotamus, camel, and ruminants and by taking into account the information from third codon positions in the DNA based analyses. The evolution of sequence features of ribonucleases such as the distribution of positively charged amino acids and of potential glycosylation sites is described with regard to increased double-stranded RNA cleavage that is observed in several cetacean and artiodactyls RNases which may have no role in ruminant or ruminant-like digestion protein synthesis takes place at the ribosome, a ribonucleoprotein complex divided into two subunits that in total contains one third protein and two thirds RNA.

According to the arrangement of these genes of *E. coli*, RPS10 are followed by RPL3, RPL4, RPL23, RPL2, RPS19, RPL22, RPS3, RPL16, RPL29, RPS17 and these genes are separated by some bases spacer, but because of no genome of alpaca, it is a pity that we can not arrange those genes on genome. However, RP genes' high conservation may decide these genes' location like that of *E. coli*. As to the absent genes, they might not exit at the expected position on the genome, therefore, they are not always functional in alpaca skin, as some of them demonstrated in spinach. Another, they might exit and are functional, which had less copies and were not found.

The phylogenetic position of the alpaca became more interesting. Commonly used mitochondrial rDNA is one of the best molecular marker to resolve closer kinship between the species [13]. However, it is difficult to objectively phylogenetic analysis when only used a few genes as molecular marker because of the existence evolutionary rate difference between different genes. Therefore, it is necessary to find new molecular marker.

In our study, there was at least one species of ruminantia and suiform used as representatives to be aligned. Alpaca is monophyletic based on the phylogenetic tree of RPL17 and divergent closer to pig which appear to support the relationship with pig (suiform). As to the relationship of them, the consensus trees constructed with NJ and ML exhibited the same topology. Pig was the first divergent member of artiodactyla and alpaca can not really cluster into a group with pig in this tree because the divergence of the pig occurred before the gene duplication event that happened in an ancestor. In the analysis of 8 RP deduced amino acid sequences constructing phylogenetic trees, none can support the relationship with ruminantia. Therefore, using RP genes to support the close relationship between tylopoda, suiforms and ruminantia was not complete.

The placenta is essential for the success of therian mammalian reproduction. Intense selective pressure has shaped changes in placental anatomy and function during mammalian cladogenesis. Among placentals, rodents with their great fossile and extant diversities appear as a model group to understand the variance between dates derived from fossils and sequences. Molecular studies usually make the palaeontological dates for the origin of rodent clades older than about 25 - 55 million years [14]. These species were shown to have faster rates of sequence evolution [15] and it is known that contrasted substitution rates can severely affect divergence estimate. Rodents are also the most diversified mammals [16]. Using phylogenetic and statistical analyses of molecular and morphological data, it is demonstrated that the ancestral eutherian mammalian placenta had the distinctive features of 1) hemochorial placental interface, 2) a discoid shape, and 3) a labyrinthine maternofetal interdigitation. Alpaca and rat or mouse share these common features. These results reveal that the first eutherians had a deeply invasive placenta existed throughout the eutherian lineage that descended from the last common ancestor of placental mammals. The ancestral state reconstructions

demonstrate both clade-specific patterns of placentation and specific cases of convergent evolution within individual eutherian clades. In our study, of interest was that alpaca and Rodent were clustered into a group in RP genes phylogenetic trees. The high conservation of 8 RP genes from prokaryotes to eukaryotes suggested that this protein might have been subjected to a strong selective pressure during evolution, and its role might have a substantial biological meaning from prokaryotes to eukaryotes [17]. It is because of gene duplication of RP nearly at the same time and both of them face the approximate same evolutionary pressures. These data, therefore, point towards a very close genetic affinity between the alpaca and rodent. The implications of these data are potentially important for the way in which these genetic resources are managed in the future.

Like other genes of a gene family at some stage of evolution, RP genes have arisen by gene duplication. The evolution of genes belonging to the same gene family does not occur at random, but is controlled by a mechanism which is termed gene conversion resulting in concerted evolution of the genes. This meant that if a mutation occurs in one of the genes, it can be repaired by homologous recombination with the other genes [18].

5. CONCLUSION

The goal of this study was to add both more molecular data and analytical rigor to the phylogenetic study of basal relationship of alpaca and ruminant and pig. To the best of our knowledge, this is the first report describing the alpaca evolution by the RP genes. While it pointed towards a very close genetic affinity between the alpaca and rodents. The implications of these data are potentially important for the way in which these genetic resources are managed in the future.

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