Morphology and Viruslike Particle Content of *Helminthosporium victoriae* Colonies Regenerated from Protoplasts of Normal and Diseased Isolates

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ABSTRACT

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Protoplast suspensions were prepared from cultures of two normal and two diseased isolates of *Helminthosporium victoriae*. Protoplasts from the normal isolates regenerated and formed colonies morphologically identical to the original cultures. Colonies regenerated from protoplasts of diseased isolates were of three morphologically-distinct types: type 1–normal vigorous colonies with dense aerial mycelium; type 2–colonies resembling the original diseased cultures; and type 3–severely stunted colonies with very sparse aerial mycelium. Diseased isolates previously were reported to contain two distinct types of isometric viruslike particles (VLPs) designated 145S (Svedberg units) and 190S VLPs. Normal isolates, on the other hand, either were VLP-free or contained only the 190S VLPs. The normal colonies, type 1, obtained from protoplasts of diseased isolates either were devoid of or contained only traces of 145S VLP. The 145S VLP content of mycelia from the diseased colonies (type 2 or 3) correlated directly with disease severity; the highest yields of 145S VLPs were obtained from mycelia with the most pronounced disease symptoms. These results suggest that the 190S VLP is not deleterious to its host and that the 145S VLPs are associated with the disease.

A disease of the fungus *Helminthosporium victoriae* Meehan & Murphy, the causal agent of Victoria blight of oats, was described by Lindberg in 1959 (4). The disease was transmitted to normal isolates by hyphal anastomosis (5). Diseased isolates were characterized by reduced rates of growth, collapse of aerial mycelium, excessive branching of hyphae with usually short branches, and reduced spore production (4). Examination by light microscopy revealed that hyphae from diseased isolates had swollen, distorted cells and varying degrees of lysis (6).

Although certain features of the disease suggested a viral etiology (4,6), no supportive evidence was presented. Recently, we reported the isolation of two serologically and electrophoretically distinct types of isometric viruslike particles (VLPs) from diseased isolates of *H. victoriae* (7). Based on their Svedberg sedimentation coefficients, these two particle types were designated as the 145S and 190S VLPs (7). Normal isolates, on the other hand, were either VLP-free or contained only the 190S VLP (7).

The present study was undertaken to determine whether normal colonies could be obtained from diseased isolates of *H. victoriae* through the preparation and regeneration of protoplasts and if so, whether the VLP content of such colonies was different from that of the original isolates.

MATERIALS AND METHODS

Fungus isolates, culture media, and VLP extraction. Four isolates of *H. victoriae* were used for protoplast preparations. Isolates A-9, B-1, and B-2 were subcultures of isolates used in a previous investigation (7). The fourth isolate, designated H.v. 408, was obtained from O. C. Yoder, Cornell University. Stock cultures of all isolates were maintained on slants of potato-dextrose agar supplemented with 0.5% yeast extract (PDAY). For extraction of VLPs, the fungal isolates were grown in stationary cultures for 10–14 days at room temperature on potato-dextrose broth containing 0.5% yeast extract (PDBY). Purification of VLPs was as previously described (7).

Preparation and regeneration of protoplasts. Protoplasts were isolated either by the procedure of Anderson and Yoder (1) or by a modification of the method described by Ferenczy et al (2). The latter yielded greater numbers of protoplasts and finally was adopted. In this procedure, 0.7 M KCl was the osmotic stabilizer during protoplast preparation and mycelium regeneration. Mycelial mats, grown for 2–3 days on a medium containing 0.5% yeast extract and 1% glucose (YG), were transferred to a solution containing 0.7 M KCl and 10% (v/v) Millipore-filter-sterilized β-glucuronidase Type H-2 (Sigma Chemical Company) in a 125-ml Erlenmeyer flask. The mixture was shaken at 80 rpm for 3 hr at 25 C in a Metabolite water bath shaker (New Brunswick Scientific Company). The protoplasts were separated from mycelia by filtration through 20 μm nylon mesh and the filtrate was centrifuged at 180 *g* for 10 min in an International Model HN centrifuge. The pellets (protoplasts) were washed three times with 0.7 M KCl and serial dilutions of the washed protoplasts were made using 0.7 M KCl as a diluent. Each of the various dilutions was mixed at 42 C into YG medium containing 0.7% KCl and 0.8% agar and poured over YG medium containing 0.7% KCl and 2% agar. Regenerated protoplasts, 24–48 hr after plating, were transferred individually to PDAY plates and the cultural characteristics of the developing colonies were observed.

RESULTS

Properties of the fungal isolates. Four isolates of *H. victoriae*, two normal and two diseased, were used for protoplast preparation (representative cultures of these isolates are shown in Fig. 1). One of the two normal isolates, B-2, previously was shown to contain the 190S type of VLP (7) whereas the other, H.v. 408, was found to be VLP-free. Extracts from isolate H.v. 408, prepared as described before (7), were subjected to rate zonal sucrose density gradient centrifugation (7). No VLPs were resolved in the centrifuged gradients of at least five different preparations. The two normal isolates grew rapidly on PDAY and produced uniform mycelial

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mats and abundant aerial mycelium.

The two diseased isolates used were A-9 and B-1; each has been shown to contain both the 145S and 190S VLPs (7). The diseased isolates grew at a slower rate on PDAY than the normal isolates and produced irregular mycelial mats which had a large number of sectors. The aerial mycelium was sparse and often collapsed.

Morphology of colonies produced from protoplasts. Protoplasts of all four isolates varied greatly in size (5–15 μm) and in number and size of vacuoles (Fig. 2A). The protoplasts regenerated readily in regeneration medium osmotically stabilized with either 0.7 M KCl or 20% sucrose. In each experiment 50–100 protoplasts showing hyphal initiation (Fig. 2B) were transferred individually, 24–48 hr after plating, to PDAY plates and the cultural characteristics of the developing colonies were observed. Colonies produced from protoplasts of the normal isolates B-2 (Fig. 3) or H.v. 408 were morphologically identical to those of the respective original cultures. Protoplasts from diseased isolates, on the other hand, regenerated into three morphologically-distinct types of colonies (Fig. 3,4). Type 1 colonies showed vigorous growth with abundant aerial mycelium and had the cultural characteristics of normal isolates. Type 2 colonies essentially resembled the original diseased cultures; they grew at a slower rate than type 1 colonies and produced sparse aerial mycelium which often collapsed. Type 3 colonies grew extremely slowly; colony diameters did not exceed 5–10 mm after 10 days of growth.

Hyphae at the edges of type 1 colonies had the morphological and cytological characteristics of normal isolates (6). Hyphae were long with few branches (Fig. 5A), and the cells were uniform in size and in appearance of the cytoplasmic contents (Fig. 5B). Hyphae of type 3 colonies were short, swollen, and highly branched (Fig. 5C), and the cells varied in size and appearance (Fig. 5D). The majority of cells were swollen and had disorganized or granular cytoplasmic contents. The cytoplasm of some cells was extruded and appeared as globules attached to the sides of hyphae (Fig. 6). These characteristics are typical of the symptoms described by Lindberg (4) for the disease of *H. victoriae*. Young hyphae at the edge of type 2 colonies initially appeared to be normal, but as the colony grew older, areas of diseased hyphae developed along the margins. This was followed by collapse of aerial mycelium. Cells with extruded cytoplasmic contents were observed less frequently in type 2 than in type 3 colonies.

Type 1 and 2 colonies remained unchanged throughout successive transfers to PDAY. However, in some older cultures of type 1, scattered areas of aerial mycelium would collapse. This was not observed in cultures of B-2 or H.v. 408 (normal isolates) of comparable age. Subculturing of type 3 colonies on PDAY generally gave rise to colonies with type 3 morphology. However, after several transfers a segment of normal-appearing hyphae occasionally would develop along the edge of some colonies. Subculturing of these hyphae on PDAY resulted in a culture with the morphology of type 2 colonies.

VLP content. Mycelia from type 1 and 2 colonies of isolate B-1 were grown on PDBY in flasks for the purpose of VLP extraction. The results of density gradient centrifugation of VLP preparations from these types of mycelia are shown in Fig. 7A and B. VLP preparations from type 1 mycelium contained predominantly the 190S VLP and only trace amounts, if any, of the 145S VLP. Type 2 mycelium was similar to the original diseased culture in that it contained both 145S and 190S VLPs. The 145S VLP-titer varied with different preparations, but generally was lower than that of the 190S VLP. The slowest sedimenting component, designated 113S (Fig. 7), previously was shown to be composed primarily of empty capsids of both 145 and 190S VLPs (7).

The growth of type 3 colonies on PDYB was very limited and adequate mycelium for VLP extraction could not be obtained. Thus, the following modification was made so that type 3 mycelium would be included as a source of VLPs: mycelial fragments obtained from type 3 colonies as well as segments of swollen, highly branched hyphae from type 2 mycelium were transferred to PDBY in petri dishes and their growth was observed with a stereomicroscope. The process of selecting mycelial fragments exhibiting typical disease symptoms from these cultures was continued and eventually mycelium from 50–80 plates (100–150 g wet weight) was harvested and processed for VLP extraction. The VLP titer of this mycelium, estimated from UV absorbancy at 260 nm of purified preparations, was two- and five-fold greater than that obtained from equivalent weights of type 2 and type 1 mycelia, respectively. When analyzed by density gradient centrifugation, VLP preparations from this mycelium showed a remarkably high 145 S: 190S ratio (Fig. 7C).

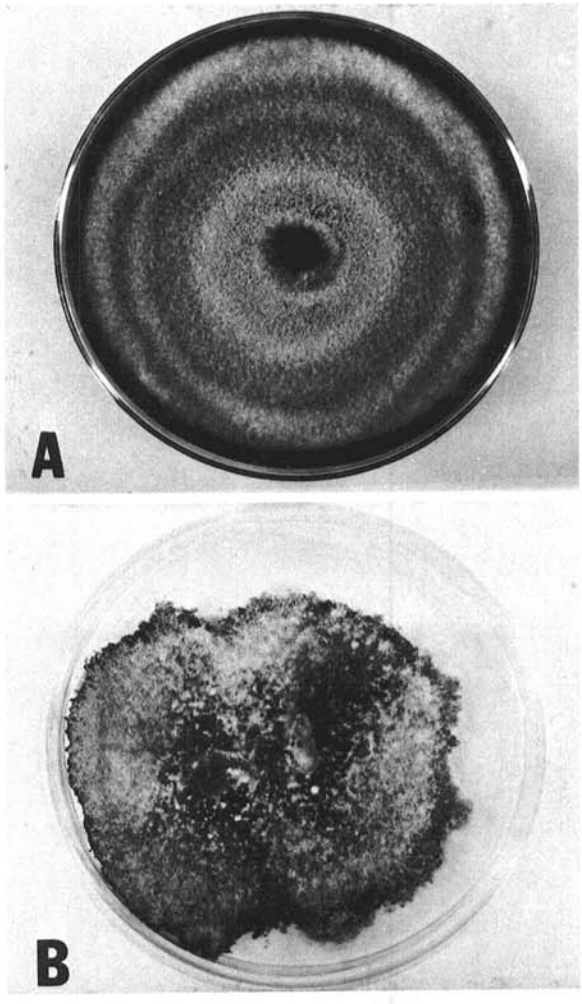

Fig. 1. Morphology of two isolates of *Helminthosporium victoriae*: **A)** normal isolate, B-2, and **B)** diseased isolate B-1, grown on potato-dextrose agar-yeast extract for 7 days.

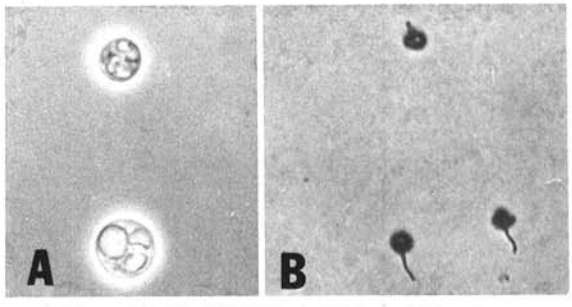

Fig. 2. A) Protoplasts from isolate B-1 of *Helminthosporium victoriae* (×800, phase-contrast). **B)** Regenerating protoplasts from isolate B-1 of *H. victoriae* on yeast extract-glucose medium containing 0.7 M KCl and 2% agar. Photographed 20 hr after plating (×60, stereomicroscope).

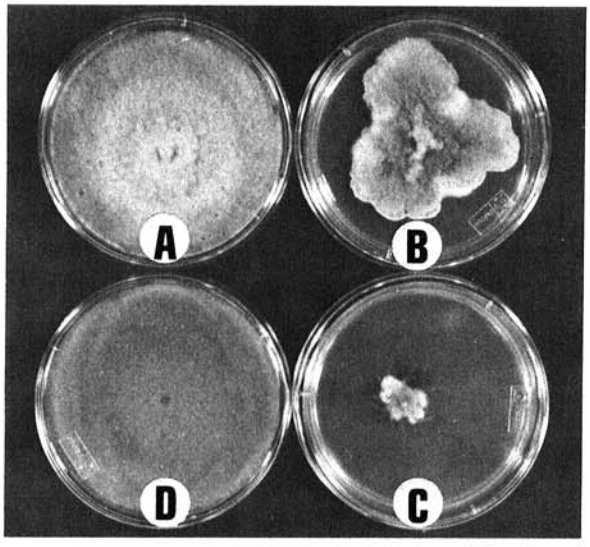

Fig. 3. Morphology of colonies produced from regenerated protoplasts of (A to C) a diseased isolate, A-9, and (D) a normal isolate, B-2, of *Helminthosporium victoriae*. A to C show three morphologically-distinct colony types produced from protoplasts of isolate A-9 and designated: **A)** type 1, **B)** type 2, and **C)** type 3. **D)** shows a representative colony of those derived from protoplasts of isolate B-2; the colonies are morphologically identical to the original culture. All colonies are 7 days old on potato dextrose agar yeast extract medium in 60 × 15 mm petri plates.

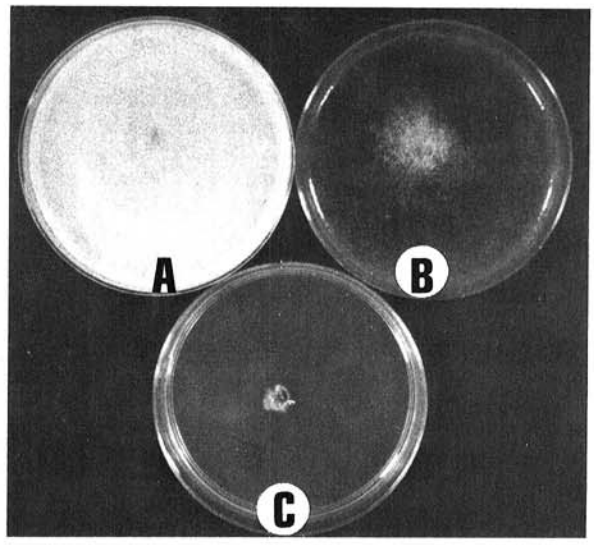

Fig. 4. Colony types produced from regenerated protoplasts of a diseased isolate (B-1) of *Helminthosporium victoriae*. Three morphologically distinct types of colonies, designated type 1, 2, and 3 are shown in **A, B,** and **C,** respectively.

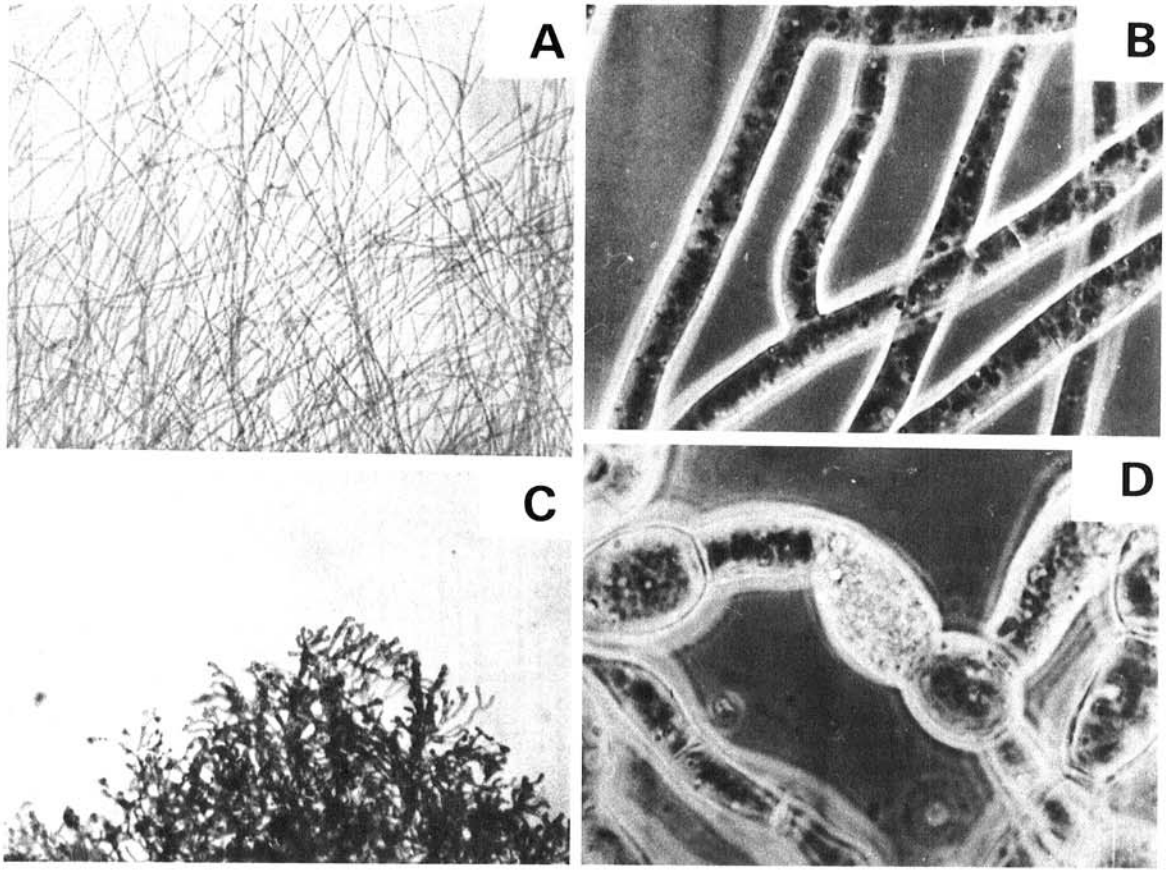

Fig. 5. Morphology of hyphae at the edges of colonies produced from protoplasts of a diseased isolate (B-1) of *Helminthosporium victoriae* grown on potato dextrose broth yeast extract medium for 5 days. Hyphae from type 1 colonies examined in **A)** the stereomicroscope (×43), and **B)** with phase contrast microscopy (×1,027). Hyphae from type 3 colonies, similarly examined, are shown in **C** and **D**.

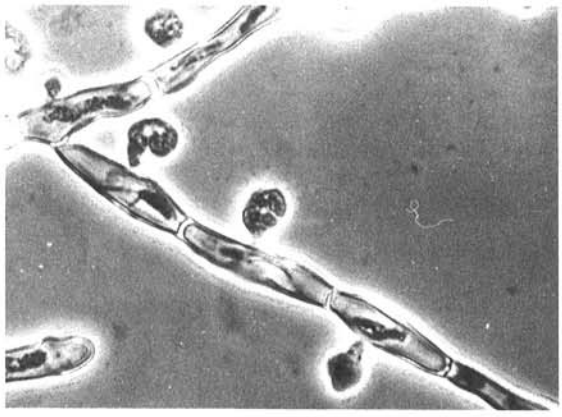

Fig. 6. Morphology of hyphae from type 3 colonies of a diseased isolate (B-1) of *Helminthosporium victoriae* showing cells that have extruded some of their cytoplasmic contents (×1,000, phase contrast).

DISCUSSION

Previous data (7) indicated that diseased isolates of *H. victoriae* contained two distinct types of isometric VLPs designated 145S and 190S VLPs, and that normal isolates were either VLP-free or contained only the 190S VLP. The present finding that normal colonies, containing predominantly the 190S VLP, could be produced from diseased isolates by the preparation of protoplast suspensions and the regeneration of colonies from those protoplasts lends support to a previously stated hypothesis (7) that the 190S VLP does not have deleterious effects on its host, whereas the 145S VLP is associated with the disease. Further support for this was obtained in experiments in which severely diseased cultures appeared to have an increased titer of the 145S VLP. That the 145S VLP is the causal agent of disease can only be documented if the disease is transmitted to normal isolates using purified preparations of this particle type. Research is now in progress to develop infectivity assays utilizing protoplasts from normal VLP-free isolates and purified VLP preparations.

Even though isolates containing only the 190S VLP appear as healthy as the VLP-free isolates, a role for the 190S VLP in disease development cannot be ruled out. All diseased isolates contained both types of particles and no isolate was ever found which contained only the 145S VLP. If these two types of VLPs are indeed viral in nature, the replication of the 145S and/or the development of the disease might be dependent on the presence of the 190S VLP in the same cell.

The production of morphologically-distinct colonies from regenerating protoplasts of diseased isolates of *H. victoriae* apparently reflects the uneven distribution of 190 and 145S VLPs in different regions of the mycelium. Fluctuation in the relative titers of two VLPs in a strain of *Penicillium citrinum* was reported to occur spontaneously resulting in phenotypic segregation in the host for growth and sporulation (8). Lemke (3) reasoned that replication of a virus in a fungal mycelium made up of an extensive, semi-continuous, asynchronous network of cells, would be expected to be asynchronous.

The 190S VLP from *H. victoriae* was apparently more uniformly distributed throughout the mycelium than was the 145S VLP. The 145S VLP was reported to be a multicomponent system containing four species of double stranded(ds-)-RNA distributed among four classes of particles, whereas the 190S was shown to be a single component VLP containing a single species of ds-RNA (7). The multipartite nature of the 145S VLP genome might decrease its efficiency of infection and, hence, affect its distribution in the fungal mycelium.

Preparation and regeneration of protoplasts from fungi may represent an alternative method to thermotherapy, chemotherapy, or hyphal-tip subculturing for production of virus-free or partially free fungal cultures.

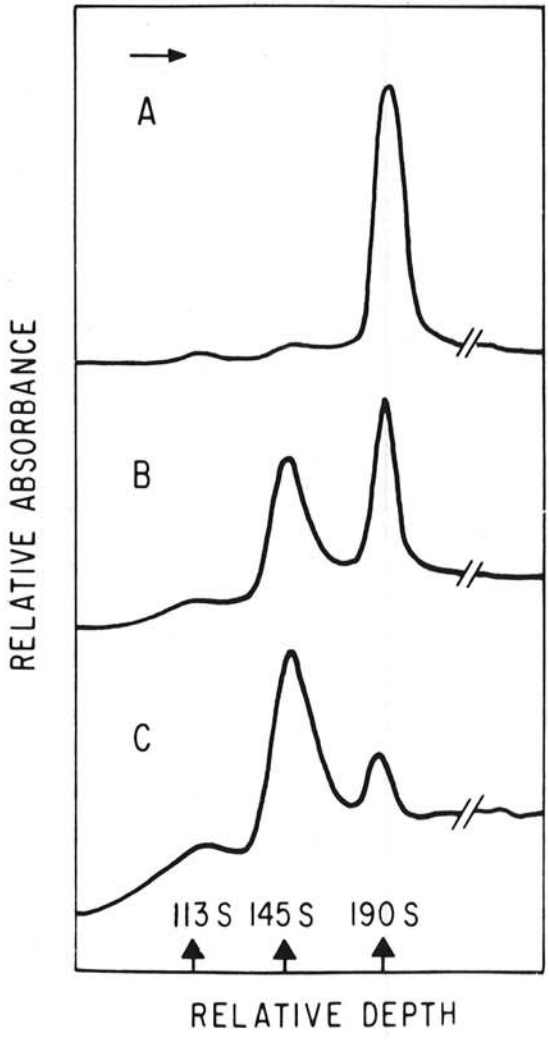

Fig. 7. Ultraviolet absorbancy profiles after sucrose density gradient centrifugation of viruslike particles preparations from mycelia regenerated from protoplasts of a diseased isolate (B-1) of *Helminthosporium victoriae*. A, B, and C represent VLP preparations from mycelia subcultured from type 1, type 2, and a combination of type 2 and 3, respectively. Sucrose gradients (100-400 mg/ml) in 0.1 M phosphate buffer, pH 7.0, were centrifuged for 150 min at 24,000 rpm in a Spinco SW 27 rotor. Arrow indicates direction of sedimentation.

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