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Xenopsin: The neurotensin-like octapeptide from *Xenopus* skin at the carboxyl terminus of its precursor

(mixed oligodeoxynucleotide primer/cloned cDNA/DNA sequence/amphibian skin peptide)

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ABSTRACT **We have synthesized two oligodeoxyribonucleotide mixtures that contain sequences complementary to different parts of the hypothetical mRNA sequence of xenopsin, a biologically active octapeptide found in skin extracts from *Xenopus laevis*. The two primer pools were independently used to initiate reverse transcription on skin poly(A)$^+$ RNA and the resulting cDNAs were then used to screen in parallel a cDNA library prepared from skin poly(A)$^+$ RNA. One of the clones that hybridized with both probes was subjected to sequence analysis. It contains a nearly full-length DNA copy of a mRNA of ≈490 nucleotides that encodes a xenopsin precursor protein. The deduced precursor is 80 amino acids long, exhibits a putative signal sequence at the NH_2 terminus, and contains the biologically active peptide at the COOH terminus. The region corresponding to the NH_2-terminal portion of the xenopsin precursor shows a striking nucleotide and amino acid sequence homology with the precursor of PYL^a, another recently described peptide from *Xenopus* skin.**

Peptides with different biological activities have been isolated from the skin of amphibians (1, 2). Their chemical and biological characterization has allowed them to be grouped into distinct families. Similar or even identical peptides have been identified in mammalian nervous and gastrointestinal tissue (3, 4). Whereas the functional importance of most of the peptides in amphibian skin is not known, their mammalian counterparts have been shown to act as chemical mediators—for example, as releasing factors, hormonal messengers, or neurotransmitters. The occurrence of similar peptides in tissues as different as brain, gut, and amphibian skin has led to the proposal (5) that the cells producing these peptides are of neuroectodermal origin and possibly share a common neuroendocrine program.

It is well established that most peptide factors are first synthesized as larger protein precursors that are subsequently processed to the final product. However, the molecular mechanisms that operate in the expression of the peptide coding genes and in the processing of the initial translation product are not fully understood. There is now accumulating evidence that a given gene may be involved in the production of several neuroactive peptides (6–10). Moreover, the recent report by Scheller *et al.* (11) has clearly shown that in *Aplysia* a family of genes can give rise to a family of protein precursors that are then processed to different, although related, sets of active peptides.

Xenopsin is one of the peptides found in amphibian skin. First isolated by Araki *et al.* (12), it consists of eight amino acids and is present in relatively high amounts in skin extracts from *Xenopus laevis*. The octapeptide bears a striking sequence resemblance to mammalian neurotensin and shares a number of its biological properties (13, 14).

We have used the known amino acid composition of xenopsin to design two sets of oligodeoxynucleotide mixtures complementary to different portions of its presumptive mRNA. These primer pools were synthesized and subsequently used to isolate xenopsin-specific sequences from a cDNA library constructed from skin poly(A)$^+$ RNA.

In this paper, we characterize a cDNA clone that corresponds to a mRNA encoding a xenopsin precursor protein of M_r 10,000.

MATERIALS AND METHODS

Preparation of RNA. Dorsal skin from *X. laevis* females was removed immediately after decapitation of the animals and frozen in liquid N_2. Ten grams of tissue was disrupted in a mortar under liquid N_2 in the presence of dry ice, suspended in 100 ml of 4.5 M guanidine thiocyanate/50 mM EDTA, pH 8/25 mM sodium citrate, pH 7/0.1 M 2-mercaptoethanol/2% lauroyl sarcosine (15). The viscous solution was homogenized in a loose-fitting Potter homogenizer, insoluble material was removed by centrifugation at 8,000 × *g* for 10 min. After addition of CsCl at 0.2 g/ml to the supernatant, the RNA was sedimented through a 5-ml layer of 5.7 M CsCl/50 mM EDTA, pH 7.3, in an SW 27 rotor (25,000 rpm, 22 hr, 15°C). The RNA pellet was dissolved in 1.5 ml of 10 mM Tris·HCl, pH 7.4/1 mM EDTA/0.5% $NaDodSO_4$ and further purified by extracting three times with 2 vol of phenol/$CHCl_3$ (1:1) followed by one $CHCl_3$ extraction. Ten grams of tissue generally yielded 3 mg of total RNA. Poly(A)$^+$ RNA was then isolated by chromatography on oligo(dT) cellulose (16).

cDNA Synthesis Primed with Synthetic Oligonucleotides. Oligonucleotide mixtures were synthesized in one step on a solid support by the triester method (17) using protected dinucleotides and a nonautomated bench-type DNA synthesizer, both purchased from Bachem Fine Chemicals. Other chemicals were either from Merck or Fluka and if necessary were dried over activated molecular sieve 4 A. After removal of protecting groups, the final products were purified by electrophoresis on preparative 25% polyacrylamide/8 M urea gels.

cDNA synthesis was carried out in a reaction mixture of 50 μl containing 10 μg of skin poly(A)$^+$ RNA/200 pM oligonucleotide primers/50 mM Tris·HCl, pH 8.3/7.5 mM $MgCl_2$/1.25 mM dithiothreitol/50 mM KCl/dATP/dGTP/dCTP (0.5 mM each)/2.5 μM [α-^{32}P]dTTP (1,000 Ci/mmol, 1 Ci = 3.7 × 10^{10} Bq). The mixture was first kept at 0°C for 30 min (18) and then incubated at 37°C for 30 min after addition of 19 units of avian myeloblastosis virus reverse transcriptase (kindly provided by J. Beard). The same amount of enzyme was added again and the incubation continued for 30 min. The reaction was stopped by addition of EDTA, and RNA was hydrolyzed at 100°C for 3 min with 0.1 M NaOH. The neutralized sample was passed over Sephadex G-50 fine to further purify the cDNA. 5'-end-labeled cDNA was generally prepared in the same way, but the reaction was scaled

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Abbreviation: kb, kilobases.

up 10-fold and done in the presence of the nonradioactive dNTPs (0.5 mM each). Prior to cDNA synthesis the synthetic oligonucleotides were phosphorylated (19) with [γ-^{32}P]ATP (7,000 Ci/mmol) and polynucleotide kinase (P-L Biochemicals) to a specific activity of 0.3–1 $\times$ 10^6 cpm/pmol.

Construction and Screening of the cDNA Library. Skin poly(A)$^+$ RNA (3.2 μg) was used to prepare a cDNA library of 1,000 transformants using the procedure described by Okayama and Berg (20). Recombinant clones were replica-plated onto duplicate nitrocellulose filters (21), and the liberated and fixed DNA hybridized to the different ^{32}P-labeled cDNA probes (2×10^5 cpm per ml of hybridization mixture) as described in ref. 22. Twelve recombinants hybridizing with both probes were selected and further analyzed using the minilysate technique (23).

Gel Electrophoresis, Transfer, and Hybridization of RNA and DNA. Southern and RNA blot experiments were carried out as described (24). When end-labeled oligonucleotides were used as a probe, prehybridization and hybridization were carried out at 15°C in 6× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate, pH 7.2)/1× Denhardt's solution (25)/10 mM EDTA/0.1% lauroyl sarcosine containing the ^{32}P-labeled probe at 3.5×10^5 cpm/ml. Filters were washed three times for 5 min with 6× NaCl/Cit at 4°C. It should be noted that a nick-translated purified cDNA insert was used as a probe in genomic blot experiments. The plasmid vector (20) itself hybridizes to a great number of DNA fragments in restricted *Xenopus* DNA, obviously because of the presence of simian virus 40 sequences in the hybrid vector.

Nucleotide Sequence Analysis of Cloned cDNA. DNA sequence analysis was carried out as described by Maxam and Gilbert (19). Restriction enzyme cleavage sites, which were end-labeled for sequence analysis, are located at the following positions in the sequence shown in Fig. 2: *Xmn*, 171–181; *Xho*, 319–324; *Hinf*, 341–345. DNA strands that were analyzed had either been phosphorylated at the 5′ end with [γ-^{32}P]ATP (3,000 Ci/mmol) or were labeled at the 3′ end with [α-^{32}P]cordycepin (New England Nuclear) and terminal transferase.

RESULTS

Design of Oligodeoxynucleotide Pools A and B. Originally we had tried to directly identify xenopsin-specific sequences in skin poly(A)$^+$ RNA. For this purpose, we synthesized a mixture of primers containing sequences complementary to the central portion (nucleotides 7–19) of the peptide coding region (Fig. 1). When this oligonucleotide pool, which contained 32 of the possible 64 coding sequences, was used to prime cDNA synthesis, we could identify various predominant extension products. However, none of them contained sequences coding for xenopsin. These results suggested that it might have been necessary to use a double-screening procedure (see below) for the isolation of xenopsin cDNA. We therefore synthesized the two oligonucleotide mixtures A and B. Pool A contained 12 (out of 32 possible) undecamers complementary to nucleotides 1–11 and pool B contained 18 (out of 32 possible) undecamers complementary to nucleotides 10–20 of the xenopsin coding region. Potential G·U mismatches were allowed for at positions 3, 5, 12, and 15. Both oligonucleotide sets were synthesized as a mixture of sequences; their composition and location with respect to the potential xenopsin mRNA sequence is shown in Fig. 1.

Isolation of cDNA Clone pXP. Poly(A)$^+$-containing RNA was isolated from *X. laevis* skin and used for the construction of a cDNA library using the plasmid vector elaborated by Okayama and Berg (20). Approximately 1,000 transformants were obtained and screened for xenopsin sequences.

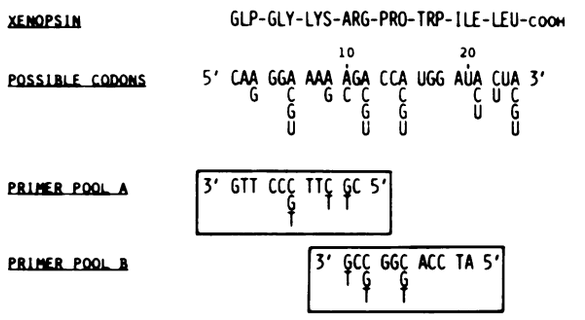

FIG. 1. Design of oligodeoxynucleotide mixtures A and B synthesized for the isolation of xenopsin-specific sequences. Glp, glutamic acid pyrollidone carboxylic acid.

Duplicate sets of colonies were transferred to nitrocellulose filters and separately hybridized with two [^{32}P]cDNA probes. These probes had been prepared by reverse-transcription of skin poly(A)$^+$ RNA using either oligonucleotide pool A or pool B as primers. About 60 colonies were found to contain sequences complementary to both cDNA preparations. Some of the positive transformants showed a great variation in hybridization intensity depending on which of the cDNA probes had been used. Twelve of the clones were selected at random and further analyzed. Restricted plasmid DNA from these recombinants was transferred from duplicate agarose gels to nitrocellulose filters that were then hybridized either to ^{32}P-end-labeled primer pool A or to a [^{32}P]cDNA probe that was synthesized by using oligonucleotide pool A as a primer. As expected, one or more restriction fragments from each plasmid appeared with the cDNA probe, but only one single clone (pXP) was found to contain a DNA fragment that also hybridized to the labeled oligonucleotide pool (data not shown). pXP was subsequently chosen for further analysis.

Characterization of pXP. The cloned cDNA in pXP has a total length of 426 nucleotides. DNA sequence analysis (Fig. 2) of the insert reveals the presence of 24 consecutive nucleotides (positions 220–244), which encode the xenopsin peptide. This sequence is part of an open reading frame of 240 nucleotides that is delimited by an AUG start signal and a UAA termination codon. The octapeptide xenopsin is thus part of a precursor polypeptide that consists of 80 amino acids and has a calculated molecular weight of 10,000. The NH_2-terminal portion of this precursor protein contains a cluster of hydrophobic amino acids, a structural feature typical of signal (or pre-) sequences of secreted polypeptides (26). Xenopsin itself is present at the extreme COOH terminus of the protein molecule. One potential proteolytic processing site Lys-Arg is located in the precursor sequence nine amino acids in front of the NH_2 terminus of xenopsin.

The protein coding region is preceded by a stretch of 62 untranslated nucleotides and followed by a 3′ noncoding region of 112 nucleotides (Fig. 2). The potential polyadenylylation signal A-A-U-A-A-A is present twice in the trailer sequence. It is located 70 and 12 nucleotides, respectively, upstream from the polyadenylylated 3′ end of the mRNA.

To determine the actual size of xenopsin mRNA, poly(A)$^+$ RNA from skin, liver, and stage 40 embryos was glyoxylated and fractionated in parallel by agarose gel electrophoresis. The RNA was transferred to nitrocellulose and hybridized to nick-translated [^{32}P]pXP DNA. Fig. 3 shows that the probe hybridizes predominantly to a mRNA of about 490 nucleotides. This RNA species is only found in skin poly(A)$^+$ RNA and does not seem to be present in detectable amounts in the other two RNA preparations.

The relatively good agreement between the size of the hybridizing RNA [490 nucleotides including the poly(A) tract] and the length of the cloned cDNA [426 nucleotides exclud-

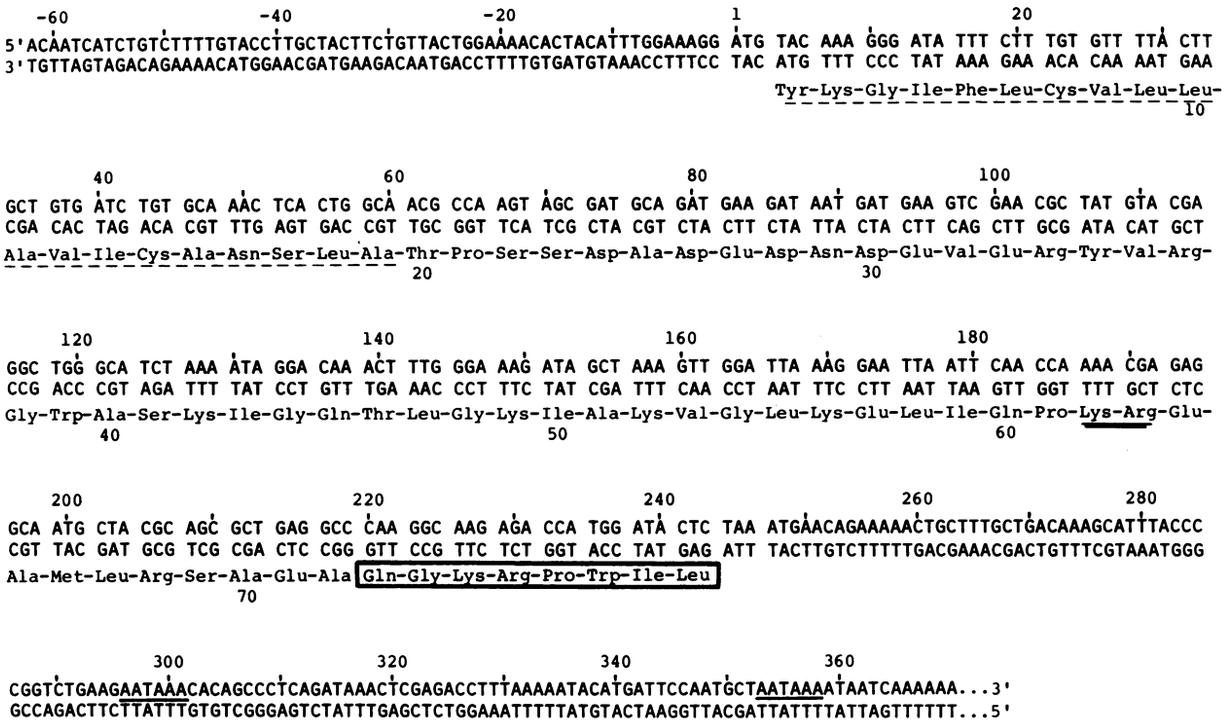

FIG. 2. cDNA sequence of the mRNA encoding the xenopsin precursor. The predicted amino acid sequence of the protein is shown below the nucleotide sequence. The putative signal sequence is underlined by a dashed line, the potential Lys-Arg processing site by a double line, and the mature peptide is boxed. Nucleotides are numbered positive from the adenosine of the initiating methionine to the 3′ end of the cDNA, and negative numbers are used in the 5′ untranslated region. The polyadenylylation signal A-A-T-A-A-A is underlined.

ing the poly(A) tract] suggests that the described cDNA represents an almost complete copy of the mRNA. In fact, the results of a primer extension experiment (data not shown) indicate that only the first 20 5′ nucleotides of the mRNA are not contained in the cloned cDNA.

An additional, much less intense, band appears just below the xenopsin mRNA after a longer exposure of the hybridized RNA blot. This band could be a degradation product of xenopsin mRNA or, alternatively, represent a different RNA species containing sequences homologous to xenopsin mRNA.

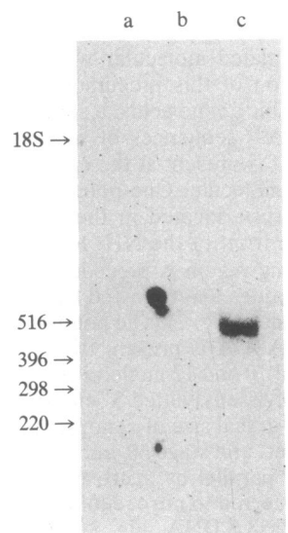

FIG. 3. Size determination of the mRNA coding for the xenopsin precursor. Four micrograms of glyoxylated poly(A)$^+$ RNA isolated from *X. laevis* liver (lane a), stage 40 embryos (lane b), and skin (lane c) were fractionated on a 1.4% agarose gel, blotted, and hybridized to nick-translated pXP DNA. Size markers were 18S RNA and glyoxylated *Hinf* restriction fragments from pBR322. Numbers denote nucleotides.

For preliminary information about the genomic representation of xenopsin or xenopsin-related sequences, we carried out a Southern blot experiment. *Xenopus* liver genomic DNA was digested with three different restriction enzymes and probed with the cDNA insert of pXP. Fig. 4 shows that two fragments of ≈10 and ≈7 kilobases (kb) appear in *Bam*HI-restricted DNA. *Hin*dIII digestion reveals two bands of 5 and 3.2 kb and an additional fragment of 2.7 kb, which hybridizes to a lesser extent. A relatively complex spectrum of fragments is observed in the *Eco*RI-digested sample, which shows a total of five fragments of various sizes hybridizing with different intensities. The appearance of several hybridizing fragments can be due to the existence of introns within the xenopsin gene. However, the rather complex hybridization pattern observed in *Eco*RI-restricted DNA might also indicate that additional sequences present in the genome show homology to xenopsin mRNA.

DISCUSSION

We have isolated and subsequently analyzed a cloned cDNA (pXP) corresponding to the mRNA that encodes the precursor for the biologically active peptide xenopsin. pXP was isolated from a cDNA library prepared from *Xenopus* skin poly(A)$^+$ RNA. We decided to use skin RNA for the construction of the library, assuming that the high level of xenopsin in this tissue might reflect a relatively large amount of the corresponding mRNA. This assumption was verified when we rescreened the cDNA library with the purified insert of pXP. Seven individual recombinants containing xenopsin sequences were identified. Taking into account that the library consisted of ≈1,000 independent transformants, one can roughly estimate that xenopsin mRNA constitutes about 0.5–1% of the skin poly(A)$^+$ RNA molecules. The RNA blot experiment indirectly confirms this result. Xenopsin mRNA

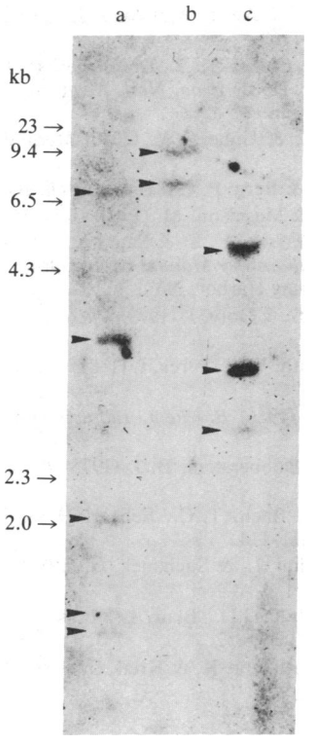

FIG. 4. Detection of genomic fragments homologous to xenopsin cDNA. Ten micrograms of *X. laevis* liver DNA was digested with *Eco*RI (lane a), *Bam*HI (lane b), and *Hin*dIII (lane c), fractionated on a 1% agarose gel, and hybridized to nick-translated xenopsin cDNA. Molecular weight marker was a *Hin*dIII digest of λ phage DNA.

could be visualized in skin poly(A)$^+$ RNA after short exposure of the hybridized nitrocellulose filter. In contrast, even after long exposure times we were not able to detect this mRNA sequence in any of the other two RNA preparations, a finding that indicates the xenopsin gene is not expressed early in development or in specialized liver tissue.

DNA sequence analysis of pXP reveals that the biologically active peptide is synthesized as part of a larger precursor protein. The predicted polypeptide is 80 amino acids long and exhibits a typical signal sequence. Fig. 2 shows that the central portion (amino acids 7–14) of this hydrophobic leader is bounded by two cysteine residues that might contribute to the secondary structure of the pre-region by disulfide bond formation. The presence of this sequence at the NH_2 terminus of the predicted xenopsin precursor suggests to us that we have correctly assigned the ATG codon at position 1–3 as translation initiation signal. This is supported by the fact that no additional ATG triplet is found further upstream in the sequence and that only very few nucleotides from the 5′ end of the mRNA are not contained in the cDNA clone.

The biologically active octapeptide is contained at the COOH terminus of the precursor molecule. Liberation of the mature peptide could theoretically be effected by one single cleavage event occurring just adjacent to its amino terminal glutamine residue. However, the amino acid sequence composition of the peptide chain preceding xenopsin does not support such a simple cleavage mechanism. It is more likely that a first processing step occurs at the dibasic residues Lys-Arg at position 62–63, provided of course that the additional Lys-Arg sequence in the xenopsin octapeptide itself (position 75–76) is somehow protected from cleavage. Processing at position 62–63 would result in the liberation of an extended xenopsin peptide consisting of 17 amino acids. The existence of such processing intermediates has already been proposed for the skin peptide caerulin (27). It has been suggested (27) that the final processing to the mature peptide proceeds via the stepwise removal of dipeptide residues, a mechanism that was shown to be involved in the biosynthesis of mellitin (28) and yeast α-mating factor (29). Examination of the sequence of the nine extra amino acids constituting the NH_2-terminal portion of the extended xenopsin peptide reveals in fact the presence of three dipeptide residues X-Ala, which are known substrates to dipeptidyl aminopeptidase. However, since they are interrupted by the sequence Met-Leu-Arg, it is evident that an additional proteolytic event would be required for the complete liberation of the octapeptide xenopsin. It should be pointed out that there is no evidence as yet that such a xenopsin processing intermediate indeed exists. We also do not yet know whether the remaining portion of the precursor molecule might give rise to additional functional peptides.

It is important to note that we have found a striking resemblance between a portion of the xenopsin cDNA and a cloned mRNA that has recently been characterized by Hoffmann *et al.* (30). The described cDNA, which is also derived from *Xenopus* skin mRNA encodes the precursor of PYL^a, a putative skin peptide that has not yet been isolated. Comparison of the two clones reveals an extensive sequence homology (70%) between both mRNAs that is confined to the area located between nucleotides 1–170 (Fig. 2). This homology is also reflected in the amino acid composition of the predicted precursor molecules, which show an internal homology of about 54% in the respective area. Whether these sequence similarities extend into the 5′ untranslated portion of the mRNAs could not be determined, because no sequences are available for PYL^a in this region. With the exception of two short blocks of 5 and 10 nucleotides, no apparent homology is observed in the remaining 3′ portion of the mRNA. However, it should be noted that a remarkable sequence similarity between the 3′ untranslated nucleotides of PYL^a mRNA and caerulin mRNA has been described (30). The three mRNAs are thus related to each other and it is reasonable to assume that the genes that encode these skin peptides represent different members of a gene family.

The described genomic blot does not allow us at present to determine whether this gene family consists of more members than those just mentioned. The observed limited number of hybridizing fragments, at least in the *Bam*HI and *Hin*dIII digests, might indeed reflect a small family size. However, other alternatives are obviously possible and it will therefore be necessary to isolate and characterize the relevant genomic loci to answer these questions. The isolation of the described cloned xenopsin cDNA will now allow us to design and carry out experiments that might help to elucidate the structural organization and ultimately the expression of the corresponding gene(s).

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