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Mutations in S-cone pigment genes and the absence of colour vision in two species of nocturnal primate

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SUMMARY

Most primates have short-wavelength sensitive (S) cones and one or more types of cone maximally sensitive in the middle to long wavelengths (M/L cones). These multiple cone types provide the basis for colour vision. Earlier experiments established that two species of nocturnal primate, the owl monkey (*Aotus trivirgatus*) and the bushbaby (*Otolemur crassicaudatus*), lack a viable population of S cones. Because the retinas of these species have only a single type of M/L cone, they lack colour vision. Both of these species have an S-cone pigment gene that is highly homologous to the human S-cone pigment gene. Examination of the nucleotide sequences of the S-cone pigment genes reveals that each species has deleterious mutational changes: in comparison to the sequence for the corresponding region of the human gene, exon 4 of the bushbaby S-cone pigment gene has a two nucleotide deletion and a single nucleotide insertion that produces a frame shift and results in the introduction of a stop codon. Exon 1 of the owl monkey S-cone pigment gene likewise contains deletions and insertions that produce a stop codon. The absence of colour vision in both of these nocturnal primates can thus be traced to defects in their S-cone pigment genes.

1. INTRODUCTION

Colour vision requires at minimum the presence of two types of photopigment. A typical arrangement among mammals includes one or two types of cone containing photopigments absorbing maximally in the middle to long wavelengths (M or L pigments) and another type of cone whose resident pigment has maximum absorption in the short wavelengths (S pigments) (Jacobs 1993). In recent years the genes that specify the opsins of these and other photopigments have come under increasing scrutiny. Structural comparisons of cone opsin genes suggest that a divergence in the cone-photopigment line occurring perhaps 200 to 300 million years before present (Ma BP) yielded an S-cone pigment and an M- or L-cone pigment (Yokoyama & Yokoyama 1989). This event could have provided the photopigment basis for dichromatic colour vision. A second, more recent (*ca.* 30 Ma BP) divergence that is limited to the primate line is believed to have yielded the two separate M and L photopigment types that characterize the retinas of many Old World primates and provide the basis for trichromatic colour vision (Nathans *et al.* 1986; Yokoyama & Yokoyama 1989; 1990).

Although most primates are diurnal, a single anthropoid species and most of the prosimians are nocturnal. It has been suggested that the early primates were probably nocturnal (Martin 1990). If that is so, these contemporary nocturnal primates are of particular interest because they might provide a means of looking back to early stages in the evolution of primate colour vision. Accordingly, we have recently used electrophysiological procedures to characterize the photopigments in two species from this group of nocturnal primates: a platyrrhine monkey, the owl monkey (*Aotus trivirgatus*), and a prosimian, the thick-tailed bushbaby (*Otolemur crassicaudatus*). Like many other mammals, each of these species was found to have a single type of M/L cone photopigment. In each case this photopigment had a spectral peak of 543–545 nm. Despite an extensive search we failed to find any evidence for the presence of an S-cone pigment in either of these primates (Jacobs *et al.* 1993; Deegan & Jacobs 1994). Our results thus supported those of an earlier study in which attempts to label S cones with opsin antibodies in these primates were unsuccessful (Wikler & Rakic 1990). The presence of only a single cone type in these species predicts that neither should have cone-based colour vision. That prediction has been verified in behavioural tests conducted on the owl monkey (Jacobs *et al.* 1993).

If the S-cone photopigment gene and its opsin product is ancient in origin and seemingly common among contemporary mammals, then why do both these primates fail to have S-cone photopigment? One possibility is suggested by recent examinations of human tritanopes. Tritanopia is a colour vision defect which can be inherited as an autosomal dominant trait (Pokorny *et al.* 1979). The details of tritanopic colour matches and results from retinal electrophysiology (Padmos *et al.* 1978) are consistent with the in-

terpretation that the S-cone mechanism is absent or ineffective in these individuals. Recently, Weitz and his colleagues examined the genes for S-cone photopigment opsins in a number of tritanopic subjects (Weitz *et al.* 1992*a*, *b*). They discovered a total of three different point mutations in these genes that segregated with tritanopia leading them to conclude that any one of the amino acid substitutions encoded by these gene alterations were sufficient to interfere with the viability or fidelity of the S cones and that, thus, these mutational changes account for the absence of S-cone function. We wondered if a similar explanation might underlie the absence of S-cone function in these two species of nocturnal primates. That possibility was encouraged by results from a hybridization analysis showing that the owl monkey in fact has a gene that is highly homologous to the human S-cone photopigment gene (Jacobs *et al.* 1993). This paper describes results from a test of the hypothesis that both owl monkey and bushbaby have S-cone photopigment genes that have been rendered non-functional.

2. METHODS

Genomic DNA was isolated from peripheral blood leukocytes obtained from a male owl monkey and from a female bushbaby using techniques previously described (Neitz *et al.* 1995). In earlier electrophysiological studies both of these subjects had been verified as lacking a viable S cone. An aliquot of DNA was used in the polymerase chain reaction (PCR) to amplify segments of the S-cone pigment gene. The PCR components have also been described previously (Neitz *et al.* 1995). The primers used in the PCR are specified in table 1. The human S-cone photopigment gene derives from chromosome 7 and comprises five exons. We amplified exons 1, 3 and 4 because these are the exons which encode amino acid substitutions that are reported to cause tritanopia in humans. Primers 1 and 2 were used to amplify exon 4 of the S-cone pigment gene from both owl monkey and bushbaby. The thermal cycling conditions for primers 1 and 2 were: one cycle at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 61 °C for 45 s, and 72 °C for 45 seconds, followed by one cycle at 72 °C for 7 min. Primers 3 and 4 were used to amplify a DNA fragment that included a region upstream of the gene and extending into exon 1 of the S-cone photopigment gene from the owl monkey. The thermal cycling parameters for primers 3 and 4 were: one cycle at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min, followed by one cycle at 72 °C for 7 min. The fragment obtained from PCR using primers 3 and 4 was used in a second round of PCR with primers 5 and 6. Thermal cycling parameters for primers 5 and 6 were one cycle at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 45 s, 72 °C for 7 min, followed by one cycle at 72 °C for 7 min. A portion of exon 1 from the owl monkey S-cone gene was obtained by PCR amplification with primers 4 and 7. Exon 3 from the owl monkey photopigment gene was obtained by amplification with primers 8 and 9. For primer pairs 4 and 7, and for primer pairs 8 and 9, the thermal cycling parameters were 5 min at 94 °C for one cycle, followed by 30 cycles at 94 °C for 1 min, 56 °C for 45 s, 72 °C for 45 s, followed by a final cycle at 72 °C for 7 min.

Amplified DNA fragments were subjected to asymmetric PCR and then sequenced directly (Neitz *et al.* 1995). The amplified fragments were also ligated into a plasmid vector, and the resulting recombinant plasmids were isolated and sequenced. DNA sequencing was carried out using Sequenase version 2.0 kit (United States Biochemical) or cycle sequencing (Perkin Elmer).

3. RESULTS

As noted, we had earlier found that the owl monkey has a photopigment gene that is highly homologous to the human S-cone photopigment gene (Jacobs *et al.* 1993). An initial hybridization analysis that used a cDNA clone of the human S-cone pigment gene as a probe led to a similar conclusion for the bushbaby. One of the mutational changes associated with tritanopia had been mapped to exon 4 (Weitz *et al.* 1992*a*, *b*), and thus this portion of the S-cone photopigment gene was the initial target for examination.

Sequence ladders for a portion of exon 4 of the S-cone photopigment genes of owl monkey and bushbaby are shown in figure 1. These have been aligned with the corresponding sequence for the human S-cone photopigment gene. The sequence for this portion of owl monkey exon 4 is very similar to the human sequence and the handful of changes that are seen are not indicative of any serious defect. By contrast, this portion of exon 4 for the bushbaby does contain a serious defect. In the bushbaby gene, in addition to a number of other nucleotide changes, nucleotides 778 and 779 are deleted and a T residue is inserted at position 793. The deletions and insertion produce a frame shift which results in the introduction of a stop codon (as indicated in figure 1). These changes in the nucleotide sequence for this portion of exon 4 of the bushbaby S-cone photopigment gene are shown in figure 2*a*.

Further examination of the owl monkey S-cone photopigment gene was undertaken by additional

Table 1. *Primers used to amplify gene segments*

primer	sequence	location	strand
1	5′TTG CAG CTC AGC AGC AGG AGT 3′	exon 4	+
2	5′ CTG CTT ATT CAT GAA GCA GTA GAT 3′	exon 4	−
3	5′ CAG TTG TGC CAG AAG CCA AAA 3′	upstream, −378	+
4	5′ AGC CCT CCA AAG CAC AAA CAT 3′	exon 1	−
5	5′ AAT CCC AAA CTT TGT CCT TGG 3′	upstream, −134	+
6	5′ AAG GAA GAC AGT GCC CAT GAA 3′	exon 1	−
7	5′ GCC ACA CTG CGC TAC AAA AAG 3′	exon 1	+
8	5′ CAG TGT TCC TGT GGC CCT GAC 3′	exon 3	+
9	5′ GGC CCT CAG GAG CTG AGT GTA 3′	exon 3	−

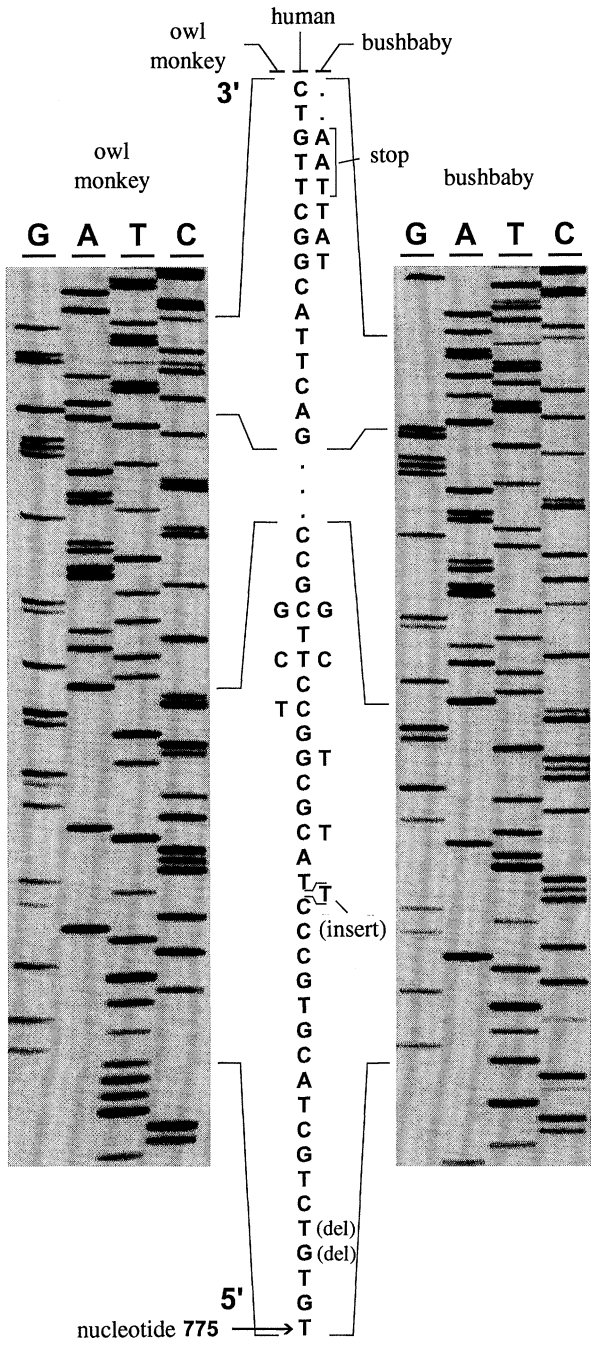

Figure 1. Sequence ladders for a portion of exon 4 from the S-cone opsin genes of bushbaby and owl monkey. Compared to the human sequence, the ladder for the bushbaby contains a region where there is a deletion of two nucleotides, a nucleotide insertion, and a stop codon. The corresponding sequence for the owl monkey is shown. Portions of the human sequence are written out in the center column. Only the differences between the human and non-human sequences are shown.

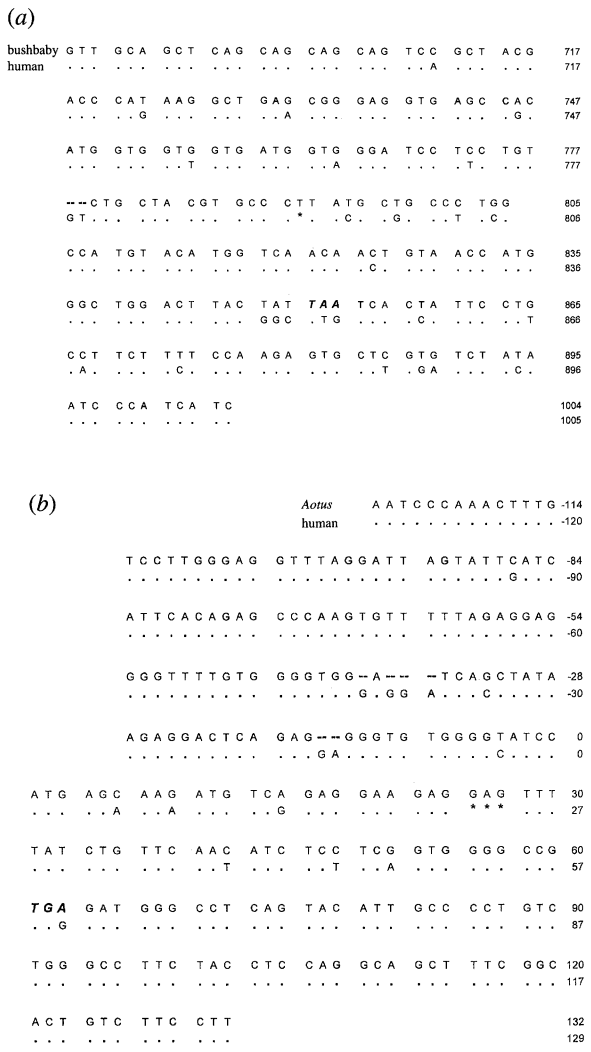

Figure 2. Defects found in the S-cone pigment gene sequences of the owl monkey and the bushbaby. Partial gene sequences are shown for both species. The sequences have been aligned with the corresponding regions of the human S-cone pigment gene sequence. Differences between the human and monkey sequences are shown and positions of sequence identity are indicated by (.). Locations of nucleotide insertions in the owl monkey and bushbaby sequence are indicated by an asterisk in the human sequences. Nucleotide deletions are denoted by (–). The nucleotide numbering system is based on the coding sequence. Figure 2*a* shows the sequence of a region of exon 4 from the human and bushbaby S-cone pigment genes. Figure 2*b* shows the sequence upstream region and part of exon 1 of the S-cone pigment gene from the owl monkey and the human.

sequencing of portions of exons 1 and 3. A number of nucleotide differences were found between owl monkey and human S-cone genes in the regions that were examined and these differences are listed in table 2. In addition, a clear defect was discovered in exon 1. Figure 2*b* shows the sequence of the upstream region and part of exon 1 that contains this defect. In the region sequenced, there were six nucleotide deletions upstream of the start codon in the monkey gene relative to the human gene. Within the coding region, there were several silent nucleotide substitutions, a three base-pair insertion, and a nonsense mutation.

4. DISCUSSION

Although S-cone photoreceptors are a characteristic feature of most primate retinas, previous results showed that the retinas of both the owl monkey and the bushbaby lack cones containing S pigment. The present investigation has revealed a proximate reason for the absence of S cones in these animals: the S-cone photopigment genes of representatives of both species

Table 2. *Nucleotide sequence differences identified between human and owl monkey S-cone pigment genes*

(The DNA fragments that were examined extend from nucleotides 172–234, 677–678, and 689–903. The numbering system is based on the human cDNA and starts with the first nucleotide of the first codon. The amino acid difference column is left blank in those cases where the nucleotide difference is silent.)

nucleotide position	human	*Aotus*	amino acid difference
256	T	C	Phe86Leu
585	C	T	
596	C	A	Ser199Tyr
630	T	C	
631	G	A	Val211Met
717	G	A	
774	C	T	
800	C	T	Ala266Val
802	T	C	Phe268Leu
804	C	G	
822	C	T	
840	G	A	
867	A	G	
883	G	T	Ala295Ser

contain deleterious mutational changes that introduce premature stop codons. Although the nature of the mutational changes is different for each species, the bushbaby and owl monkey resemble human tritanopes in the sense that there is a direct genetic explanation for S-cone failure. A significant difference between tritanopes and these two non-human primates is in the incidence of the genetic defect. Tritanopia is rare, affecting at most about 1 in 500 individuals (van Heel *et al.* 1979). In contrast, it appears that both bushbabies and owl monkeys routinely have S-cone gene defects. In the earlier electrophysiological studies each of the subjects examined (six owl monkeys, three bushbabies) were found to lack S cones (Jacobs *et al.* 1993; Deegan II & Jacobs 1994) and the failed attempts to label S cones with cone-specific antibodies involved four animals of each species (Wikler & Rakic 1990). Further, the genetic analysis indicates that these animals are homozygous for the defects and this also suggests that these defects are universal. In the present experiments the sequences for the S-cone pigment genes were determined by direct analysis of DNA amplified by PCR. The sequence so obtained is a consensus sequence representing both copies of the autosomal S-cone genes. If the two copies of the gene differ in nucleotide sequence this would be apparent in the autoradiograms as bands in two lanes at one nucleotide position in the sequence ladder. Alternatively, if the two autosomal copies of the gene are the same, then a band appears in only one lane at each nucleotide position. The latter was observed, indicating that the two autosomal copies of the S-cone pigment gene are identical over the region sequenced and that each animal must be homozygous for the genetic defects.

Hunt and colleagues have recently reported the deduced amino acid sequence of the S-cone opsin from another New World monkey, the common marmoset (*Callithrix jacchus*) (Hunt *et al.* 1995). Unlike the owl monkey, marmosets have a population of functional S cones (Travis *et al.* 1988). There are two insertions in the marmoset photopigment relative to that of the human photopigment: glutamic acid at position 9 and proline at position 28. The owl monkey has the former insertion, but not the latter. In addition, at least one copy of the marmoset S-cone gene specifies alanine instead of serine at the human position numbered 214 (Hunt *et al.* 1995). Interestingly, this is a position that is mutated in tritanopic humans. Three different amino acid substitutions, G79R, S214P, and P264S, have been shown to be associated with tritanopia. Individuals heterozygous for any one of these three mutations can be tritanopic (Weitz *et al.* 1992*a*, *b*). Apparently the S214A substitution in the marmoset does not result in a tritan defect as does a less conservative substitution (S214P) at the homologous position in humans. When they are optimally aligned, comparison of the owl monkey and human S-cone photopigment genes reveals that the owl monkey gene specifies the same amino acids as are found in normal human S-cone photopigment at all three of the positions involved in tritanopia. For the codons we examined in the owl monkey S-cone pigment gene, the sequence encoded is remarkably similar to that of the human: a 97% amino acid identity (200 identical out of 206 examined). The similarity of the owl monkey to the marmoset is slightly lower: 94.7% amino acid identity (195 identical out of 206). However, the marmoset and the owl monkey pigments do share some sequence similarities that are not found in humans or in another Old World primate, the talapoin monkey (*Cercopithecus talapoin*) (Hunt *et al.* 1995). These are at amino acid positions 199, 268, 295 (human numbering system).

Studies of the opsin genes of cavefish (*Astyanax fasciatus*) have revealed a cone-opsin defect that may bear some similarity to what is found in bushbaby and owl monkey (Yokoyama *et al.* 1995). Some populations of this fish are cave-dwelling and blind. An M cone opsin gene in one population of the blind fish has a deletion of 12 nucleotides corresponding to four consecutive codons in a region of the protein that is believed to be critical for visual pigment function. The argument in the case of these blind cavefish is that they have become adapted to a lightless environment in which vision is unnecessary, and that in the absence of selective pressure for maintenance of the capacity the visual pigment genes accumulate missense and nonsense mutations (Yokoyama *et al.* 1995).

Our results pose intriguing questions about the possible relationship between the nocturnal lifestyles of these primates and their S-cone opsin defects. Unlike the blind cavefish, where there has been a complete loss of vision, bushbaby and owl monkey have lost S-cone based vision but otherwise see well. Thus, both of these primates have been shown to be capable of making a range of acute visual discriminations in cases where the animals are provided with luminance differences as cues for spatial or temporal discriminations (Silver 1966; Ward & Doerflein 1971; Jacobs 1977; Jacobs *et*

al. 1993). The retention of these capacities in the absence of S cones presumably reflects the fact that signals from primate S cones contribute little to visual sensitivity or to spatial or temporal resolution. The role of the S cones, rather, is primarily that of providing a signal that allows for one dimension of colour vision (Mollon 1991). Both of these nocturnal primates are mostly active only under quite low light levels (Charles-Dominique 1971; Wright 1989) Consequently, colour vision might provide no advantage in these primates since the mechanisms in the visual system that underlie colour vision (cone photoreceptors and spectrally-opponent pathways) require moderately high light levels for their operation.

It is unclear how these S-cone gene defects are manifested in the retina. One possibility is that there is no gene expression at all. However, an example drawn from the study of another member of the G-protein coupled receptor family provides another possibility. The human genome contains two genes that share high homology with the dopamine D5 receptor. Each of these contain defects that render them incapable of encoding a functional receptor (Nguyen *et al.* 1991*a*; Grandy *et al.* 1991). Surveys of human brain cDNA libraries indicate that these defective genes are transcribed in the human brain (Nguyen *et al.* 1991*b*; Weinshank *et al.* 1991). The mRNA would be capable only of specifying a truncated receptor because of frame shifts relative to the functional D5 receptor gene. These foreshortened proteins could not function as G-protein coupled receptors. The S-cone pigment genes of owl monkey and bushbaby could be transcribed in a manner similar to the mutant dopamine genes so that the cells that would have been destined to become S cones do express the truncated opsins. If so, by analogy to retinitis pigmentosa (RP) in which mutations in the rhodopsin gene lead to photoreceptor death (Dryja *et al.* 1990; Humphries *et al.* 1994), it might be expected that photoreceptors attempting to express the mutant S-cone pigment gene would die. In contrast to autosomal dominant RP, in which affected individuals are heterozygous for the defect, these monkeys are homozygous for their gene defect. A doubled dose of the mutant protein might cause early cell death, perhaps while the eye is still developing. If that happened, S cones containing S-cone pigment would be absent in the adult just as the evidence from immunohistochemical studies suggests. In any case, what we have found is a defect in the gene specifying the visual pigment protein of the S cones. The identified defect obviates function in the S cones of these nocturnal primates, but it is still an open question as to the fate of the S-cone cells.

Two kinds of hypotheses might be advanced to relate nocturnality and the absence of S-cone function in these primates. By analogy to the work on the cavefish, the first would suggest that if S cones offer no advantage in nocturnal environments, deleterious mutational changes in these pigment genes do not affect fitness and there is an absence of selection against such changes. The second is that, somehow, a functional S-cone system may be detrimental in eyes specialized for nocturnal vision so that the inactivating genetic changes increase fitness. The two scenarios suggest different patterns of detrimental gene mutations. In the absence of an influence on fitness one might expect random mutations in the S-cone pigment genes with considerable variations across individuals. Alternatively, if inactivating the S cone increases fitness then a single inactivating mutation might spread to become a species trait. In the present study random mutations would show up as differences between the two autosomal copies of the gene in the consensus sequence. In the owl monkey, the subject in which a majority of the gene was sequenced, there were no differences in sequence between the two copies; rather, both copies showed the same, single, deleterious point mutation. This observation leaves open the possibility that deleterious mutations in the S-cone pigment genes increase fitness in these nocturnal primates. Examination of the genes from additional members of both species could shed light on the question. In any case, it is worth remembering that the nocturnality of bushbabies and owl monkeys are believed to have quite separate histories, the latter having had diurnal ancestors (Martin 1990). They thus could have taken quite separate routes to their current S-cone loss.

Finally, we note that there are many species of nocturnal mammals and in most cases it is not known what cone photopigments their retinas may contain (Jacobs 1993). On the basis of the results reported here it seems conceivable that among them are additional species in which gene mutations have rendered their S-cone pigments non-functional and consequently ablated any prospects for colour vision.

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