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Detection of $P$ element transcripts in embryos of $Drosophila$ melanogaster and $D. willistoni$

MONICA L. BLAUTH$^{1,2}$, RAFAELA V. BRUNO$^3$, ELIANA ABDELHAY$^4$, ELGION L.S. LORETO$^{2,5}$ and VERA L.S. VALENTE$^{2,5}$

$^1$Departamento de Ciências Biológicas, Universidade do Estado de Mato Grosso (UNEMAT) Campus Tangará da Serra, Rodovia MT-358, km 07, Bairro Aeroporto 78300-000 Tangará da Serra, MT, Brasil
$^2$Laboratório de $Drosophila$, Departamento de Genética, Instituto de Biociências Universidade Federal do Rio Grande do Sul (UFRGS), Caixa Postal 15053 91501-970 Porto Alegre, RS, Brasil
$^3$Laboratório de Biologia Molecular de Insetos, Fundação Oswaldo Cruz, Av. Brasil, 4365 Bairro Manginhos, 21045-900 Rio de Janeiro, RJ, Brasil
$^4$Centro de Transplante de Medula Óssea, Instituto Nacional de Câncer Praça da Cruz Vermelha, 23, 7$^{\text{a}}$ andar, 20230-130 Rio de Janeiro, RJ, Brasil
$^5$Departamento de Biologia, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Caixa Postal 5050, Agência Universitária, 97111-970 Santa Maria, RS, Brasil

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ABSTRACT

The $P$ element is one of the most thoroughly studied transposable elements (TE). Its mobilization causes the hybrid dysgenesis that was first described in $Drosophila$ melanogaster. While studies of the $P$ element have mainly been done in $D. melanogaster$, it is believed that $Drosophila$ willistoni was the original host species of this TE and that $P$ was transposed to the $D. melanogaster$ genome by horizontal transfer. Our study sought to compare the transcriptional behavior of the $P$ element in embryos of $D. melanogaster$, which is a recent host, with embryos of two strains of $D. willistoni$, a species that has contained the $P$ element for a longer time. In both species, potential transcripts of transposase, the enzyme responsible for the TE mobilization, were detected, as were transcripts of the 66-kDa repressor, truncated and antisense sequences, which can have the ability to prevent TEs mobilization. The truncated transcripts reveal the truncated $P$ elements present in the genome strains and whose number seems to be related to the invasion time of the genome by the TE. No qualitative differences in antisense transcripts were observed among the strains, even in the $D. willistoni$ strain with the highest frequency of heterochromatic $P$ elements.

Key words: $Drosophila$, $D. willistoni$, hybrid dysgenesis, RNAi, $P$ element, transposable element.

INTRODUCTION

The $P$ element is the most thoroughly studied transposable element (TE) in $Drosophila$. It was first discovered as the causative agent of hybrid dysgenesis in $Drosophila$ melanogaster by Kidwell 1977, after several elements, including transposons and retroelements, had been identified as also being able to promote a similar syndrome in $Drosophila$ melanogaster and in other species of the genus (Yannopoulos et al. 1987, Crozatier et al. 1988, Lozovskaya et al. 1990, Petrov et al. 1995, Evgen'ev et al. 1997). Since then, $P$ element has been shown to play a central role in the biology of these species, including its role in generating genome diversity.
tween males of strains that contain functional \( P \) elements in the genome and females that do not (Kidwell and Kidwell 1979, Engels 1989). This syndrome promotes sterility and gonadal atrophy.

The \( P \) element is a class II TE that is 2.9 kb long, including the 31-bp Inverted Terminal Repeats (ITR), and is transposed by the DNA cut and paste mechanism (Misra and Rio 1990, Laski et al. 1986). The \( P \) element contains four open reading frames (ORFs) that encode at least two proteins by alternative splicing of the third intron, named IVS3. The transposase, an 87-kDa enzyme necessary for transposition, is expressed exclusively in the germline cells (Siebel and Rio 1990). In somatic cells, the IVS3 sequence is retained in the transcript and a 66-kDa repressor protein is instead produced.

The \( P \) element is widely dispersed in Neotropical species of the subgenus *Sophophora* (Daniels et al. 1990, Clark and Kidwell 1997, Loreto et al. 1998). This sequence is present in the cosmopolitan species *D. melanogaster*, but not in other species ranked under the *melanogaster* subgroup, suggesting a recent invasion of the *D. melanogaster* genome by horizontal transfer. This hypothesis is also supported by studies showing that several strains of *D. melanogaster* collected before the 1950s do not have the \( P \) element (Bregliano and Kidwell 1983). The \( P \) element sequences of *D. willistoni* and *D. melanogaster* differ by only a single nucleotide substitution (Daniels et al. 1990), suggesting that the donor species belongs to the *willistoni* group. The first contact between the two species is calculated to have occurred around the year 1800, when it is believed that *D. melanogaster* arrived in the New World (Engels 1989). Therefore, in a period of about 200 years, the \( P \) element invaded the *D. melanogaster* genome and dispersed across the world.

The invasive capacity of the \( P \) element has been attributed to its ability to regulate its own mobility, increasing the chances of host survival in the face of its invasion (Brookfield 1991). Besides the tissue-specific splicing of IVS3, other alternative forms of splicing into this intron, such as those described for *D. melanogaster*, *D. bifasciata* and *D. hydei* by Chain et al. (1991), transposing. The KP protein, encoded by a truncated \( P \) element sequence, is one of the most widely known repressors of \( P \) element mobility. The short KP polypeptides may interact with the \( P \) transposase and inhibit its function through the assembly of inactive heteromultimers (Lee et al. 1996, 1998). Alternatively, short \( P \) polypeptides may interact with the \( P \) element promoter, thus inhibiting transcription.

Simmons et al. (2002) suggested that the nature of the female \( P \) cytotype, which refers to the maternal ability to prevent the paternally originated \( P \) element from transposing, is determined by the 66 kDa-repressor. A consensus sequence, similar to those found in the maternal genes, was identified in the IVS3 intron and appears to allow only unspliced transcripts to be maternally transmitted by the nurse cells to the oocyte, where they prevent transposase enzyme activity after egg fertilization. On the other hand, the most recent work of Josse et al. (2007) proposes the telomeric Trans-Silencing Effect (TSE), a mechanism by which a transposon inserted in subtelomeric heterochromatin or close to the centromere has the capacity to repress homologous transposons in the genome. The observed sensitivity to mutations in genes that code for the proteins AUBERGINE and PIWI suggested that the \( P \) cytotype might depend on repeat-associated small interfering RNA. The AUBERGINE and PIWI proteins link to small antisense RNAs and trigger the production of more antisense transcripts, which in turn prevent the expression of homologous sequences by sequence complementarity.

Considering these aforementioned studies, we investigated the presence of transcripts coding for transposase and potential repressors of mobilization of the \( P \) element in embryos of two strains of *D. willistoni*, which produce offspring affected by hybrid dysgenesis syndrome and whose previous description detected the \( P \) element inserted preferentially in heterochromatin in the Wip strain and in euchromatin in the 17A2 strain. These strains favor the study of the proposed hypothesis that correlates heterochromatin sequences with their transcriptions in an antisense way. Apart from this, one strain of *D. melanogaster* was included in our studies.
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**MATERIALS AND METHODS**

**Fly Stocks**

The strains used were *D. melanogaster* Harwich (H) from the USA, first collected in the 1960s and normally used as a P element-positive control in experiments; *D. willistoni* 17A2 from South Brazil (30°05′S, 51°39′W), sampled in the early 1990s, and *D. willistoni* Wip from Northeast Brazil (12°54′S, 38°19′W), sampled in the 1960s. The strains of *D. willistoni* were chosen since they were previously studied for *P* element chromosomal positioning (Regner et al. 1996). In the 17A2 strain, only 17% of the chromocenters analyzed by the authors have a *P* element inserted, while the Wip strain have 50% of the chromocenters with *P* element sites. Some hybridization signals were detected in the euchromatic arms of the Wip strain, but none had a frequency higher than 15%. The mating of 17A2 males with Wip females generates offspring with a 26% hybrid dysgenesis rate. The mating of 17A2 males with Wip females, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 2.5 min, and a final extension at 72°C for 10 min. A 1 μl sample of this first PCR amplification was submitted to a second round of amplification with internal primers combined as follows: meli1+ and mele3-, meli2+ and mele3-, meli2+ and meli3- (Table I; Figs. 1B, 1C, 1D), where meli1+, meli2+ and meli3- are intron-spanning primers. The cycle parameters for the second amplification were a denaturation step at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 2.5 min, and a final extension at 72°C for 10 min.

The flies were reared in cornmeal medium (Marques et al. 1966) at constant temperature and humidity (17 ± 1°C; 60% rh).

**Extraction of Nucleic Acids**

The DNA of 25 adult flies was extracted by the phenol-chloroform method according to Sassi et al. (2005). Total RNA of embryos between 0-18 h of development was extracted by the Trizol method (Invitrogen).

**Synthesis of cDNA**

The cDNA synthesis was done according to the protocol of M-MLV Reverse Transcriptase enzyme method (Invitrogen) using 2 pmol of the specific mele3- primer for the detection of transposase, 66-kDa repressor and truncated transcripts or meli1+ primer for the detection of antisense transcripts (Table I lists the primer sequences and Fig. 1 depicts the primers applied and their respective binding sites). The cDNA synthesized from Amd-un2 and Amd-bw primers, specific for the *mele3*- and truncated transcripts or meli1+ primer for the detection of transposase, 66-kDa repressor and truncated transcripts or meli1+ primer for the detection of antisense transcripts (Table I lists the primer sequences and Fig. 1 depicts the primers applied and their respective binding sites). The cDNA synthesized from Amd-un2 and Amd-bw primers, specific for the *mele3*- and meli1+ primer for the detection of antisense transcripts (Table I lists the primer sequences and Fig. 1 depicts the primers applied and their respective binding sites). The cDNA was extracted by the Trizol method (Invitrogen).

**Amplification of DNA and cDNA**

The reaction was carried out in a 15 μl volume containing 1 μl of genomic DNA, 1 U Taq DNA polymerase (Invitrogen) in 1X polymerase buffer, 2.5 mM of MgCl₂, 10 pmol of each primer and 0.6 μl of dNTP mix. The first PCR round of cDNA amplification was performed with 2 μl of the sample and the mele3- primer and meli1+ intron-spanning primers (Fig. 1A). The amplification parameters were a denaturation step at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 2.5 min, and a final extension at 72°C for 10 min.

The parameters of amplification of the *Amd* gene, *mele3*- and *meli1+* intron-spanning primers were as follows: a denaturation step at 94°C for 7 min, followed by 40 cycles at 94°C for 1 min, 56°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min.

The amplification parameters were a denaturation step at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 2.5 min, and a final extension at 72°C for 10 min.

**Southern Blot**

The first amplification product, with meli1+ and mele3- primers, was submitted to a Southern blot procedure. The gel preparation was carried out following Sambrook et al. (1989). The hybridization, stringency washes, and detection reaction followed the Gene Images Labeling kit (Amersham Biosciences). The gel was probed with a 32P-labeled, full-length *P. oestrus* cDNA fragment. The probe used was the complete canonized cDNA for the *P. oestrus* gene, specific for the *mele3*- and meli1+ primer for the detection of antisense transcripts (Table I lists the primer sequences and Fig. 1 depicts the primers applied and their respective binding sites). The cDNA was synthesized from Amd-un2 and Amd-bw primers, specific for the *mele3*- and meli1+ primer for the detection of antisense transcripts (Table I lists the primer sequences and Fig. 1 depicts the primers applied and their respective binding sites). The cDNA was synthesized from Amd-un2 and Amd-bw primers, specific for the *mele3*- and meli1+ primer for the detection of antisense transcripts (Table I lists the primer sequences and Fig. 1 depicts the primers applied and their respective binding sites).
TABLE I
PCR primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Position in canonical P element of D. melanogaster (GenBank accession code X06779)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meli1+</td>
<td>TACACAAACAGAGTCCTGTT</td>
<td>431–442, 501–508</td>
</tr>
<tr>
<td>Meli2+</td>
<td>GTATATCAGAATCAAAAACTTCG</td>
<td>1157–1168, 1222–1231</td>
</tr>
<tr>
<td>Meli3-</td>
<td>CATTTCTGTATTCCTGCTATT</td>
<td>2154–2138, 1947–1943</td>
</tr>
<tr>
<td>Mele3-</td>
<td>GTTTATCAACATCGACGTTC</td>
<td>2581–2561</td>
</tr>
<tr>
<td>M-IR **</td>
<td>CAAGGGTGGCTCCGTCG</td>
<td>1–31, 2907–2877</td>
</tr>
</tbody>
</table>


Fig. 1 – Map of the canonical P element showing exons, introns and primers binding sites used in RT-PCR. (A) meli1+ and mele3- primer binding sites and the probable fragments amplified in RT-PCR amplification. (B) meli1+ and meli3- primer binding sites and the probable fragments amplified in RT-PCR amplification. (C) meli2+ and mele3- primer binding sites and the probable fragments amplified in RT-PCR amplification. (D) meli2+ and meli3- primer binding sites and the fragment and the probable fragments amplified in RT-PCR amplification.

SEQUENCING

The fragments obtained by RT-PCR were sliced off the 0.8% agarose gel and purified with GFX PCR DNA and Band Gel Purification kit® (Amersham Biosciences) according to the supplier’s instructions. The purified DNA in the PCR amplification were used for dideoxy chain-termination reaction. Sequence identity was confirmed in BlastN (http://www.ncbi.nlm.nih.gov/). The confidence consensus of multiple reads was obtained by analysis of the Staden Package Gap 4 program (Staden 1996). The sequences were then aligned in the MultAlin program (Corpet 1988), according to the default system parameters.
RESULTS

DETECTION OF PUTATIVE TRANSCRIPTS OF TRANSPOSASE AND 66-KDA REPRESSOR IN EMBRYOS OF D. melanogaster AND D. willistoni

By RT-PCR, we identified a putative transcript of the transposase enzyme and of the 66-kDa repressor in D. willistoni and D. melanogaster embryos. The 743-bp fragment was amplified with the meli2+ and meli3- primers in both species (Fig. 1D; Fig. 2, indicated by a narrow arrow). This fragment corresponds to transcripts that have experienced IVS2 and IVS3 splicing, and would thus code for the functional transposase enzyme (GenBank accession codes DQ486674 and DQ486675).

Two amplification products were expected in a RT-PCR using meli2+ and mele3- primers: 1180 and 1371-bp (Fig. 1C), corresponding to the spliced and unspliced IVS3 transcripts, respectively. In Figure 2, we observe only the unspliced IVS3 fragment (indicated by an arrowhead), a result which was confirmed by partial sequencing of the D. melanogaster fragment. We infer that the transcription of the 66-kDa repressor is higher than the transcription of the transposase, previously identified with the PCR with meli2+ and meli3- primers.

ALTERNATIVE SPLICING

A 572-bp fragment was amplified by meli2+ and meli3- primers (Fig. 2, indicated by a thick arrow – GenBank accession codes DQ486676, DQ486677 and DQ486678). This sequence lost 198-bp of the 3' region of exon 2 (1742-1941 nt of canonical P element), but maintained its splicing donor site (position 1947 nt of the canonical P element) after undergoing the IVS3 splice.

DIFFERENTIAL TRANSCRIPTION BETWEEN SPECIES

The transcriptional differences between the species were more evident when PCR primers that amplify longer sequences were used. Figure 3 displays the amplification products obtained with the meli1+ primer, combined with the mele3- or the meli3- primers (Figs. 1A and B display the expected amplification products).

When meli1+ and meli3- were used in the RT-PCR amplification, the transposase fragment was visible only in D. willistoni strains (Fig. 3, indicated by an arrow), not in D. melanogaster. In contrast, truncated transcripts were more evident in D. melanogaster, both combinations of primers were used, as compared to D. willistoni (Fig. 3).

This differential expression between the species appears to result from the larger number of truncated P element sequences in the D. melanogaster genome as compared to D. willistoni, determined by a genomic screen.
PCR with the M-IR primer (Fig. 4, M-IR). This primer anneals in the ITRs and amplifies the element across the entire length.

The greater number of truncated sequences related to P element in D. melanogaster also promotes PCR primer competition, as the complete sequence is not realized in the agarose gel (Fig. 4, indicated by an arrowhead).

Since truncated sequences of the P element are potential repressors of transposition (Pinser et al. 2001), three of the highly expressed fragments in D. melanogaster were sequenced. They are indicated as i1e31, i1e32 and i1i34 in Figure 3 and 377, 611 and 284 nt of their sequence were determined, respectively (GenBank accession codes DQ486679, DQ486680 and DQ486681). The sequences align with 3' exon 1 region, which is also present in the KP repressor sequence (Black et al. 1987), but they show little nucleotide conservation. The i1e32 sequence has a duplication of the 5' exon 1 region, and the disparity of the fragment size estimated in the agarose gel and the sequence determinants can be the result of a secondary structure acquired by the sequence.

**Presence of Antisense P Element Transcripts During Embryogenesis**

The PCR procedure using total embryo cDNA, synthesized starting from a sense primer (meli1+), suggested the presence of antisense transcripts (Fig. 5) in embryos of both species.

Unlike the P element, no transcript amplification was observed in the total embryo cDNA synthesized starting from the sense primer Amd-un2 of the Amd gene.
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DISCUSSION

The present work aimed to characterize the transcription of P element sequences during embryogenesis of one recently invaded species, D. melanogaster, as well as in D. willistoni, a long-standing host of this transposable element, and obtain data about the antisense transcription of P element comparing specifically the two strains of D. willistoni. The work of Haring et al. (1998) proposes the utilization of a combination of four primers to detect the transposase and the 66-kDa repressor. Three of these primers anneal only in spliced sequences, assuring that only cDNA sequences are used as template and also makes it possible to distinguish the transcripts of the transposase and the 66-kDa repressor by the alternative splicing of the IVS3 intron. Using this procedure, we detected transcription of the transposase gene and the 66-kDa repressor in embryos of the studied strains. The absence of transposase transcripts in the amplification using meli2+ and mele3- primers, which was designed to reveal transcripts of both the transposase and the 66-kDa repressor, suggests that there is a greater number of transcripts of the 66-kDa repressor available, as this transcript was more readily used as the template in the RT-PCR reaction. However, transcription of the transposase is specific to germline cells, at least in adults, and these cells are less well represented in the sample than somatic cells.

It is believed that the 66-kDa repressor can bind to P element cleavage sites and block subsequent binding of the transposase enzyme, thus avoiding excision of the TE (Gloor et al. 1993, Laski et al. 1986). Additionally, antisense transcripts and polypeptides encoded by truncated copies offer alternative hypotheses to explain how P element mobilization can be silenced (Rasmusson et al. 1993, Simmons et al. 1996). These authors tested a group of antisense P element constructs and showed that these are bona fide repressors of pupal lethality, a condition established by the existence of P element sequences in which the IVS3 intron is deleted. These mutated sequences allow transcription of the transposase only, not the 66-kDa repressor, even present work, we suggested the synthesis of an antisense P element in un-manipulated organisms.

Antisense transcriptional silencing or RNA interference (RNAi) has been described for other TEs (Kleckner et al. 1996, Joanin et al. 1997, Jensen et al. 1999a). In D. hydei (Lankenau et al. 1994) and all of the repleta group (Almeida and Carareto 2000), the antisense transcripts of TE microopia, starting from an internal promoter, can be expressed in a testis-specific fashion. These antisense transcripts are not expressed in D. melanogaster, suggesting that D. melanogaster has developed a different mechanism of regulation than D. hydei. In our work, two distantly related species of the melanogaster group and the willistoni group, shared an antisense P element transcript of a similar size, suggesting the conservation of the transcription of the antisense sequence and implied importance for the organism.

In further support of the idea that antisense transcripts regulate transposon mobilization in Drosophila embryos, Blumenstiel and Hartl (2005) proposed siRNAs (small interfering RNA, composed of dsRNA of 21-25 nt) homologous to the Penelope TE are normally loaded into embryos of D. virilis and act as a silencing machinery against Penelope and other unrelated TEs. Moreover, P element regulation relies on a non-coding non-mosomal component. Ronssery et al. (1991, 1992) suggested that the insertion of the P element in telomeric-associated sequence on the X chromosome of D. melanogaster strongly promotes the induction of the cytotype inhibitor. The authors also showed that this activity is sensitive to mutant alleles of the gene Su(v) 

which encodes the Heterochromatin Protein 1, involved in the heterochromatin formation, and of the gene aubergine, which encodes the AUBERGINE protein, a protein that favors P element silencing in a non-loci-dependent manner, through a mechanism triggered by RNAi (Aravin et al. 2001, Reis et al. 2004). Also, Josse et al. (2007) determined the sensitivity of the P cytotype to mutations in the gene Su(var) 

which encodes proteins that trigger a silencing mechanism on P element sequences.


The TEs gypsy, Zam and Idefix of Drosophila are also regulated by heterochromatic loci: the flamenco locus of gypsy (Sarot et al. 2004), and the COM locus of Zam and Idefix (Desset et al. 2003). PIWI is one of the proteins involved in the RNAi-mediated mobilization silencing of these TEs (Desset et al. 2003, Sarot et al. 2004). Through immunopurification, various kinds of TEs were observed as being linked to PIWI, as were sense and antisense sequences of the TE roo (Saito et al. 2006). It is interesting that this protein is strongly expressed in ovaries and early embryos, including the pole cells where mobilization of the P element promotes the gonadal atrophy that characterizes hybrid dysgenesis.

When evaluating the two strains of D. willistoni with regard to their heterochromatic sites and the silencing mechanisms described previously, we expected that the Wip strain would have a more efficient RNAi silencing mechanism than the 17A2 strain, in which the UP1 and NP2 sequences, with 212 and 159 amino acids similar to the KP, do not silence P element mobilization (Simmons et al. 1996). In addition, we observed a truncated sequence of TEs were already sequenced. The most conserved region among them is an exon 1 sequence, also present in the KP repressor. However, we do not know whether these transcripts contain the same DNA binding and dimerization motifs of the KP repressor (Lee et al. 1996, 1998), two important sites for the inhibition of transcription, because the ORF0, which encodes these motifs, was not amplified and sequenced. Independent of the sequence conservation, the activity of the truncated transcripts as repressors of mobility should be tested, as the UP1 and NP2 sequences, with 212 and 159 amino acids similar to the KP, do not silence P element mobilization (Simmons et al. 1996).

In the present work, truncated transcripts related to the P element and amplified by RT-PCR were sequenced. These truncated copies could function as a stable source of repressive proteins (Pinsker et al. 2001), by creating heteromultimers (Rio 1999), or by triggering a mechanism of homology-dependent silencing (Jensen et al. 1999a, b). It is also possible to conjecture that we detected a deletion hotspot by creating heteromultimers (Rio 1999), or by triggering a mechanism of homology-dependent silencing (Jensen et al. 1999a, b).
$sites$, however, lie within the IVS3 intron sequence, while our data suggest an alternative splicing site into exon 2, at 1741 nt of the canonical $P$ sequence (GenBank accession code X06779). The transcripts described by Haring et al. (1998), originated by variant splicing, are 120 and 105-bp longer than the transposable transcript, and were not observed in embryos of either species examined in our study, in spite of their nucleotide conservation. The ability of these splicing variant sequences to transpose and their capacity to function as a repressor are still unknown.

The presence of a putative transposase transcript during embryogenesis of a non-hypermutable strain indicates the existence of a post-transcriptional control mechanism, possibly including the 66-kDa repressor, the truncated copies, mainly in $D. melanogaster$ mechanism, possibly including the 66-kDa repressor, indicates the existence of a post-transcriptional control during embryogenesis of a non-hypermutable strain.

The ability of these splicing variant sequences to transpose and their capacity to function as a repressor are still unknown.

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