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Activation of Two *Cynops* Genes, *Fork Head* and *Sonic Hedgehog,* in Animal Cap Explants[1]

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We have isolated cDNAs of *sonic hedgehog* (*shh*) and *fork head* from *Cynops* (Japanese newt) embryo. Their expression was investigated in relation to mesoderm induction by activin and basic fibroblast growth factor (bFGF). Different from these homologs in *Xenopus,* they are activated not only by activin but also by bFGF in animal cap explants, showing a difference of animal pole cells in responsiveness to bFGF between *Cynops* and *Xenopus.* We also investigated the involvement of *fork head* in *shh* activation. The expression of *shh* was activated in animal caps which overexpressed either of two *Xenopus fork head* homologs, *pintallavis/XFD-1* or *XFKH-1/XFD-1′,* indicating that *fork head* up-regulates the transcription of *shh* in *Cynops* embryo.

Amphibian embryos have been frequently used in the study of developmental biology. Studies of the *Xenopus* over the last decade have revealed several important molecules and phenomena involved in mesoderm induction (1). Among them, the effects of activin and bFGF on animal caps have been intensively investigated. Activin can induce dorsal mesoderm, while bFGF can induce only ventral mesoderm. However, little is known about the molecular interactions during embryogenesis of newt. In this study, we have focused on the activation of two genes, *fork head* and *shh,* in *Cynops* embryos. In *Xenopus,* they are initially expressed in the mesodermal region (2–6), and the expressions of *fork head* homologs can be induced by activin in animal cap explants (2–5).

It is currently believed that *shh* displays a wide range of activities in vertebrate development. *Shh* regulates dorso-ventral patterning of the neural tube and the somites, the anterior-posterior axis of the limb bud (7,8), and the proximo-distal axis of developing eyes. (9). With respect to the mechanism of the transcriptional activation of *shh,* it is supposed that *fork head* homologs (mouse *HNF-3β, Xenopus pintallavis/XFD-1,* and zebrafish *axial*) expressed in the notochord activate the expression of *shh,* which in turn activates *fork head* homologs in the floor plate of the ventral neural tube in a positive feedback loop (10). There is enough direct evidence to support the notion that *shh* activates the transcription of *fork head* homologs (11–14). Although there are several indicative observations of the reversed regulation control (activation of *shh* by *fork head*) (11, 15–18), no direct evidence showing the activation of *shh* transcription by expressing *fork head* homologs has been described. In the present study, we examined whether *fork head* activates the expression of *shh* indeed.

MATERIALS AND METHODS

Embryos. Eggs of *Cynops pyrrhogaster,* which were obtained from the oviducts of females injected with human chorionic gonadotropin, were fertilized *in vitro* with seminal duct homogenates. Embryos were dejellyed manually and were cultured in 0.2×MMR (1×MMR is 100mM NaCl, 2mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM Hepes, pH 7.4). They were staged

[1] Sequences of the genes described can be accessed in GenBank (*shh,* D63339; *fork head,* D64021; *goosecoid,* D64022; *brachyury,* D64018; EF1-α, D64019).

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according to Okada and Ichikawa (19). Eggs of *Xenopus laevis*, which were obtained from females injected with human chorionic gonadotropin, were fertilized *in vitro* with testis homogenates. They were dejellyed by treatment of 2% cystein (pH 8.0), and were cultured in 0.2×MMR. They were staged according to Nieuwkoop and Faber (20)

cDNA cloning. The PCR product of *shh* was initially isolated from cDNAs originated from *Cynops* neurula RNA as template. The primer sequences used were: shh-U, 5′-CC(ACGT)GA(CT)AT(ACT)AT(ACT)TT(CT)AA(AG)GA(CT)GA(AG)GA(AG)-3′; shh-D, 5′-GA(CT)TC(AG)TA(AG)TA(ACGT)ACCCA(AG)TC(AG)AA-3′. A 1.88 kb cDNA of *shh* was isolated from a *Cynops* neurula cDNA library using the modified enrichment procedures described by Takabatake, et al. (21). In short, the desired plasmid in the sublibraries was identified by PCR amplification, instead of the two-dimensional gel electrophoresis of the proteins transcribed and translated *in vitro.* The primer sequences used for RT-PCR assays were: shh-1, 5′-ACAGGCTGATGACCCAGAGG-3′; shh-2, 5′-GGCCAGCATGCCATACTTGC-3′. Information about the nucleotide sequences of *Xenopus shh* were obtained from GenBank database (U26314, L39213, L35248). The primer sequences used for the amplification of *shh* in *Xenopus* were: Xshh-1, 5′-TCCGTGATGAACCAGTGGCC-3′; Xshh-2, 5′-GCCACT GAGTTCTCTGCTTT-3′. The primer sequences used for the amplification of *fork head* are described in Fig. 1. Primers used for the other markers, EF1-α, *goosecoid* and *brachyury,* were: EFI-α-1, 5′- GTCATTGGACA(CT)GT(ACGT)GA(CT)TC(ACT)GG(ACGT)AA-3′; EF1-α-2, 5′-GGGGGTTC(AGT)GT(AGT)GA(AG)TCCAT(CT)TT(AG)TT-3′, gsc-1, 5′-CACCAGTGCCTCACCAGATG-3′; gsc-2, 5′-GTGCCCACATCTGGGTACTT-3′; bra-1, 5′-GAGCTCACCAA(CT)GA(AG)ATGAT-3′; bra-2, 5′-GAGACAGG(AG)TC(CT)TTCATCCA-3′. They were used in both cases of *Cynops* and *Xenopus.* Besides *shh,* more than three independent clones for each homolog were isolated and used for sequence confirmation. To obtain the cDNAs corresponding to the open reading frames of *Xenopus pintallavis/XFD-1* (3,4) and *XFKH-1/XFD-1′* (2,4), the cDNA mixture prepared from *Xenopus* early gastrula RNA was amplified by PCR with the following primers corresponding to the conserved sequences between *pintallavis/XFD-1* and *XFKH-1/XFD-1′*: pfk-1, 5′-CGGAATTCTTGTTGGAGAAAC-CATACCTC-3′; pfk-2, 5′-CGGGGTACCTGTTTAAAGGGAGCTGAGGAT-3′. Amplified fragments were cloned into pGEM3Zf(−) using the sites for the restriction enzymes of *Eco*R I and *Kpn* I. After sequence analysis of the isolated clones, plasmids containing the fragments of *pintallavis/XFD-1*and of *XFKH-1/XFD-1′* were selected.

RT-PCR assays. Total RNAs were isolated by acid guanidinium thiocyanate-phenol-chloroform method (22). After precipitation by isopropylalchol, they were dissolved in water, containing *E. coli* ribosomal carrier RNAs (Boehringer) in the cases for animal cap explants, and mixed with an equal volume of 8M LiCl. After a four hour incubation on ice, precipitates obtained by centrifugation were further treated by DNase I (Boehringer). Total RNAs (0.2μg) were used as templates to generate first strand cDNA except for the animal cap assays, in which RNAs from one animal cap equivalent were used. Reverse transcription was carried out in a 20 μl reaction using MMLV-reverse transcriptase (BRL) and oligo dT as the primer. One tenth of each cDNA reaction mixture was used in each set of PCR reactions. PCR reactions were carried out in a 25 μl reaction volume in the presence of trace [α-^{32}P] dCTP. About 25% of each reaction were resolved by electrophoresis on 9% polyacrylamide gels. After an initial 3 minute denaturation at 94°C, reactions were cycled through 1 minute at 55°C, 2 minutes at 72°C, and 1 minute at 95°C. After 21 cycles for EF1-α or 24 cycles for other primer sets, the final extension was carried out for 5 minutes at 72°C. Linearity was tested on serial dilutions of cDNAs by analyzing radioactive signals with a Fujix BAS2000 analyzing system.

Animal cap assays. Cynops and *Xenopus* embryos were placed at 20°C or 23°C, respectively, for the periods indicated in the results. Animal caps were dissected and incubated in 1xMMR containing 25 μg/ml gentamycin on a bed of 1% 0agarose. To study induction by activin and bFGF, 0.1% BSA was included in the medium. Caps from *Cynops* embryos were incubated for 3 hr in 50 ng/ml of activin A or bFGF. Caps from *Xenopus* embryos were incubated for 40 min in 10 or 100 ng/ml of bFGF. Human activin A was a gift from Dr. Yuzuru Eto. Human and bovine recombinant bFGF were purchased from Amasham (Lot 14) and Toyobo (Lot 7457) respectively.

In vitro transcription and translation. The template plasmids for *pintallavis/XFD-1* and *XFKH-1/XFD-1′* were digested with *Kpn* I, blunt ended by T4 DNA polymerase and transcribed by T7 RNA polymerase to generate sense capped transcripts. The template plasmid for Lac Z mRNA (23) was digested with *Xho* I and transcribed by SP6 RNA polymerase. *In vitro* translation was performed using rabbit reticulocyte lysate (Amasham). 0.25 μg of rabbit globin mRNA (BRL) and capped transcripts of *pintallavis/XFD-1* and *XFKH-1/XFD-1′* were translated, in the presence of trace [^{35}S]-methionine, according to the manufacture's recommendations. Equal amount of them were separated by 12% SDS polyacrylamid gel electrophoresis.

RESULTS

To obtain the PCR-generated fragment of *Cynops* homolog to *pintallavis/XFD-1*, we used cDNAs prepared from *Cynops* gastrula RNA as templates and primers corresponding to the highly conserved nucleotide sequences between *pintallavis/XFD-1* and *XFKH-1/XFD-1′* within the *fork head* domain. *Pintallavis/XFD-1* and *XFKH-1/XFD-1′* are closely related and probably representing pseudo-allelic variants (4). The amino acid sequence predicted from isolated clones (Figure 1A) had the highest similarity (94%) with *pintallavis/XFD-1* among the *Xenopus* homologs (2–5)

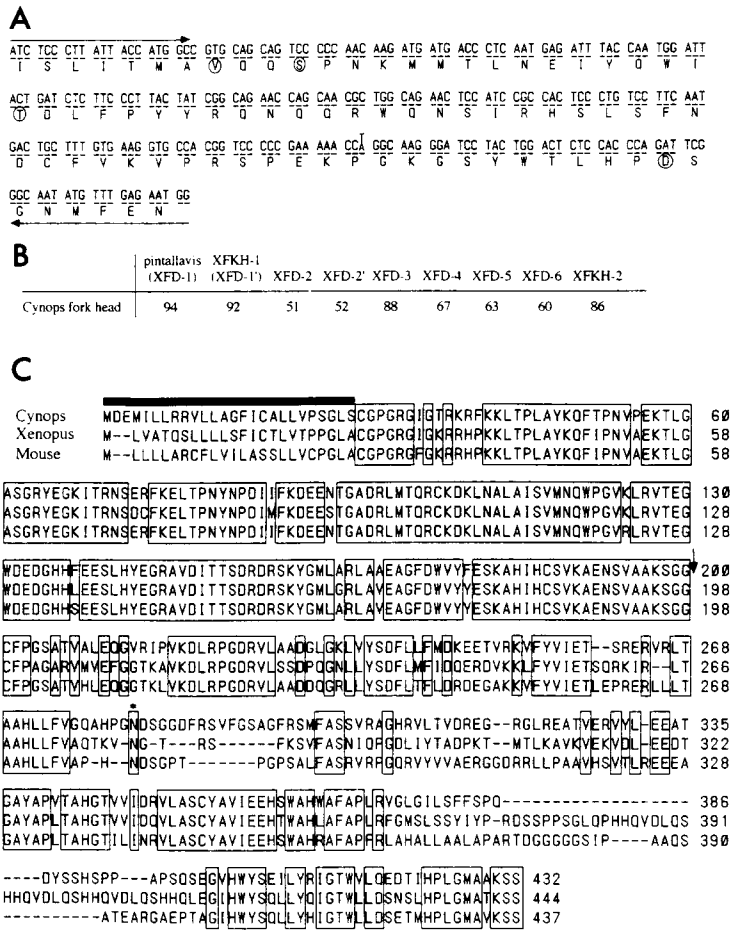

FIG. 1. (A) Nucleotide and deduced amino acid sequence of *fork head.* Arrows show the primer sequences used for the amplification by PCR. Two nucleotides drawn in one position indicate heterogeneity among the clones obtained from PCR-generated fragments and the lower one shows the minority. Positions of the amino acids enclosed with a circle indicate differences with the *pintallavis/XFD-1*. (B) Percentages of aa identity between *Cynops fork head* and *Xenopus* homologs (2–5). (C) Predicted amino acid sequences of *shh* of *Cynops, Xenopus* (6) and Mouse (12) are aligned. Residues, carboxy-terminal to the signal sequence, identical in all three sequences are boxed, and a dash indicates a gap in the alignment. The predicted amino-terminal signal sequence indicated by the solid black bar, ends just before the highly conserved CGPGRG sequence. Predicted site of N-linked glycosylation is indicated by asterisk, and the site of autoproteolytic cleavage presumed in *X-shh* is indicated by the arrow (6).

(Figure 1B). For *shh*, a 1.88 kb cDNA containing a single long open reading frame was isolated from a neurula cDNA library. Conceptual translation of this open reading frame predicted a protein of 432 amino acids that is closely related to the *shh* of other species (Figure 1C). Comparing the residues carboxy-terminal to the signal sequence with mouse homologs, it had 72, 59, and 56% identities with *Sonic*, *Indian*, and *Desert* classes, respectively. Furthermore, RT-PCR products for EF1-α, *goosecoid* and *brachyury* were also obtained as described in "Materials and Methods" and were used as control markers.

To address the issue of stage-specific expression, we performed RT-PCR analyses of the total RNA from various stages of whole embryos (Figure 2). *Cynops* genes show expression profiles essentially predicted from data obtained from *Xenopus* and other species. In brief, *fork head*, *goosecoid*, and *brachyury* are activated at, or shortly after, the midblastula transition. They peak

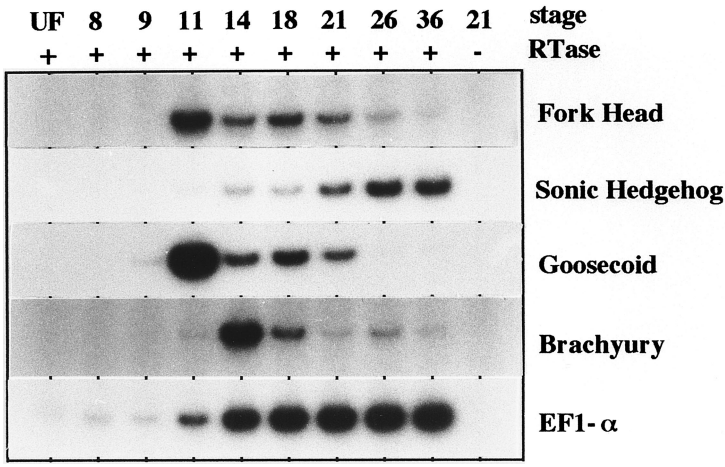

FIG. 2. Temporal expression of *fork head, shh, goosecoid, brachyury,* and *EF1-α* during embryonic development assayed by RT-PCR. "UF" and numbers refer to embryonic stages. UF, unfertilized egg; 8, morula; 9, midblastula; 11, early gastrula; 14, late gastrula; 18, mid-neurula; 21, late neurula; 26, tail bud; 36, larva with fore limb bud.

during gastrulation, with a delay of *brachyury,* and the levels decline at the following stages. *Shh* mRNA gradually increases during and after gastrulation. EF1-α mRNA is visible at a low level in the first three tracks (egg to blastula stages) and then increases during the gastrula stages.

The expressions in animal caps treated with activin or bFGF were examined by RT-PCR (Figure 3A). Caps were dissected from late blastula embryos (39 hr after fertilization), incubated for 3 hr in 50 ng/ml of activin A or bFGF, and harvested when sibling control embryos reached early gastrula (st.12b) or mid-neurula (st.18). Both activin and bFGF induced the expression of *fork head* and *shh.* Comparing the levels of activation, *fork head* and *brachyury* responded to bFGF earlier than *shh* and *goosecoid. Fork head* and *shh* were faintly expressed in untreated caps harvested at mid-neurula and early gastrula, respectively. Judging from the fact that *goosecoid* and *brachyury* were not detected in these untreated caps and from the reproducibility of patterns, we believe that these slight expression were not artifacts caused by the contamination of mesodermal or endodermal cells during the dissection. These expressions may correspond to the following observations in *Xenopus: shh* showed a low level of expression throughout the animal pole in the early gastrula embryo (6) and one of *fork head* homologs, *XFKH2,* was expressed in untreated caps after a certain period of cultivation (5).

The activations of *fork head* and *shh* by bFGF were unexpected results, because it had been reported that *Xenopus fork head* homologs, *pintallavis/XFD-1* and *XFKH-1/XFD-1′,* are not activated by bFGF (2–4) and they are considered as the transcriptional regulator of *shh* (10). To know whether *shh* in *Xenopus* is also induced or not by bFGF, the expression of *shh* in animal caps of *Xenopus* treated with bFGF was also examined (Figure 3B). Caps were dissected from st.9 blastula embryos (7 hr after fertilization), incubated for 40 min in 10 or 100 ng/ml of bFGF, and harvested when sibling control embryos reached mid-gastrula (st.11) or neurula (st.15). While bFGF could increase the expression of *brachyury,* it did not activate the expression of *shh* at neither concentrations.

To investigate the effect of *fork head* on *shh* expression, the overexpression experiments of two *Xenopus fork head* genes, *pintallavis/XFD-1* and *XFKH-1/XFD-1′,* were carried out. The template plasmids to synthesize messenger RNAs of these genes were constructed as described in Materials and Methods. The capped transcripts prepared from the plasmids were actively translated in rabbit reticulocyte lysates. The calculated molecular weights of *pintallavis/XFD-1* and *XFKH-1/XFD-1′* are 44.51 kd and 44.48 kd, respectively (Figure 4A). Animal caps were dissected from the injected

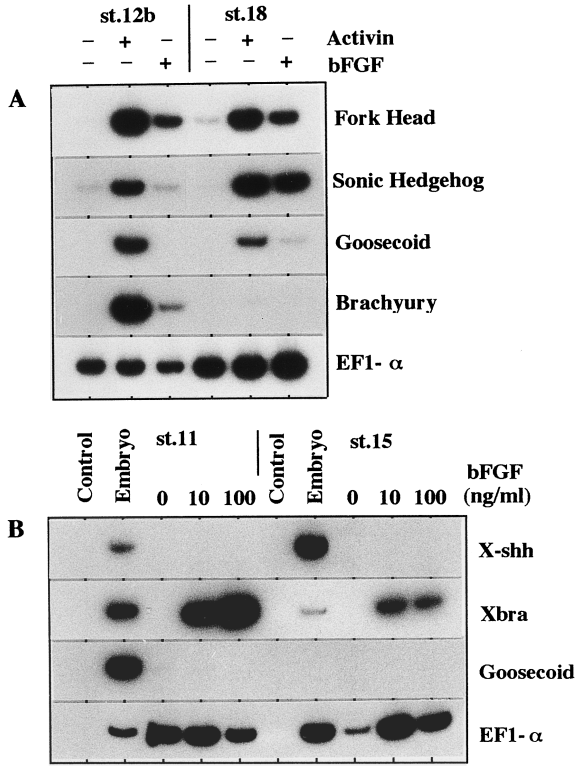

FIG. 3. A: Effects of activin and bFGF on *Cynops* ectoderm. Late blastula stage (39 hr postfertilization) *Cynops* animal caps were exposed to 50 ng/ml of activin A, bFGF, or a control solution for 3 hr. After four exchanges of the medium, they were cultured until the sibling control embryos reached early gastrula (st.12b) or midgastrula (st.18). Then they were harvested and analyzed by RT-PCR assay. B: Effects of bFGF on *Xenopus* ectoderm. Animal caps dissected at stage 9 were exposed to 0, 10, or 100 ng/ml of bFGF for 40 min. After four exchanges of the medium, they were cultured until the sibling control embryos reached midgastrula (st.11) or neurula (st.15). Then they were harvested and analyzed by RT-PCR assay. The lane labeled "Embryo" is 0.2 μg of total RNA from sibling control embryos (st.12b and st.18) used as a positive control. The lane marked "Control" contains all the ingredients of "Embryo" except for reverse transcriptase.

embryos at midblastula (33 hr after fertilization), harvested when sibling control embryos reached midgastrula (st.13a) and analyzed by RT-PCR. As shown in Figure 4B, the transcriptional level of *shh* was clearly up-regulated in the animal caps injected with *pintallavis/XFD-1* and *XFKH-1/XFD-1′*, but not in the caps from uninjected and Lac Z mRNA injected embryos.

DISCUSSION

We showed that *fork head* and *shh* in *Cynops* are induced in animal caps both by activin and bFGF. Their activation by bFGF is not in line with those of *Xenopus.* We confirmed the reproducibility using another bFGF as described in "Materials and Methods" (data not shown). In addition, *Cynops* genes were also induced by bFGF in a case of animal caps dissected from midblastula embryo (data not shown), and all of the predicted amino acid sequences of *fork head* obtained from six independent clones (three were originated from activin-induced caps and the remaining three from bFGF-treated caps) were completely identical to the sequence in Figure 1A. Furthermore, the activation of *shh* by bFGF were also confirmed using additional two sets of other primers in RT-PCR (data not shown). From these observations, we concluded that *Cynops* genes can respond to bFGF differently from those of *Xenopus* in animal cap explants.

It is noticeable that *goosecoid* was also slightly activated by bFGF in contrast to the observation

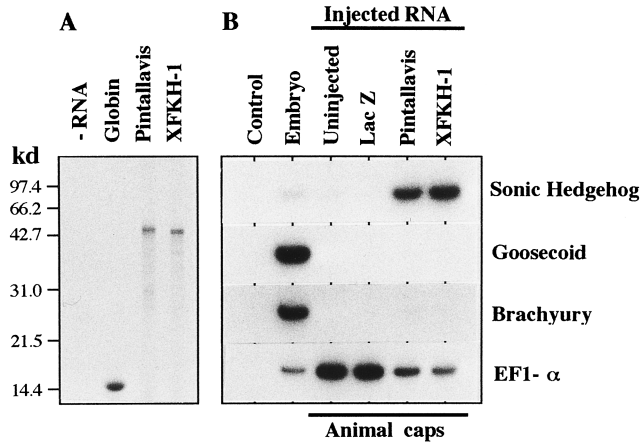

FIG. 4. A: *In vitro* translation of capped synthetic RNAs encoding *pintallavis/XFD-1* and *XFKH-1/XFD-1′* using rabbit reticulocyte cell free system. Rabbit globin mRNA was also translated as a positive control. They were fractionated by SDS-PAGE. B: Expression of *pintallavis/XFD-1* or *XFKH-1/XFD-1′* in *Cynops* animal caps leads to the activation of *shh.* Embryos were injected in the animal pole of both blastomeres at the 2-cell stage with 1 ng of control Lac Z, *pintallavis/XFD-1* or *XFKH-1/XFD-1′* RNA. At the blastula stage (33 hr postfertilization), animal caps were isolated, cultured until the sibling control embryos reached midgastrula (st.13a), and the expression of *shh, goosecoid* and *brachyury* was assessed by RT-PCR. The lane labeled "Embryo" is 0.2 μg of total RNA from sibling control embryos (st.13a) used as a positive control. The lane marked "Control" contains all the ingredients of "Embryo" except for reverse transcriptase.

in *Xenopus* (24). This can be explained by the preceding expression of *fork head* and *brachyury* in bFGF-treated caps, because *pintallavis/XFD-1* and *brachyury* cooperatively cause a transition from the formation of ventral mesoderm to that of more dorsalized tissues in *Xenopus* (25).

One possible explanation about these activations by bFGF is that bFGF lowered the threshold of activin concentration required for the activation of *fork head*, and then endogenous activin existed at low level in animal caps induced the expression of *fork head*, then *shh* and *goosecoid* was activated consequently. We observed minimal activin concentration to induce *fork head* and *shh* was low (about 0.5 ng/ml) both in animal caps from mid- and late-blastula embryos (data not shown), and we presented data showing the positive regulation of *shh* expression by *fork head* homologs in animal caps of *Cynops* embryos. At present, we can not present clear explanation about the different responsiveness in between *Cynops* and *Xenopus* ectoderms. However, it is noteworthy that *Cynops* and *Xenopus* have differences in the morphogenetic movements of mesodermal cells during gastrulation (26) and in the regenerative ability in the limb and lens.

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