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EXPERIMENTAL ARTICLES

Identification of *Chlorella* Viruses in *Paramecium bursaria* Clones by Pulse-Field Electrophoresis

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Abstract—The ciliates *Paramecium bursaria* contain endosymbiotic green algae *Chlorella* spp. in their cytoplasm. The algae isolated from *P. bursaria* are sensitive to large DNA-containing viruses of the family *Phycodnaviridae.* The type virus of this family is PBCV-1 (*Paramecium bursaria Chlorella* virus). Investigation of the total DNA of *P. bursaria* clones by pulse-field electrophoresis (PEGE) revealed a pronounced band on PEGE profiles of some *P. bursaria* clones; the band was formed by DNA molecules of approx. 300 kb. This band probably contained the DNA of *Chlorella* virus. Two approaches were used in the present work to confirm this hypothesis. Microbiological tests were used to scan a collection of *P. bursaria* clones for specific types of viruses; the 300-kb band was revealed only in the PEGE profiles of virus-containing clones. Blot hybridization of *P. bursaria* total DNA separated by pulse-field electrophoresis with the virus-specific probe revealed that the band under study was formed by the DNA of a *Chlorella* virus. Paramecium clones were shown to contain approx. 10^5 copies of nonintegrated viral DNA.

Key words: Paramecium bursaria, Chlorella, PBCV, *Phycodnaviridae*, symbiotic *Chlorella*.

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The ciliates *Paramecium bursaria* contain *Chlorella* green algae as endosymbionts [1]. The average number of algal cells per paramecium is 200 to 600; each is enclosed in an individual perialgal vacuole formed by the host's membrane [2]. *Chlorella* may be isolated from *P. bursaria* and maintained in pure culture. These chlorellas of symbiotic origin are sensitive to large DNA-containing viruses of the family *Phycodnaviridae* [3]. *Paramecium bursaria Chlorella* virus-1 (PBCV-1) is the type virus of this family. Viruslike particles in the *P. bursaria–Chlorella* system were originally revealed by electron microscopy [4]. The particles were found on the surface of *P. bursaria* cells, in the cytoplasm of *Chlorella* in the environment, and in digestive vacuoles of the paramecium [4].

At least two types of *Chlorella* exist, with different physiological and genetic characteristics and different sensitivity to viruses, i. e., type NC64A and type Pbi [5, 6]. The viruses specifically infecting two types of *Chlorella* are termed NC64A and Pbi viruses, respectively [5]. The distribution of viruses was suggested to depend on latitude and on the climate [7, 8]. *Chlorella* and viruses of the NC64A and Pbi types were therefore termed "northern" and "southern," respectively [9–11]. Each type of virus is specific against one of the two type strains of symbiotic *Chlorella*; other symbiotic or free-living *Chlorella* species are not infected [12]. The viruses were shown to infect and lyse only the cultures of ex-symbiotic *Chlorella*. Lysis of the symbiotic *Chlorella* within *P. bursaria* cells has never been observed; paramecia probably protects *Chlorella* cells from the lytic action of the virus [3].

Pulse-field electrophoresis is one of the approaches used to study symbiotic multicomponent systems; it has been previously adapted for *P. bursaria* [13]. This method enables differentiation between genomes of different size belonging to the symbiotic partners. Most of the PEGE profile of the total *P. bursaria* DNA contains the macronuclear DNA, a spectrum of relatively short molecules (from 30–40 to 120–150 kb). The high-molecular fraction (over 2000 kb) probably contains the chromosomal DNA of the micronucleus and the DNA of symbiotic *Chlorella*. In the PEGE profiles of some *P. bursaria* clones, a distinct narrow band was revealed; it contained DNA molecules of approx. 300 kb, the size of a viral genome [13].

The goal of the present work was to investigate *P. bursaria* clones for *Chlorella* viruses by standard microbiological methods [14], as well as by blot hybridization of the total DNA PEGE profiles with a virus-specific probe. The band in question was shown to contain viral DNA. The genomes of *Chlorella* viruses were for the first time studied in a complete symbiotic system (*P. bursaria–Chlorella*). The work was carried out using 34 clones collected worldwide.

MATERIALS AND METHODS

***Paramecium bursaria* clones.** A total of 34 green *P. bursaria* clones (containing *Chlorella*) from the col-

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P. bursaria clones used in the present work

Clone	Geographyc origin and year of isolation	Results of testing for NC64A viruses (southern)	Results of testing for Pbi viruses (northern)	Band on the PEGE profile
Mit B	–	Absent	Absent	Absent
Cs 2	–	Absent	Absent	Absent
T 316	–	Present	Absent	Present
OK 1	–	–	–	–
97 JR 16	Japan, 1997	Absent	Absent	Absent
GoB 4	Georgia, 1988	Absent	Absent	Absent
AL 2-10	Lake Sevan, Armenia, 1983	Absent	Absent	Absent
87 MC 1	St. Petersburg, Russia, 1987	Absent	Present	Present
94 BS 3	St. Petersburg, Russia, 1994	Absent	Absent	Absent
PP 1	Pskov oblast, Russia, 2000	Absent	Absent	Absent
DP 1	Leningrad oblast, Russia, 2000	Absent	Absent	Absent
DP 2		Absent	Absent	Absent
MP 3	Leningrad oblast, Russia, 1991	Absent	Present	Present
02 RA 5-1-2	Altai krai, Russia, 2002	Absent	Absent	Absent
AZ 7-15	Astrakhan Reserve, Russia, 2002	Absent	Absent	Absent
AZ 1-1		Absent	Absent	Absent
AZ 1-2		Absent	Absent	Absent
AZ 8-2		Absent	Present	Present
AZ 11-10		Absent	Present	Present
AZ 12-9		Absent	Present	Present
AZ 17-2		Absent	Present	Present
AZ 17-5		Absent	Absent	Absent
AZ 17-6		Absent	Absent	Absent
AZ 17-7		Absent	Present	Present
02 B 1-2	Volgograd oblast, Russia, 2002	Absent	Present	Present
88 T 1-3	Tajikistan, 1988	Absent	Absent	Absent
88 T 4-1		Absent	Absent	Absent
88 T 4-2		Absent	Absent	Absent
88 T 24-5		Present	Absent	Present
BP 37	Far East, Russia, 1984	Absent	Absent	Absent
BA 1-6	Dal'nie Zelentsy, Russia, 1987	Absent	Absent	Absent
AB 6-51	Boston, United States, 1994	Absent	Absent	Absent
AB 6-65		Absent	Absent	Absent
AB 6-70		Absent	Absent	Absent
Ard 16	Oklahoma, United States, 2006	Present	Absent	Present

lection of the Laboratory of Protozoan Karyology Biological Research Institute, Faculty of Biology and Soil Science, St. Petersburg State University were used. Clones T 316, Mit B, and Cs 2 were obtained from I. Miva (Ibaraki, Japan) in 1989. Other clones have been isolated from geographically remote natural populations (table). Clone OK 1 (also obtained from I. Miva) is a natural asymbiotic clone not containing *Chlorella* in the cytoplasm. Paramecia were maintained on lettuce medium inoculated with *Klebsiella cloacae*. The clones were cultivated according to the standard procedure [15].

Viral isolates. The following pure viral isolates were used: PBCV-1 southern type (obtained from J. Van Etten, Nebraska University, Lincoln, United States), and PBCV-2L, northern type (isolated in 1984 from natural water bodies of the Soviet Far East). The southern type of the virus T316-1S was isolated in 2006 from the *P. bursaria* clone T316. The strains were isolated and purified by the standard procedure (filtration of the lysate through a Nucleopore 0.4 μm filter) [14].

Testing the *P. bursaria* clones for viruses. The following ex-symbiotic *Chlorella* sp. strains from the collection of the Biological Research Institute, St. Petersburg State University were used as test cultures: strain NC64A of the southern type (obtained from J. Van Etten, Nebraska University, Lincoln, United States); strain OCH1 of the northern type (isolated in 1985 in Karelia, USSR); and strain Pbi (obtained from W. Reisser, Gettingen, Germany). Strain NC64A is sensitive to the southern type viruses; strain Pbi is sensitive to the northern type viruses. According to the standard procedure for water testing [14], the culture liquid of a *P. bursaria* clone was filtered through Nucleopore 0.4 μm, and an aliquot was added to an actively growing *Chlorella* culture. While in the absence of viruses, *Chlorella* growth was the same in experimental and control test tubes (Fig. 1b), in the presence of a specific virus, *Chlorella* cells lysed, and the medium became transparent (Fig. 1a). All the clones were tested for the presence of the northern and southern type viruses. In accordance with the standard procedure [14], lysate dilutions (dilution series of $10–10^{-8}$) were mixed with soft (0.75%) agar and a *Chlorella* suspension (NC64A or Pbi) and plated on dishes with agarized medium (1.5%); typical viral plaques were registered after 24–48 h.

Preparation of *P. bursaria* DNA samples for pulse-field electrophoresis. Paramecium cultures were used to prepare the DNA samples for PEGE. The cells were concentrated by centrifugation at 600 *g* to $10^4–10^5$ cells per 0.5 ml. The pellet was resuspended and mixed (1 : 1) with melted (cooled to 50°C) agarose (SeaKem, FMC, United States) on 0.125 M EDTA, pH 8.0. The mixture was pipetted and distributed into the moulds (between the bottoms of two 40-mm plastic petri dishes fixed with adhesive tape). Agarose plates 1 mm thick were obtained, with a final cell concentration of 0.7–1.5×10^4 per 1 ml. The plates were cooled for 5 min at

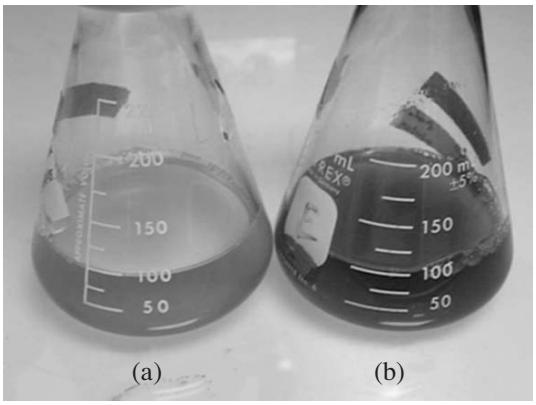

Fig. 1. Testing the *P. bursaria* clones for viruses. Addition of an aliquot of a virus-containing *P. bursaria* clone into a thick *Chlorella* suspension (b) causes its lysis; the medium becomes transparent (a).

4°C. The integrity of the cells was determined under a binocular microscope. The plates were then incubated for two days at 37°C in the ESP lysing buffer (0.5 M EDTA, pH 9.5; 1.5% sodium lauroylsarcosine, 200 μg/ml pronase E). The blocks were stored in the same lysing solution at 4°C. For PEGE, block-inserts were excised to fit the size of the gel pockets. *Saccharomyces cerevisiae* 15B-P4 chromosomes of the previously determined size [16] were used as size markers for PEGE. The agarose blocks with *S. cerevisiae* DNA were prepared according to the standard procedure [17].

Preparation of *Chlorella* and pure viruses samples for pulse-field electrophoresis. *Chlorella* cells were removed from the agar with a loop and resuspended in the Bristol medium [18]. The cells were concentrated by centrifugation for 10 min at 600 *g* to a volume of 0.5 ml. The subsequent procedure was the same as for the *P. bursaria* DNA. The purified viral particles were resuspended in 100 mM Tris–HCl, pH 7.4 and mixed with molten agarose (1 : 1). The subsequent procedure was the same as for the *P. bursaria* DNA.

Pulse-field electrophoresis. The device designed by the authors of [19] was used for PEGE; it enables creation and alternation of two electric fields with a realignment angle of 120°. PEGE was carried out in 1% agarose gel (Sea Kem, FMC, United States) on a 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 2 mM EDTA, pH 8.0). Gel thickness was 4 mm. Electrophoresis was carried out in a 0.5× TBE buffer at 16°C. The field strength was 10 V/cm. For the separation of *P. bursaria* DNA, the optimal mode was as follows: 30 s–7 h; 50 s–7 h; 70 s–7 h; 120 s–7 h; and 150 s–8 h, with the seconds indicating the time of action of each electric field (pulse time) and the hours the duration of device operation at a given pulse time.

Southern blot hybridization. After PEGE, DNA was transferred to nylon filters (Amersham Pharmacia Biotech Nylone Membranes) by the capillary method

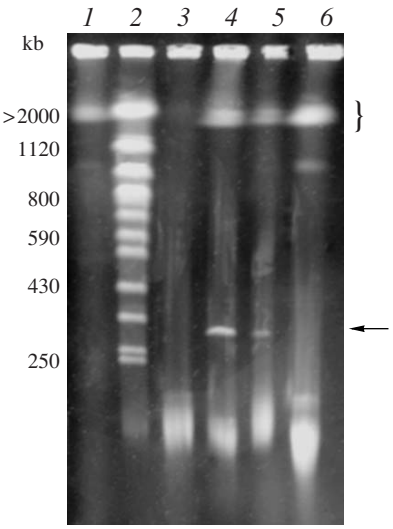

Fig. 2. Results of PEGE separation of the total DNA of *P. bursaria* clones and a *Chlorella* strain. Lanes: *1*, *Chlorella*, strain OCH1 (northern type); *2*, size marker (*S. cerevisiae* chromosomes); *3*, *P. bursaria*, clone OK 1, *Chlorella*-free; *4*, *P. bursaria*, clone 87 MC 1, contains the northern type of the virus; *5*, *P. bursaria*, clone T316, contains the southern type of the virus; *6*, *P. bursaria*, clone Mit B, virus-free. Location of the band of approx. 300 kb in virus-containing strains is marked by a black arrow. A curled bracket indicates the zone of high-molecular DNA (2000 kb and more) where *Chlorella* DNA is localized.

using the standard procedure with 20× SSC [20] and immobilized by ultraviolet irradiation.

The hybridization probe was obtained by polymerase chain reaction (PCR); the PCR mixture was supplemented with digoxygenin-11-dUTP (Roche Diagnostics, Mannheim, Germany). A conservative part of the viral DNA polymerase gene corresponding to the polymerase domain fragment was used as a hybridization probe. Synthetic oligonucleotides 5'-GA(A/G)GGIGGIACIGTI(T/C)TIGA(T/C)GC-3' (primer I) and 5'-GCIGC(A/G)TAIC(G/T)(T/C)TT(T/C)TTI(A/T)(A/G)TA-3' (primer II) were used as primers (see [21]). The total DNA of PBCV-2L and T316-1S viruses was used as the template. The PCR reaction mixture (100 μl) contained 1.5 U *Taq* polymerase (SibEnzyme, Russia), 20 pmol of the nucleotidetriphosphate mixture, 10 pmol dioxygenin-11-dUTP, 50 pmol of the primer mixture (1 : 1), and 1–5 ng of template DNA. The PCR parameters for the probe synthesis were as follows: 94°C, 45 s; 57°C, 45 s; 72°C, 45 s for 30 cycles with annealing time in the first cycle increased to 4 min. The PCR output was controlled by electrophoresis in 1% agarose in 0.5× TBE buffer.

The Southern hybridization was carried out at 60°C according to the standard procedure. The signal was detected using the colorimetric label detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. Thermal washing in 0.5× SSC [20] was carried out at a temperature 1°C higher than the hybridization temperature.

RESULTS AND DISCUSSION

PEGE profiles of some *P. bursaria* clones revealed a clear narrow band formed by DNA molecules of approx. 300 kb. A suggestion was made that this band was formed by a *Chlorella* virus DNA. To confirm this hypothesis, PEGE profiles of the total DNA of 34 *P. bursaria* clones were obtained. All the clones were tested for the presence of viruses of the northern and southern type using the standard microbiological techniques [14]; the results are presented in the table. Eleven clones contained viruses, and 25 were virus-free. Three clones contained the southern type viruses (T 316, Ard 16, and T 24-5), while eight clones contained the northern type viruses (AZ 8-2, AZ 12-9, AZ 17-2, AZ 17-7, 02 B 1-2, MP 3, and 87 MC1). PEGE profiles of eleven clones contained a band of approx. 300 kb (Fig. 2, lanes *4, 5*; Fig. 3, lanes *2, 5*). The presence of the viruses in these clones was confirmed by microbiological tests. The clones without the band in this part of the PEGE profile were virus-free (Fig. 2, lane *6*). Thus, a relation was established between the presence of viruses in the clones and the band in their PEGE profiles (table).

Experiments were performed on blot hybridization of PEGE profiles of the total DNA from *P. bursaria* clones with the virus-specific probe. A fragment of the viral DNA polymerase gene sequence was used as the probe. The hybridization pattern of the PEGE profiles of total DNA from the virus-containing paramecium clones always contained a clear signal corresponding precisely to the 300-kb DNA band (Fig. 3a, lanes *2, 5*; Fig. 3b, lanes *2, 5*). The DNA of purified viruses used as a control also formed a band in the 300-kb region of the PEGE profiles (Fig. 3a, lanes *1, 3, 4, 6*) and produced a bright hybridization signal (Fig. 3b, lanes *1, 3, 4, 6*). No hybridization signal was detected when the total DNA of the virus-free *P. bursaria* clones was hybridized with the virus-specific probe. Thus, two different approaches uniformly confirmed that the band of interest in the PEGE profiles of the total DNA of *P. bursaria* clones was formed by genomic DNA of *Chlorella* viruses.

Two probes were used in hybridization experiments; one of them was obtained with the southern type virus (PBCV-1) as template and the other with the northern type virus (OCH-1). Hybridization signals were stronger when the viral DNA of the same type as the one present in *P. bursaria* clones was used as a template (Fig. 3b, lane *2*). Hybridization with the probe specific for another type of the virus resulted in a relatively weak signal (Fig. 3b, lane *5*). A similar signal was observed in the case of hybridization of the total viral DNA. The signal was stronger when both the template and the total DNA sample belonged to the same type of virus, northern (Fig. 3b, lane *6*) or southern (Fig. 3b,

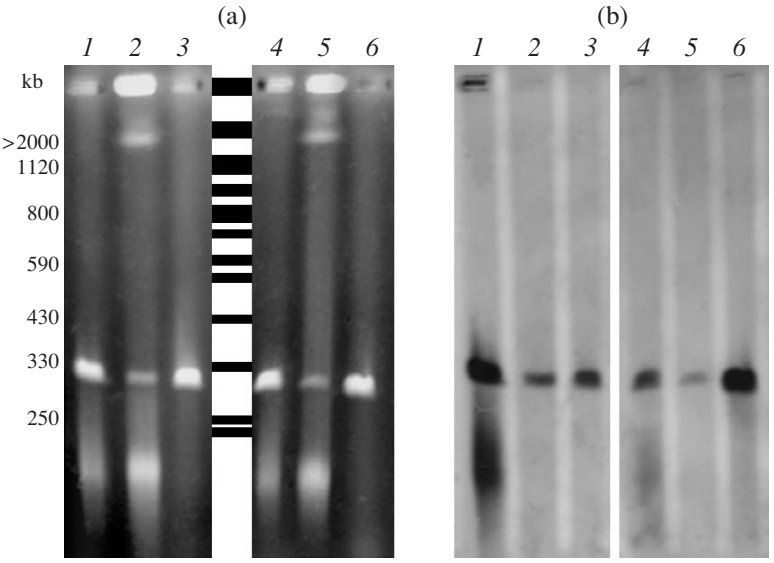

Fig. 3. Results of PEGE separation of the total DNA of a *P. bursaria* clone and DNA of the viral isolates (a) and blot hybridization (b) with the probe synthesized using the southern type of the virus as template (lanes *1, 2*, and *3*) and with the probe synthesized using the northern type of the virus as template (lanes *4, 5*, and 6). Lanes: *1* and *4*, DNA of PBCV-1 virus (southern type); *2* and *5*, results of PEGE separation of the total DNA of *P. bursaria*, clone T 24-5, containing the southern type virus; *3* and *6*, DNA of OCH1 virus (northern type).

lane *1*). Cross hybridization resulted in a weaker signal (Fig. 3b, lanes *3*, *4*). Dependence between the hybridization efficiency and the virus type used to construct the probe is most probably due to the variability of the DNA polymerase gene. For 42 studied NC64A (southern type) and PBCV-1 viruses, the homology of the fragment of the DNA polymerase gene fragment used as a probe was 93 to 99.7% [22]. For different types of the virus, these differences are more pronounced; homology between five Pbi viruses and PBCV-1 (NC64A virus) was only 70% [22].

These data demonstrate that nonintegrated viral DNA is present in virus-containing *P. bursaria* clones. Since the complete symbiotic system was studied (*P. bursaria*–*Chlorella*–virus), the possibility of integration of the viral genome into the genome of its host (*Chlorella*) was also considered. Viruses of brown algae *Ectocarpus siliculosus* EsV, phylogenetically the closest relatives of *Chlorella* viruses [23], have a lysogenic phase in their life cycle (the viral genome is incorporated into the host genome) [24]. Lytic viruses have been reported for the symbiotic *Chlorella*, though not for the free-living *Chlorella* and *Chlamydomonas* [12]. The life cycle of *Chlorella* viruses may also contain a lysogenic phase. The viral DNA incorporated into the host genome should be located in the high-molecular region (over 2000 kb) where *Chlorella* chromosomal DNA migrates [25]. However, hybridization signals in this region were never detected in our experiments. Thus, neither PEGE nor Southern hybridization revealed the copies of the viral DNA incorporated into *Chlorella* genome.

Thus, the presence of nonintegrated copies of the viral genome in *P. bursaria* strains was demonstrated. Two other tasks to be achieved were determination of the amount of viral DNA in the clones and its localization in the system. Qualitative estimation of the copy number of the viral genome forming the 300-kb band was carried out by visual comparison of the intensity of the bands formed by viral DNA on *P. bursaria* PEGE profiles and the bands of the "pure" viral DNA of a known concentration. The biological titer of the virus in the solution used for subsequent dilutions was 1×10^6 plaque-forming units per 1 ml. Since only half of the particles are believed to be infective [12], the estimated total number of viral particles in this solution was 2×10^6 viral particles per 1 ml. Using the serial dilutions of this suspension containing 10^4, 5×10^4, 2.5×10^5, 5×10^5, and 10^6 viral particles per 1 ml, the number of copies of the viral genome forming the band on *P. bursaria* PEGE profiles was estimated as 10^5.

It was further investigated whether the viral DNA revealed on *P. bursaria* PEGE profiles belonged to free viruses or to those associated with the paramecia cells. The virus-containing *P. bursaria* clone T 316 was used for the purpose. The sample for PEGE were prepared according to the standard procedure; however, prior to centrifugation (See Materials and Methods), the cell suspension was divided into two subsamples. The cells from one subsample were removed by filtration, and only the filtrate was used for PEGE. The other subsample was used for the standard (cell-containing) preparation. The PEGE profile of the standard sample revealed the typical *P. bursaria* pattern (picture not shown) with a viral DNA band of approx. 300 kb. No viral DNA was

detected in the PEGE profile of the cell-free sample. Since free viral particles are not precipitated at the centrifugation mode applied [14], the viral DNA revealed by PEGE profiles is evidently concentrated together with *P. bursaria* cells. Viral particles are certainly present in the medium, as was confirmed by microbiological tests [14]; their concentration, however, is not sufficient for detection of viral DNA by PEGE. According to our quantitative estimates, approx. 300 paramecium cells and 10^5 copies of the viral genome are contained in a block-insert. Thus, approx. 300 viral particles are associated with each paramecium cell.

Thus, the presence of viral DNA on the PEGE profiles of virus-containing *P. bursaria* clones indicates physical interaction between viruses and paramecium cells. It is still unclear whether the viral DNA detected in the system is contained within the viral particles or is represented otherwise. The cell compartment with which the viral particles are associated is also unknown. The particles are most probably located on the surface of paramecium cells, as has been demonstrated by Kawakami [4]. Association of algae with the infusorians prevents the development of lytic infection; the contact between *Chlorella* and viruses that occurs in the case of paramecium death and liberation of *Chlorella* into the medium. A definite low concentration of the viruses is therefore constantly maintained in the medium [3].

Thus, viral DNA in virus-containing *P. bursaria* cells can be revealed by PEGE. In *P. bursaria* clones, a considerable part of the viral DNA is not integrated in the *Chlorella* genome. Approx. 300 viral particles are associated with one paramecium cell. Direct interaction between the viral particles and paramecium cell probably occurs; its mechanisms require further investigation.

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