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Nucleotide sequence of the leader RNA of the New Jersey serotype of vesicular stomatitis virus

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ABSTRACT

Sequence for the leader RNA Synthesized by the New Jersey serotype of vesicular stomatitis virus is presented and its complementary sequence representing the 3'-terminal sequence of the genome RNA is deduced. Comparison with the leader RNA sequence of the serologically distinct Indiana strain reveals that the 3'-terminal region of the genomes of two viruses is highly conserved.

INTRODUCTION

Vesicular stomatitis virus (VSV), a prototype of negative strand viruses, contains a single-stranded genome RNA of molecular weight of 4×10^6 that serves as template for mRNA transcription in vitro by an RNA polymerase associated with the purified virions (1,2). Five distinct mRNA species are synthesized in vitro that code for the five structural proteins of VSV designated L, G, M, NS and N (3,4). Recent studies have shown that transcription initiates in vitro at the 3'-end of the genome RNA with synthesis of a 48 nucleotide long leader RNA followed by the sequential synthesis of the five mRNA species (5). From the order of transcription, the gene order of the viral genome is established as 3' N-NS-M-G-L 5' (5). A model for the biosynthesis of the mRNAs in vitro has been proposed that involves cleavage at specific sites on a growing precursor RNA molecule as the RNA polymerase transcribes the genome RNA (5). Recently, the complete nucleotide sequence of the leader RNA synthesized by the Indiana serotype of VSV has been determined (6). From the complementary sequence of the leader RNA the 3'-terminal sequence of the genome RNA, which presumably serves as the binding and initiation site of the virion-associated RNA polymerase, was deduced. In this communication, we present the complete nucleotide sequence of the leader RNA synthesized by the New Jersey serotype of VSV which is serologically distinct from the Indiana strain. The results have enabled us to compare for the first time the 3'-terminal sequences of these two genomes in relation to their related-

ness during evolution.

MATERIALS AND METHODS

Purification of VSV

The seed stock of the Ogden strain of New Jersey serotype of VSV was kindly provided by Dr. R. A. Lazzarrini, National Institutes of Health, Bethesda, Md. VSV was grown in baby hamster kidney cells (BHK-21, clone 13), adapted to suspension culture, and purified as described previously (7).

Synthesis and Purification of Leader RNA in Vitro

In vitro RNA polymerase reactions (1 ml) contained 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 5 mM $MgCl_2$, 4 mM dithiothreitol, 0.05% triton N101, 0.1 mM UTP, 1 mM ATP, CTP, and GTP. Radioactive labeling of product RNA was as follows: for AMP labeling, 500 µCi/ml [α^{32}P] ATP (11.21 Ci/mmole) was added to the reaction, and the amount of unlabeled ATP added was reduced to 0.4 mM. For CMP, GMP, and UMP labeling, 250 µCi of [α^{32}P] CTP (22.8 Ci/mmole) or 250 µCi of [α^{32}P] GTP (35.6 Ci/mmole) or 250 µCi [α^{32}P] UTP (8.6 Ci/mmole) or 10 µCi [^{3}H] UTP (35 Ci/mmole) were added and the corresponding concentration of unlabeled triphosphate added was lowered to 30 µM.

Reaction mixtures were incubated with 0.1 mg/µl of purified VSV for 6 hr at 30°. The reaction was terminated by the addition of SDS to 0.5% and the product RNA was directly extracted with phenol and purified by Sephadex G-50 chromatography, oligo(dT) cellulose chromatography, and electrophoresis on a 20% polyacrylamide slab gel as previously described (8,9). The leader RNA was recovered from the gel as described previously (10).

Determination of the Nucleotide Sequence of Leader RNA

Purified leader RNA which was separately labeled internally with each of the [^{32}P]-labeled ribonucleoside triphosphate, was digested with a 1:20 ratio of RNase T_1 to RNA in 2 µl of 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) for 1 hr at 37°. The digest was spotted directly on a cellulose-acetate strip and subjected to high voltage electrophoresis and following direct transfer to a DEAE thin layer chromatography plate, the oligonucleotides were separated in a second dimension by homochromatography and identified by autoradiography (6). The oligonucleotides were recovered from the DEAE plate and subjected to secondary digestions with RNase A followed by RNase T_2 and analyzed as described previously (6).

For ladder sequencing of the leader RNA, 0.5 µg of [^{3}H] UMP labeled leader RNA was labeled at the 5'-terminus with [γ^{32}P] ATP (2600 Ci/mmole) using polynucleotide kinase as described by Donis-Keller, Maxam, and Gilbert (11). Following repurification on a 20% polyacrylamide slab gel (10) the

5'-[^{32}P]-labeled RNA was divided into 6 aliquots and digested in 10 µl with 40 µg of carrier RNA as follows:

RNase Phy I - 40 µg/ml in 10 mM Na acetate (pH 4.5) for 7 min at 37°; RNase A, 0.5 µg/ml in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) for 10 min at 37°; RNase U_2, 2.5 units/ml in 10 mM Na acetate (pH 4.5) for 12 min at 37°; RNase T_1, 50 units/ml in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) for 3 min at 0°; RNase T_2, 10 units/ml in 10 mM Na acetate (pH 4.5) for 5 min at 37°.

Reactions were terminated by adding 10 µl of 10 M urea in 50 mM Tris-borate, 1 mM EDTA (pH 8.2) electrophoresis buffer and heating in a boiling water bath for 1 min. The digests were loaded directly onto a 20 x 40 cm 20% polyacrylamide slab gel and analyzed by electrophoresis as described previously (6).

For 3'-terminal labeling of the leader RNA with [^{32}pCp] the modified method of England et al. (12) was used as detailed elsewhere (6).

Chemical and Enzymes

All radioactively labeled compounds were purchased from New England Nuclear, Boston, Massachusetts. Bacterial alakaline phosphatase was purchased from Worthington Biochemical Corp., Freehold, N. J., and T_4-RNA ligase and polynucleotide kinase from P. L. Biochemicals, Inc., Milwaukee, Wisconsin. RNases A, T_1, T_2, and U_2 were purchased from Calbiochem, La Jolla, California.

RESULTS

Two-Dimensional Fingerprint of Leader RNA

Product RNA was synthesized in vitro by purified virions of the New Jersey serotype of VSV and purified by SDS phenol extraction followed by Sephadex G-50 chromatography as previously described (9). Leader RNA was then separated from the poly(A) containing mRNAs by oligo(dT)-cellulose chromatography and from the remaining product RNA by electrophoresis on a 20% polyacrylamide gel. A distinct RNA band representing less than 5% of the total radioactivity applied to the gel migrated midway into the gel (Fig. 1), and was recovered and used in subsequent studies.

Following recovery from gels, leader RNA labeled individually with each of the four [α^{32}P] ribonucleoside triphosphates, was digested with RNase T_1 as described previously (6) and the resulting oligonucleotides resolved by two-dimensional fingerprinting using high voltage electrophoresis on cellulose acetate strips for the first dimensional separation and homochromotography on DEAE thin layer plates for separation in the second dimension.

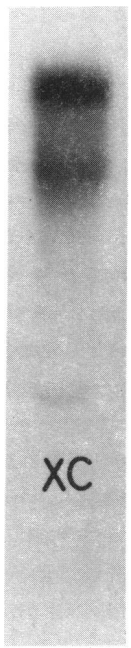

Fig. 1. Isolation of [α^{32}P] GMP-labeled leader RNA by polyacrylamide gel electrophoresis. Product RNA was synthesized in vitro in a 1 ml reaction mixture as detailed in Materials and Methods. The reaction mixture was incubated at 30° for 6 hr with 0.1 mg purified virions of clonally purified Ogden strain of New Jersey serotype of VSV. RNA synthesis was terminated by addition of SDS to 0.5% and the product RNA purified by phenol extraction and Sephadex G-50 chromatography (9). Poly(A) containing mRNA was removed by oligo(dT)-cellulose chromatography and the leader RNA isolated from non-poly(A)-containing product RNA by electrophoresis on a 20% polyacrylamide slab gel (140 V for 16 hr) as described previously (6,9). The band migrating in the center of the gel was located by autoradiography and recovered as previously described (6). The position of the xylene cyanol dye included in the sample is indicated by XC.

RNase T_1 digestion of [α^{32}P] GMP-labeled leader RNA yielded 6 oligonucleotides (numbered 1 to 6) as shown in Fig. 2. To further characterize the 6 oligonucleotides, each was recovered from the DEAE plate and further digested with pancreatic RNase A to produce oligonucleotides terminating with a 3'-pyrimidine which were separated by high voltage electrophoresis on DEAE paper. The resulting oligonucleotides were again eluted and treated with RNase T_2 and labeled bases were analyzed by paper electrophoresis. Similar analyses of the leader RNA labeled with [α^{32}P] ATP, [α^{32}P] CTP, and [α^{32}P] UTP were carried out to determine the nearest-neighbor transfers of [^{32}P] and are summarized in Table I. For comparison, the sequence of the corresponding oligonucleotides

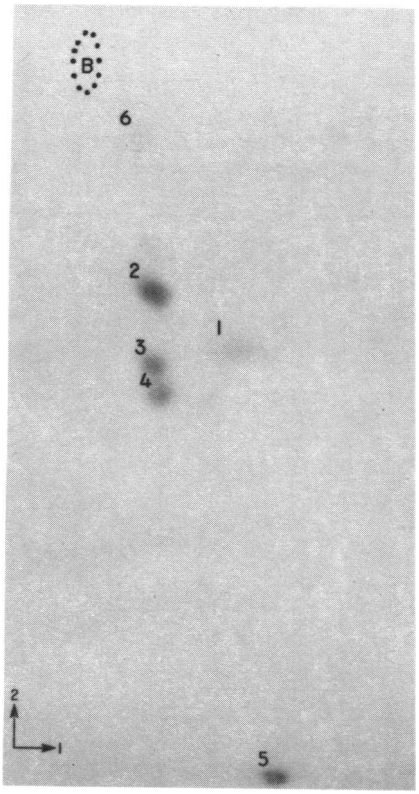

Fig. 2. Two-dimensional RNase T_1 fingerprint of [α^{32}P] GMP-labeled leader RNA. [^{32}P] GMP-labeled leader RNA was isolated as in Fig. 1, digested with RNase T_1, and characterized by high voltage paper electrophoresis on cellulose acetate strips for the first dimension and homochromatography on DEAE thin layer plates for the second dimension as detailed elsewhere (6). The B surrounded by dots represents the position of the blue dye.

obtained from the leader RNA synthesized by the Indiana serotype (6) are included in Table I.

It can be seen that oligonucleotides numbers 1, 2, 3, and 6 are identical in sequence with the leader RNAs of both the serotypes. Oligonucleotide 4 has two bases transposed, and oligonucleotide 5 contains 28 bases in both cases but has differences in a few positions.

The secondary digestions of oligonucleotide number 5, shown in Table I, were consistent with the results of ladder sequencing described below.

Sequence Analysis on Polyacrylamide Gels

To determine the precise order of the T_1 oligonucleotides in the leader RNA, we utilized the recently reported sequencing gel techniques of Donis-

TABLE I

ANALYSIS OF T1 OLIGONUCLEOTIDES FROM VSV NEW JERSEY LEADER RNA

Oligo-nucleotide	Proposed Sequence	Input NTP	Composition	Pancreatic RNase Products	Corresponding** Indiana Sequence
1	ppACG(A)	A	ppA,G	ppAC,G	ppACG(A)
		C	ppA	ppAC	
		G	C	ppAC	
		U	---	---	
2	AG(G)	A	---	---	AG(A)
		C	---	---	
		G	G	AG	
		U	---	---	
3	AAG(A)	A	A,G	AAG	AAG(A)
		C	---	---	
		G	A	AAG	
		U	---	---	
4	CCUAG(A)	A	G,U	U,AG	CUCAG(G)
		C	C	C	
		G	A	AG	
		U	C	C	
5	$AC(A)_7C_2(AUU)_2$- $AC(AAUU)_2G(G)$*	A	8A, 3C, 3U	A_XC^{Ψ},AAU,AC,AU,U,C	$AC(A_7C)_2C(AUU)_2$
		C	3A, 1C	AC,A_7C	$AUCAUUA_4G(G)$
		G	G,U	G,U	
		U	A,U	AAU,AU	
6	G(A/C/G)	A	G	G	G(A/C)
		C	G	G	
		G	G	G	
		U	---	---	

* nucleotide sequence determined in combination with sequence gel results (Fig. 3).

Ψ number of A residues not determined.

** From Reference 6.

Keller, Maxam and Gilbert (11). New Jersey leader RNA was synthesized in the presence of [^{3}H] UTP and purified as described previously (9). The 5'-terminal phosphates were removed with alkaline phosphatase and the terminus was labeled with [γ^{32}P] ATP using polynucleotide kinase (11). Following re-purification on 20% polyacrylamide gels, the 5'[^{32}P]-labeled leader RNA was subjected to partial digestion with RNase A to locate pyrimidines, RNase T_1 to locate G residues, RNase U_2 to locate A residues, Phy I RNase to visualize C residues and RNase T_2 to form a ladder from which to read the sequence. The digests were analyzed on a 20 x 40 cm 20% polyacrylamide gel under conditions which would resolve oligonucleotides which differ in chain length by only one nucleotide. Results of such analyses are shown in Fig. 3. Starting at the bottom of Panel B, one can visualize the first C cut followed by a T_1 cut in the G lane which represents the 5'-terminal (ppA)CG. The abundance of bands down in this region of the gel represents contaminating RNA fragments which were copurified with leader RNA and subsequently labeled by

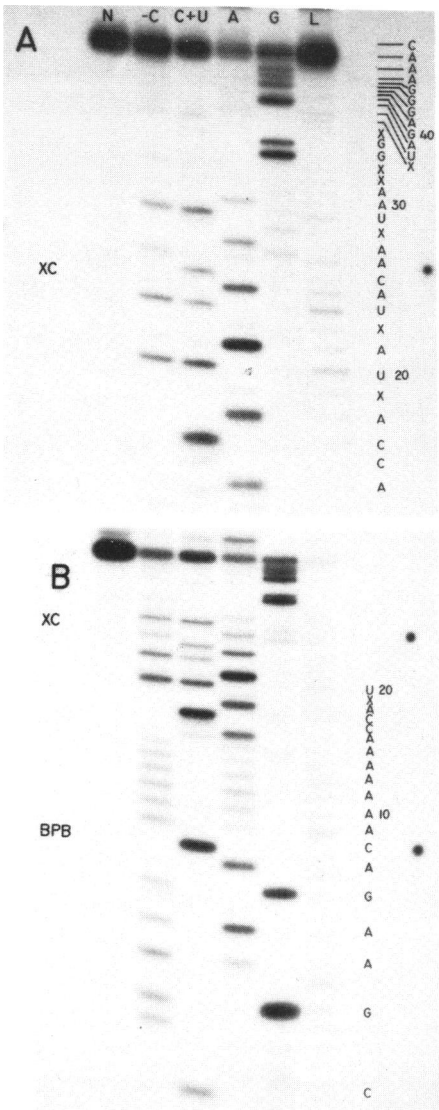

Fig. 3. Polyacrylamide gel electrophoresis of 5'[^{32}P]-labeled leader RNA following partial RNase digestions. Leader RNA (0.5 µg) synthesized in vitro in the presence of [^{3}H] UTP was isolated as in Fig. 1 and labeled specifically at the 5'-terminus with [γ^{32}P] ATP using polynucleotide kinase as described by Donis-Keller, Maxam and Gilbert (11). Following repurification on polyacrylamide gels, aliquots of 5'[^{32}P]-labeled leader were enzymatically digested as detailed in Materials and Methods. (N) No enzymes added; (-C) Phy I RNase; (C + U) RNase A; (A) RNase U_2 (G) RNase T_1; (l) RNase T_2. Positions of xylene cyanol (XC) and bromophenol blue (BPB) dye are as indicated.

polynucleotide kinase. Continuing up on the sequence gel, the nucleotide sequence (ppA)CGAAGACAAAAAAACCAXU can be read followed by AXUACAAXUAAXXGGXC XUAGAGGGAXXX in Panel A. The X's represent covalent nucleotide bonds which were not cleaved by any of the base specific RNases. The X's occurring at nucleotide positions 19, 22, 28, and 32 (Fig. 3) are UMP because based on nearest-neighbor transfers from T_1 oligonucleotide number 5 (Table I), [^{32}P] UMP-labeled leader RNA transfers [^{32}P] only to either AU or AAU. The X occurring in position 33 is also U because in oligonucleotide number 5, [^{32}P] GMP-labeled leader transfers [^{32}P] to U in addition to G, therefore, the 3'-proximal base must be U in the sequence UG(G). The nucleotides occurring at positions 36 and 37 are both C because this is the only position where the T_1 oligonucleotide number 4 can exist. From the above results, the sequence of the leader RNA can be depicted as ppACGAAGACAAAAAAACCAUUAUUACAAUUA AUUGGCCUAGAGGGAXXX with the order of T_1 oligonucleotides (Table II) being 1, 3, 5, 6, 4, 2, 6, 6.

Characterization of the 3'-Terminal Sequence

In order to characterize the last three unknown nucleotides in the leader, the following method was used. Leader RNA was labeled at the 3'-end with [^{32}pCp] using RNA ligase as described previously (6). Such a reaction results in each leader RNA molecule being labeled with one pCp molecule at its 3'-end. Such studies using pCp labeled leader RNA were used successfully to determine the 3' terminal nucleotide sequences of the Indiana leader RNA (6). The 3'[^{32}P]-labeled New Jersey leader RNA was digested with a combination of RNase A and T_1. The resulting 3'-terminal fragment was purified on DEAE-cellulose column chromatography and digested with RNase T_2 and RNase U_2 separately. The resulting mononucleotides were separated by high voltage paper electrophoresis, the results of which are shown in Fig. 4. RNase T_2 digestion (Panel A) showed that the 3'-termini of New Jersey leader RNA is heterogenous since the RNA terminates in either with A (60%) or C (40%). Panel B shows that RNase U_2 gives the same result as RNase T_2 digestion confirming that the 5'-proximal base of A and C is A. Moreover, the net negative charges of the 3'-terminal fragments produced by RNase A and T_1 digestions and analyzed by DEAE-cellulose chromatography (data not shown) supported the sequence GGGAAAC with C residue being absent 60% of the time.

DISCUSSION

The complete nucleotide sequence of the leader RNA synthesized by the New Jersey serotype of VSV is presented in Table II. The complementary sequence of the leader RNA representing the 3'-terminal sequence of the

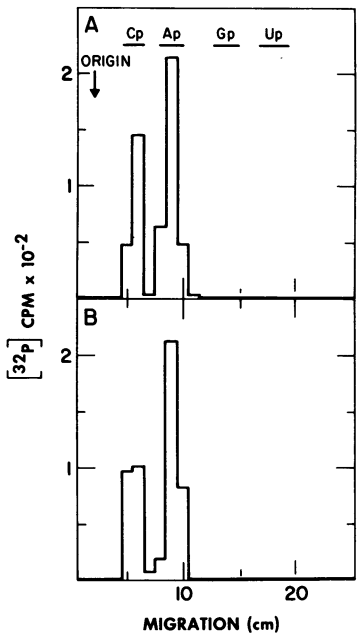

Fig. 4. Paper electrophoresis of RNase T_2 digested 3'[^{32}P]-labeled leader RNA. Purified leader RNA labeled with [^{3}H] UMP was labeled specifically at the 3'-terminus using 5'[^{32}pCp] and RNA ligase (6). Following repurification, the 3'[^{32}P]-labeled RNA was digested with a combination of RNase A and RNase T_1, and the 3'-terminal oligonucleotide isolated by a DEAE-cellulose column chromotography prior to digestion with RNase T_2 (6). The resulting mononucleotides were separated by high voltage paper electrophoresis on Whatman 3 mM paper at 2600 volts for 75 min (6). One cm strips were assayed for radioactivity in scintillation cocktail. The position of the four ribonucleoside monophosphate markers, located by UV light, are as indicated.

genome RNA of the New Jersey serotype of VSV is also presented. For comparison, the corresponding leader RNA and its complementary genome RNA sequence of the Indiana serotype of VSV (6) are also presented in Table II.

There are some striking similarities in the sequences between the leader RNAs synthesized by two serologically distinct VSV; (a) both contain 48 nucleotides which are adenine-rich (50%) and terminate variably at the 3'-terminus with cytosine (68%) and adenosine (32%) in Indiana and cytosine (40%) and adenosine (60%) in New Jersey leader RNA; (b) the first 24 nucleotides from the 5'-termini of the two RNAs are identical except for one base change at the 12th nucleotide with A for Indiana and C for New Jersey leader RNA. Beyond the 24th nucleotide, there are also extensive homologies except for the bases 25 and 26, 32 and 33, 37 and 38, 41 and 42, 44 and 45 (Table

TABLE II

NUCLEOTIDE SEQUENCES OF VSV RNA

LEADER RNA*

Indiana 5' ppA C G A A G A C A A A C A A A C C A U U A U U A U C A U U A A A A G G C U C A G G A G A A A A_{OH} C_{OH} 3'

10 20 30 40

N.J. 5' ppA C G A A G A C A A A A A A A C C A U U A U U A C A A U U A A U U G G C C U A G A G G G A A A A_{OH} C_{OH} 3'

GENOME RNA

Indiana 3' $_{HO}$U G C U U C U G U U U G U U U G G U A A U A A A U A G U A A U U U U C C G A G U C C U C U U U U G...5'

10 20 30 40

N.J. 3' $_{HO}$U G C U U C U G U U U U U U U G G U A A U A A A U G U U A A U U A A C C G G A U C U C C C U U U G...5'

*The underlined bases denote the changes occurring in two VSV RNAs.

II); (c) the locations of the guanosine residues are such that both leader RNAs generate identical sized T_1 oligonucleotides including the large RNase T_1 oligonucleotide containing 28 bases (Table I). Moreover, the T_1 oligonucleotides occur at identical locations in the nucleotide sequence through the first 40 nucleotides. (d) Both the leader RNAs terminate with a polypurine sequence $AGAGGGAAAC_{OH}$ for New Jersey and $AGGAGAAAAC_{OH}$ for Indiana leader RNA.

The above results clearly show that the leader RNAs synthesized by two serotypes of VSV share extensive sequence homology and correspondingly similar homologies exist between the 3'-terminal sequence of their genome RNAs. It is important to mention that previous hybridization studies with the leader RNA of New Jersey with Indiana genome RNA showed virtually no sequence homology (9). It is now apparent from the sequences of two leader RNAs that few base differences between these RNAs are spaced such that RNase digestion at the mismatched positions after hybridization under the conditions employed, would result in melting of the hybrids into smaller lengths with complete digestion of the resulting single-stranded RNA. Thus, the previous results showing lack of sequence homologies between the mRNAs of these two serotypes of VSV (13,14) should be re-investigated using less stringent conditions.

The sequence homology at the 3'-terminal region of the genomes of both viruses suggests that they possibly share common binding and initiation sequence at their 3'-terminal region for the virion-associated RNA polymerase. In fact, the homology of the polymerase binding site may account for the

fact that the New Jersey serotype of VSV interfere with the replication of VSV Indiana (15). In addition, by in vivo complementation studies, it has been shown that the L protein of VSV New Jersey can complement the transcription defect of a VSV Indiana mutant that is temperature sensitive in the L protein gene (16). By reconstituion experiment, it has been shown that both L and NS proteins of VSV are required for in vitro transcription using N-RNA protein complex as the template (17,18). Thus it seems that the L and NS proteins of both the viruses may recognize common sequence on the genome RNA for transcription or replication. It should then be possible using purified L and NS proteins of both viruses to retore transcription of the N-RNA complex of either virus. However, preliminary results using this approach were ambiguous (19); nevertheless, using more rigid experimental conditions, it may be possible to demonstrate this interaction.

It is interesting to note that the leader RNAs of both serotypes of VSV contain a polypurine sequence at their 3'-termini. These sequences have been implicated in the recognition site for the processing enzyme present in the virion (6). Thus, it appears that both viruses may share not only a common strategy for biosynthesis of leader RNA and mRNAs, but also they may indeed share a common processing mechanism.

Finally, it appears from the extensive sequence homologies between the 3'-terminal portion of the genome RNA that these viruses may have evolved from a common ancestor. Since this portion of the genome RNA is involved in the binding and initiation by the RNA polymerase, the conservation of this sequence during evolution may be essential.

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