Structural Components of Oriboca Virus

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Received for publication 15 November 1973

Analysis of purified Oriboca virions by neutral, sodium dodecyl sulfate polyacrylamide-gel electrophoresis indicated the presence of three structural polypeptides designated V-1, V-2, and V-3 on the basis of their relative electrophoretic mobilities in 8% gels. Polypeptides V-2 and V-3 are glycopeptides associated with the virion envelope as demonstrated by the preferential incorporation of labeled glucosamine into the polypeptides and by release of the polypeptides from the intact virion by the nonionic detergent NP-40. Polypeptide V-1 is the protein component of the nucleoprotein core of Oriboca virus as evidenced by the specific incorporation of uridine into the nucleoprotein, its release from the intact virion by NP-40 treatment, and its separation by both rate-zonal and isopycnic density gradient centrifugation from both the intact virion and envelope components. Molecular weights have been tentatively assigned to the polypeptides by extrapolation from the structural polypeptides of Sindbis virus when both are run in the same gel. Polypeptide V-1 has an apparent molecular weight of 20,000 to 23,000; V-2, 30,000 to 32,000; and V-3, 83,000 to 85,000.

The prototype strain BE An 17 of Oriboca virus was isolated from a sentinel monkey in the Oriboca forest, Belem, Brazil in 1954 (1, 7). This virus was soon incorporated into the new, previously undescribed arbovirus serological group C which also contained Apeu, Caraparu, Marituba, and Murutuca viruses (6, 19). Group C viruses are now classified as a separate serologically defined group within the Bunyamwera supergroup (23, 24). Electron microscopy has defined a virion 90 to 100 nm in diameter which consists of a central, electron-dense "core" 60 to 70 nm in diameter surrounded by a 15- to 20-nm electron-dense outer portion with spikes protruding 5 to 6 nm from the surface (2, 4, 10, 15, 16). Oriboca virus obtained from mouse brain, serum and liver, and cell culture consists of four physically distinct components separable by buoyant and rate-zonal density gradient centrifugation. Two of the components, of medium (MDCF) and light (LDCF) densities, exhibit complement-fixing activities, whereas the remaining two, HA-2 and VHA, have hemagglutinating characteristics, the latter being associated also with virion infectivity (11).

Oriboca virus contains three structural polypeptides with tentatively assigned molecular weights of 83,000 to 85,000; 30,000 to 33,000; and 20,000 to 23,000. However, these structural proteins have not been characterized as to

functional roles or locations within the virion. Similar structural proteins were also reported for Bunyamwera, BFS-283, Tahyna, and Murutuca virions (Rosato et al., *Acta Virol.*, in press), thus further indicating the homogeneity of the Bunyamwera supergroup viruses with respect to serological (6, 7, 19), morphological (2, 4, 10, 15), and morphogenetic (15, 16) characteristics.

Our knowledge of the molecular structure of Oriboca virus is limited, since little is known concerning the structural composition of the mature virion. An initial step in providing such information is the determination of the number, size, gross composition, and location of the structural polypeptides in the virion and in subvirion particles. The present studies were undertaken to determine some physical and chemical properties of Oriboca virions.

MATERIALS AND METHODS

Virus. Oriboca virus, strain Be An 17, was obtained from the American Type Culture Collection (ATCC), Rockville, Md., and carried the following designations: ATCC no. VR 310, SM/8 lot no. 1, Nov. 1968. It was passed once in 1- to 3-day-old suckling mice by intracerebral inoculation, the brains were harvested and a 20% suspension was prepared, lyophilized, and designated as seed stock virus. Working virus stocks were prepared in a similar manner from the seed stocks but were wet-frozen at -70°C . Working virus stocks were determined to contain 3.63×10^6 PFU/ml,

and were verified to consist of Oriboca virus by plaque reduction-neutralization tests.

Preparation and purification of radioactive virions. Oriboca virus was propagated in 64-oz. roller-bottle cell cultures of LLC-Mk₂ maintained in medium 199 containing 10% fetal calf serum (FCS), penicillin G (100 U/ml), streptomycin sulfate (100 µg/ml), and amphotericin B (fungizone, 50 µg/ml). Prior to infection, monolayers were washed twice with 0.9% NaCl, once with Hanks balanced salt solution (HBSS), and once with medium 199-deficient medium (Earle or Hanks salts) containing 2% dialyzed FCS and antibiotics. Medium 199 (Grand Island Biologicals, Grand Island, N.Y.) was deficient in either all amino acids or all amino acids and glucose. Depending on the radioactive compound to be incorporated, the concentration of the unlabeled amino acids or glucose added was adjusted to 1/10 to 1/20 the normal concentrations. Virus was diluted in deficient medium, added at a multiplicity of inoculum of 0.01 to 0.1, and allowed to adsorb for 60 to 90 min at 36 C. After the adsorption period the inocula were removed and the infected monolayers were washed as described above. A 30- or 40-ml amount of deficient medium was added and the cultures were returned to 36 C. Labeled substances were added 24 h after the adsorption period at 10 µCi of ³H(G)-L-amino acid mixture per ml containing 15 amino acids (specific activity 23.6 Ci/mmol), ³H(6)-D-glucosamine-hydrochloride (specific activity 7.3 Ci/mmol), and at ³H(4,5)-L-lysine (specific activity 20 to 40 Ci/mmol) in singly labeled experiments, and at 10 µCi/ml for tritiated compounds and 2 µCi of ¹⁴C(U)-L-amino acid mixture per ml (specific activity 3.5 Ci/mmol) in doubly labeled experiments. All labeled substances were obtained from New England Nuclear Corp., Boston, Mass. Cultures requiring pH adjustment were returned to pH 7.0 to 7.2 by the addition of 7.5% NaHCO₃ or by direct gassing with CO₂. Culture fluids were harvested 2 to 3 days after infection when cytopathic effects were distinctly visible. The infected fluid was clarified by centrifugation at 380 × g for 10 min at 4 C (International PR-2, no. 253 rotor) and the supernatant was centrifuged at 12,000 × g for 30 min at 4 C (Sorvall RC2-B, SS-34 rotor). The final supernatant was mixed with cold, saturated (NH₄)₂SO₄ solution, previously buffered to pH 7.2 to 7.4 by the addition of 1 M Tris. The buffered (NH₄)₂SO₄ was added dropwise to the virus suspensions (final concentration of 60% [vol/vol] ammonium sulfate) to precipitate the virus. The suspension was held at 4 C for 30 min, during which time it was swirled at 10-min intervals. The suspension was centrifuged at 10,400 × g for 30 min at 4 C (RC2-B, GSA rotor) and the resulting pellet was resuspended in 1.0 to 1.5 ml of TSE buffer (0.01 M Tris-hydrochloride, 0.15 M NaCl, 0.001 M EDTA), pH 7.2. Purified virus was obtained by processing this suspension by rate-zonal density gradient centrifugation in which virions were obtained as a single peak in linear sucrose gradients formed over a 70% sucrose cushion. All sedimentation patterns are presented from right to left in the Results section. The first fraction is at the bottom of the tube.

Plaque assay of Oriboca virus. Confluent mono-

layers of LLC-MK₂ cells were infected with 10-fold serial dilutions of Oriboca virus prepared in normal growth medium. After adsorption for 60 to 90 min, they were overlaid with 5 ml of medium containing final concentrations of 1.0% agarose (Sigma Chemical Co., St. Louis, Mo.), 1 × M-199 medium, 10.0% heat-inactivated fetal calf serum, 0.3% sodium bicarbonate, 0.02% DEAE, and antibiotics at previously stated concentrations. On the second or third day of incubation at 36 C, 5 ml of a second overlay of the same composition as the primary plus 4.0% final concentration of a 1:300 dilution of neutral red in water was added. After incubation for 2 to 4 h at 36 C, the cells were left at room temperature overnight; plaques were counted the next day.

Sucrose density gradient centrifugation. Linear 15 to 50% (wt/vol) sucrose gradients prepared with ribonuclease-free sucrose (Schwarz/Mann, Orangeburg, N.Y.) in TSE buffer were formed in mixing chambers of the type originally described by Britten and Roberts (3). Large gradients of 28 or 29 ml, required for initial virus purification, were centrifuged at 64,000 × g in the Spinco SW25.1 rotor for 3 h at 4 C. Small volumes were applied to 4.4- or 4.8-ml gradients of identical composition and the tubes were centrifuged at 204,000 × g in the Spinco SW50L rotor for 60 min at 4 C.

For isopycnic experiments, samples were applied to gradients of 20 to 70% sucrose (wt/vol) in deuterium oxide (Isomet Corp., Oakland, N.J.) and centrifuged at 204,000 × g in the Spinco SW50L rotor for the indicated time intervals. Density was determined by the direct weighing of ice-cold 50-µliter portions of each 0.20-ml fraction. All gradients were collected from the tube bottom in an apparatus designed to allow accurate displacement of a set volume of the gradient by the addition of an equal volume of mineral oil overlay. Usually 0.20-ml fractions were collected from small gradients and 1.0-ml volumes from large gradients.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed essentially according to Maizel (13) as modified by Shapiro et al. (17) with the following exceptions. Recrystallized, electrophoresis-grade acrylamide and *N,N'*-bis-methylene acrylamide (Bio-Rad Laboratories, Richmond, Calif.) were used without further purification. Samples, which normally contained sucrose and varied in volume from 25 to 100 µliters, were degraded by the addition of both SDS and 2-mercaptoethanol (2-ME), each to a final concentration of 1%, then heated in sealed glass tubes at 100 C for 10 min. After the addition of bromophenol blue to a final concentration of 0.05%, samples were applied to the gels, overlaid with 1:5 diluted electrophoresis buffer, and electrophoresed. Electrophoresis was performed at 20 V until the sample entered the gel, then at 40 V for 10 min, and at 60 V until the dye front migrated about 5 to 6 cm, which usually required 90 to 120 min. The gels were then removed, placed in a metal trough (Scoopula, Fisher Scientific Co., Pittsburg, Pa.) frozen on a block of dry ice, and pressed while frozen against a transverse slicer containing razor blades at 1-mm intervals. The slices were removed with a

dissecting needle, placed in scintillation vials, and counted as described in the section on the counting of radioactive samples. All the electropherograms are presented with migration from cathode (left) to anode (right).

Degradation of virions. Purified virions were degraded by the addition of the detergent Nonidet P-40 (NP-40, Shell Oil Co., Tulsa, Okla.) to a final concentration of 1%. Samples were mixed on a Vortex mixer and held at 4 C for 20 min, after which the degraded virions were separated into their component parts by rate-zonal centrifugation on small gradients.

Counting of radioactive samples. Liquid 25- to 50- μ l samples were assayed in scintillation fluid (10 ml) consisting of 150 ml of Liquifluor and 100 ml of Protosol (both from New England Nuclear Corp., Boston, Mass.) per 3 liters of spectral grade toluene. Gel slices were treated identically and then incubated overnight at 36 C to allow for swelling of the slices before counting. Radioactive samples were counted in either a Packard no. 3375 (^3H efficiency, 40%) or a Nuclear-Chicago, Mark II liquid scintillation counter (single-label ^3H efficiency 61%; double-label ^3H efficiency 52% with 11% ^{14}C to ^3H spillover). After the start of the project a computer program was developed for direct correction of the data from the tape for background, spillover from ^{14}C to ^3H channels, and conversion of counts per minute to disintegrations per minute; therefore results are given in either counts per minute or disintegrations per minute.

RESULTS

Preparation of radiolabeled Oriboca virions. When Oriboca was grown and labeled in LLC-MK₂ cell culture, concentrated by ammonium sulfate precipitation, and centrifuged on a rate-zonal, 15 to 50% (wt/vol) linear sucrose gradient, one principal area of radioactivity was observed which appeared in the center of the gradient and corresponded to the area of maximum infectivity.

Isopycnic centrifugation of purified labeled virions indicated an equilibrium density of 1.187 to 1.190 g/cm³ for Oriboca virus (Fig. 1 inset, fractions 16 and 17). Polyacrylamide gel analysis of the virions at density 1.187 to 1.190 g/cm³ (fractions 16, 17) indicated a characteristic polypeptide profile for Oriboca virus as illustrated by the ^{14}C -amino acid-labeled portion of the double-labeled preparation in Fig. 4A. On occasion, the density of Oriboca virions varied from a minimum of 1.185 g/cm³ to a maximum of 1.221 g/cm³. Electropherograms of the more dense samples indicated that the virions contained a slightly larger proportion (10%) of V-1 polypeptide than is found in virions from the middle density range. There was also a comparable decrease in the amount of V-3 polypeptide.

Polypeptides of Oriboca virus. Purified vi-

FIG. 1. Isopycnic centrifugation of rate-zonal purified Oriboca virions. Centrifugation was for 18 h at $204,000 \times g$ at 4 C in an SW50L rotor. The linear gradient was 20 to 70% (wt/vol) sucrose in deuterium oxide. Sedimentation is from right to left. Symbol: ●, ^3H -amino acids.

rons, labeled with glucosamine, lysine, uridine, or an amino acid mixture, were degraded and analyzed by electrophoresis on polyacrylamide gels. The electropherogram (Fig. 4A) depicting the ^{14}C -amino acid-labeled portion of intact virions shows that Oriboca virus contains three polypeptides designated V-1, V-2, and V-3.

On the assumption that all structural polypeptides are synthesized at the same rate during the labeling period, and that incorporation of isotope is uniform, the distribution of the radioactivity between the polypeptides might be used to estimate the mass distribution of the three polypeptides in the purified virion when labeled with a mixture containing 15 amino acids. Analysis of electropherograms of polypeptides labeled with the ^3H -amino acid mixture is given in Table 1. Comparable experiments in which ^3H -lysine and ^3H -glucosamine were also used to determine the distribution of the polypeptides are also given. The amino acid distribution provides a base line from which comparisons with the lysine and glucosamine distributions could be made. There is a slight increase in the lysine content of the V-1 polypeptide above base-line levels which, when coupled with the very low glucosamine content of the same polypeptide, indicates that V-1 may

be the polypeptide component of the nucleoprotein core.

The data for percent distribution of the amino acid mixture were also used to approximate the molar ratios of the polypeptides in which the ratios were determined by dividing the estimated molecular weight of the polypeptide into the total disintegrations per minute under the polypeptide peak as visualized by the electropherograms. The estimated molar ratios were V-3, 1.00; V-2, 1.09 ± 0.06 standard error; V-1, 2.42 ± 0.08 standard error.

In an attempt to determine the location of the polypeptides within the virion, purified labeled virions were degraded with NP-40 and the components were separated by rate-zonal cen-

trifugation. We found that dissociation of the virion envelope from the core was complete in 20 min at 4 C. Degraded virions, labeled with ^3H -lysine or ^3H -amino acids, sedimented as a clearly defined peak 5 to 6 fractions higher in the gradients than the undegraded control virions (Fig. 2A). This will be referred to as the "peak" component. A new area of radioactivity also appeared at the top of the gradient to be referred to as "top component." Degraded ^3H -glucosamine-labeled virions did not produce a peak; the bulk of the radioactivity was found at the top of the gradient (Fig. 2B). Polyacrylamide gel electrophoresis of the undegraded control virions gave the expected structural profile. Similar analysis of the peak and top component of degraded ^3H -labeled virions and the calculation of percent distribution from the electropherograms demonstrated that the peak component contain ~85% of the incorporated ^3H -amino acid label and ~75 to 100% of the ^3H -lysine label.

The top component contained V-3 plus V-2 at base-line or slightly increased levels, and a decreased amount of V-1 polypeptide.

A 31-h isopycnic centrifugation of pooled ^3H -lysine-labeled peak component showed an equilibrium density of $\geq 1.310 \text{ g/cm}^3$, i.e., the material passed through the 20 to 70% deuterium oxide gradient. Polyacrylamide gel analysis

TABLE 1. Distribution of the structural polypeptides in Oriboca virus^a

Polypeptide	Distribution (%)		
	V-3	V-2	V-1
^3H -amino acid mixture	45-50	18-25	22-32
^3H -lysine	42-48	20-22	32-36
^3H -glucosamine	58-65	35-36	<5

^a Determined as the total counts under a specific polypeptide peak divided by the total counts under the three polypeptide peaks as observed in electropherograms.

FIG. 2. Rate-zonal centrifugation of NP-40-degraded purified Oriboca virions. Centrifugation was at $204,000 \times g$ for 60 min in an SW50L rotor on 15 to 50% (wt/vol) linear gradients. Symbols: ●, Undegraded virions; ○, NP-40-degraded virions. A, Sedimentation pattern is characteristic for ^3H -lysine- and ^3H -amino acid-labeled virions. B, ^3H -glucosamine-labeled virions. Sedimentation is from right to left.

confirmed that this material was V-1 polypeptide. Pooled, NP-40-released, ^3H -lysine-labeled top component did not band but was found throughout the top two-thirds of the gradient, emphasizing the heterogeneity of NP-40-released top components.

Although the data derived from the preceding experiments are indicative of the possible location of the polypeptides in the virion, they cannot be considered conclusive since the nucleoprotein core material has not been specifically labeled.

To specifically label both the RNA and protein portions of the nucleoprotein component, virions were prepared, doubly labeled with ^3H -uridine and a ^{14}C -L-amino acid mixture, concentrated, and purified as described in Materials and Methods. The relative amounts of

label incorporated into the virion are shown in Fig. 3A. Infectivity as PFU was determined for odd-numbered fractions and corresponded to the area of radioactivity. An electropherogram of the ^{14}C -amino acid-labeled polypeptides of virions from the major area (Fig. 3A, fraction 13) showed a characteristic profile. The ^3H -uridine-labeled RNA did not significantly enter the gel, and more than 80% was found in the first two slices.

The purified virions from fraction 13 (Fig. 3A) when degraded by NP-40 gave characteristic sedimentation patterns on sucrose gradients (Fig. 3B). The percentage of ^3H -uridine in the peak compared to the percentage in the control in which both were approximately 80 to 85%, and the low value in the region of top component supported the assumption that peak mate-

TABLE 2. Distribution of the doubly labeled Oriboca polypeptides released by NP-40 degradation^a

Labeled material	Distribution (%)							
	^3H -uridine				^{14}C -amino acids			
Polypeptide	Top ^b	V-3	V-2	V-1	Top	V-3	V-2	V-1
Control (undegraded)	77-90	6-21 ^c	0	0	0	50-51	17-18	22-28
Peak component	90-98	0-8 ^c	0	0	0	0-4 ^c	0	83-92
Top component	4	0-10	0-7	0	0	64-70	19-24	0

^a Determined as percentage of the total disintegrations per minute recovered in the polyacrylamide gel.

^b Activity in the first 1 to 2 slices of the gel.

^c Activity was 1 to 2 slices to the left of the V-3 peak and are not considered to be part of V-3 but a separate entity.

FIG. 3. Rate-zonal centrifugation of doubly labeled Oriboca virions. A, Primary rate-zonal purification; centrifugation was at $64,000 \times g$ for 180 min in an SW25.1 rotor at 4 C. Symbols: $\circ$, ^{14}C -amino acids; $\bullet$, ^3H -uridine; $\square$, PFU. B, NP-40-degraded purified virions, centrifugation as in Fig. 2: ($\blacktriangle$) ^{14}C -amino acid- and ($\square$) ^3H -uridine-labeled degraded virions; ($\bullet$) ^{14}C -amino acid- and ($\circ$) ^3H -uridine-labeled undegraded control virions.

rial was in fact nucleoprotein and therefore contained the V-1 polypeptide.

Electropherograms of the polypeptides released by NP-40 treatment and separated by rate-zonal density gradient centrifugation are presented in Fig. 4A-C. The percent distributions calculated from the electropherograms are summarized in Table 2. In the untreated control (Fig. 4A) more than 85% of the ^3H -uridine remained on top of the gel and a minor peak representing about 10% of the total activity appeared in slices 6 to 8. The ^{14}C -amino acid polypeptide profile of the control was normal (Fig. 4A). Analysis of the peak component showed more than 95% of the ^3H -uridine-labeled portion on top of the gel and more than 85% of the total activity of the ^{14}C -amino acid-labeled protein portion in the V-1 polypeptide (Table 2). The calculations of the percent distribution of the peak component clearly show its purity. Essentially all of the ^3H -uridine remains on top of the gel, and the protein moiety is not contaminated with V-3 and V-2 components (Fig. 4B). The top component (Fig. 4C) contained relatively little ^3H -uridine, and the bulk of the ^{14}C -amino acid label was in V-3 and V-2; V-1 contained very little of the total ^{14}C -amino acid activity.

DISCUSSION

Our data indicate that Oriboca virions obtained from LLC-MK₂ cell cultures have an equilibrium density in sucrose-D₂O of 1.187 to 1.190 g/cm³, a value which differs slightly from that obtained with virions from mouse brain or liver (intracellular virus) and virions derived

from BHK-21 cell culture or mouse serum (extracellular virus) (11). As we have indicated, virions from the more dense samples (1.221 g/cm³) contained a slightly larger proportion of the V-1 polypeptide than is found in virions from the middle density range. There is also a comparable decrease in the amount of V-3 polypeptide. This might be expected if, in fact, the virion had lost a portion of the outer membrane or possibly the spike-like projections reported to be present on Bunyamwera supergroup viruses (10, 15, 16). V-1 is the polypeptide portion of the nucleoprotein core of Oriboca virus; therefore, an increase in the ratio of V-1 to the other polypeptides in a partially degraded virion could lead to an apparent increase in virion density. The data on percent distribution of radioactive lysine and glucosamine incorporation indicate that the V-1 polypeptide is associated with the nucleoprotein portion and V-2 and V-3 with the envelope components of the virion. Normally, a high lysine content is indicative of the presence of the protein moiety of the nucleoprotein core (12, 20) and glucosamine of envelope components (5, 21). The same distribution data when used to calculate the molar ratios of the polypeptides correspond reasonably with those reported (1:1:2) for La Crosse virus, a member of the California group of the Bunyamwera supergroup (14).

The previously reported (R. Rosato et al., in press) molecular weight estimates of the structural polypeptides of Oriboca virions of V-3, 83,000 to 85,000; V-2, 30,000 to 33,000; and V-1, 20,000 to 23,000, correspond to those reported by McLarran et al. (14) for La Crosse virus of V-3,

FIG. 4. Polyacrylamide gel electropherograms of NP-40-degraded purified Oriboca virions. Samples were obtained from the rate-zonal centrifugation run of Fig. 3. A, Undegraded control; B, peak component; C, top component; ●, ^{14}C -amino acids; ○, ^3H -uridine.

85,000; V-2, 45,000; and V-1, 25,000. We have on occasion assigned a molecular weight of 34,000 to 35,000 to V-2 when extrapolated from the standard curve based on the molecular weights of the polypeptides of Sindbis virus (21) but have not observed a molecular weight greater than 35,000. If, in fact, the inability to detect the 45,000-dalton polypeptide is due to propagation of virions in amino acid-deficient medium as suggested by McLarren et al. (14) for Uukuniemi virus, it does not explain the presence of the 30,000- to 32,000-dalton glycosylated V-2 polypeptide reported here.

Polypeptides V-3 and V-2 are associated with the envelope structure of the virion as demonstrated by the preferential incorporation of labeled glucosamine into the polypeptides and by the release of envelope components from the virion by NP-40. Polypeptide V-1 is the protein moiety of the nucleoprotein core of Oriboca virus as evidenced by the incorporation of uridine, its release from the intact virion by NP-40 in a relatively pure state, and its separation by both rate-zonal and isopycnic density gradient centrifugation from both intact virions and envelope constituents.

Similar results as to the number and relative sizes of the structural polypeptides have been obtained for Kaeng Khoi virus (W. A. Neill, personal communication). Kaeng Khoi virus is an ungrouped bat virus, morphologically similar to Bunyamwera supergroup viruses but as yet not shown to be related serologically to the supergroup. Preliminary polyacrylamide gel analysis of Pacui virus, an ungrouped arbovirus morphologically indistinguishable from Bunyamwera supergroup viruses, indicated a similar polypeptide structural profile as to the number and relative sizes of its structural polypeptides (unpublished data).

The data concerning La Crosse virus polypeptides when added to those previously reported for Bunyamwera, BFS-283, Tahyna, and Murutucu virions (Rosato et al., in press) and Oriboca virus reported here support the contention that arboviruses classed together on the basis of serological relationships (1, 6, 7, 9, 23, 24) also tend to be alike in general morphology (2, 4, 10, 15, 16), mode of morphogenesis (15, 16) and the number, site of virion origin, and apparent molecular weights of their structural polypeptides (8, 12, 18, 22).

Further study of many viruses of the various serogroups of the current arbovirus classification will be necessary to determine in a finite way if, in fact, the antigenic configuration, which is a biologic expression of chemical structure, correlates also with the similarities in

structural polypeptides, morphology, and mode of morphogenesis.

ACKNOWLEDGMENTS

We wish to acknowledge the technical assistance of Michael D. Douglas and Keith M. Hendrix. R. R. submitted portions of this work in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The George Washington University.

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