Characterization of the Genome of Cytoplasmic Polyhedrosis Virus

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It is possible to oxidize *in situ* the 3'-terminals of the double-stranded ribonucleic acid (RNA) contained in structurally intact cytoplasmic polyhedrosis virus. The number of 3'-terminals subsequently found by reduction with tritiated sodium borohydride was equivalent to the number observed in RNA first isolated from the virus and then oxidized and reduced. The viral RNA appears to occur in 10 distinct polynucleotide segments present in equimolar amounts.

The genome of cytoplasmic polyhedrosis virus (CPV) is similar to that of reovirus in that it consists of double-stranded ribonucleic acid (dsRNA) (6) and cannot be isolated as one piece from purified virus preparations (1, 2). The observation that the dsRNA of reovirus appears as 10 pieces when analyzed by polyacrylamide gel electrophoresis is believed to be a result of discontinuities that exist in the dsRNA genome of the intact virion (4, 5). Because of the similarities between the genomes of reovirus and CPV, it was of interest to determine if the distinct dsRNA which can be isolated from CPV (2) may also reflect the state of the viral genome as it exists in the intact virion of CPV.

The technique described by Millward and Graham (4) was used to examine the structure of the viral RNA. The method involves modifying the 3'-terminal nucleosides of the RNA by oxidation with sodium metaperiodate followed by reduction with ³H-sodium borohydride. This procedure permits one to estimate the number of 3'-terminal nucleosides available both in free RNA and in RNA *in situ* in the virion.

MATERIALS AND METHODS

CPV was grown in *Bombyx mori* and purified as described in an earlier report (3). The oxidation and reduction procedures were those of Millward and Graham (4) with the modifications noted in the legend to Fig. 1. The polyacrylamide gel analysis followed the method of Watanabe et al. (7). All other experimental details can be found in the figure legends.

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RESULTS

RNA was extracted from purified CPV with sodium dodecyl sulfate-phenol (2). This RNA was oxidized with periodate, reduced with ³H-sodium borohydride, and analyzed by polyacrylamide-agarose gel electrophoresis. A representative electropherogram is shown in Fig. 1. While nine distinct peaks of radioactivity were resolved, peak number 2 contained approximately twice as much ³H as each of the other eight. This suggested that the second electrophoretic fraction contained either two RNA components or a single component with twice the number of oxidizable 3'-terminals as was present in each of the other eight fractions. Staining a parallel gel with methylene blue, however, allowed the visual resolution of this second peak into two distinct components migrating at approximately the same rate.

When the reduction with ³H-borohydride was performed by using unoxidized CPV RNA, no incorporation of label into the RNA could be observed. This result demonstrated the absence of any reducible component in the RNA preparation as well as the specificity of the label for oxidized 3'-terminals only. Furthermore, pretreatment of the dsRNA with alkaline phosphomonoesterase, which cleaves terminal phosphates, did not appreciably alter either the total level of incorporation per given amount of RNA or the relative amounts of ³H incorporated into the individual fractions, suggesting that none of the 3'-terminals was phosphorylated.

To examine the structure of the RNA inside the virion, a portion of purified virus was oxidized

FIG. 1. Polyacrylamide gel electrophoresis of dsRNA terminally labeled with ^3H . Purified cytoplasmic polyhedrosis virus (equivalent to approximately 100 μg of dsRNA) in 0.05 M sodium cacodylate, 0.001 M ethylenediamine tetraacetic acid (EDTA), pH 7.0, was adjusted to pH 5.5 with 1 M acetic acid. An equivalent amount of dsRNA was obtained by extraction of purified virus and treated similarly. Both mixtures were oxidized with excess sodium metaperiodate for 60 min at 0 C in the dark. Excess periodate in both samples was destroyed by the addition of propylene glycol. The oxidized dsRNA sample was precipitated by ethanol, whereas the oxidized virus sample was layered on a preformed CsCl gradient and centrifuged for 3 hr at 44,000 rev/min and at 10 C in the Spinco SW50.1 rotor. The virus was collected, and, after passage through Biogel P-10 to remove CsCl, dsRNA was extracted from the oxidized virus and recovered by ethanol precipitation. The two dsRNA samples were then dissolved in 0.5 M potassium phosphate buffer (pH 7.0), cooled to 0 C, and reduced by the addition of ^3H -sodium borohydride in small quantities during 4 hr. Excess borohydride was decomposed by the addition of glacial acetic acid, and then the RNA samples were passed through Sephadex G-25 equilibrated in 0.3 M STE (0.3 M sodium chloride, 0.01 M tris(hydroxymethyl)aminomethane, 0.001 M EDTA, pH 7.4) containing 7 M urea. The urea was removed by dialysis, and the ^3H -dsRNA was recovered by precipitation with ethanol. Figure 1 represents the electropherogram of dsRNA freed from the virus and then oxidized and reduced as noted above. Migration was for 26 hr at 8 ma/tube in 2.5% polyacrylamide-1% agarose gel (7) with 1-mm slices analyzed for their radioactive content. Direction of migration is from left to right.

with periodate and then sedimented on a preformed CsCl gradient as noted in the legend to Fig. 1. Such differential sedimentation removed any RNA which was no longer associated with virus particles. The portion of the gradient containing the virus was collected and then desalted by passage through Biogel P-10. The dsRNA was subsequently extracted and recovered by ethanol precipitation. This RNA was then

TABLE 1. Relative amount of ^3H radioactivity incorporated in double-stranded cytoplasmic polyhedrosis virus RNA oxidized before and after extraction from the virion^a

Component ^b	Double-stranded RNA oxidized after extraction ^c	Double-stranded RNA oxidized in virion ^c
1	0.96	0.98
2-3	1.89	2.15
4	0.96	0.92
5	1.00	0.87
6	1.00	0.81
7	1.05	0.96
8	0.92	1.09
9	1.00	1.00
10	0.66	0.72

^a Design of this experiment is detailed in the legend to Fig. 1.

^b Numerical classification of the components refers to Fig. 1.

^c ^3H counts per minute in component 9 (Fig. 1) were arbitrarily set equal to 1.00. The ^3H counts per minute in the remaining eight peaks were then compared to component 9.

reduced with ^3H -borohydride, and it too was analyzed by electrophoresis as described for Fig. 1. Table 1 shows that the relative amounts of tritium in each electrophoretic RNA fraction was the same whether the oxidation was performed on free dsRNA or on dsRNA inside the virus. This suggests that, inside the virion, the double-stranded RNA has approximately as many oxidizable 3'-terminals as does the RNA after extraction.

It should be noted that under the current oxidation and reduction conditions approximately 50% of the theoretical number of 3'-terminals of CPV were labeled during the reduction step, whether the RNA was oxidized before or after extraction from the virion. Although not yet thoroughly understood, this inability to effect 100% theoretical labeling is in agreement with results previously obtained with reovirus (4, 5) and with unpublished control values obtained in our laboratory for single-stranded R-17 RNA.

Examination of the data in both Fig. 1 and Table 1 shows that the relative amount of radioactivity in the fastest migrating component is appreciably below that observed in the other dsRNA segments. To determine if this discrepancy in molar ratios was due to selective loss of the smallest subunit during handling procedures, the following control was performed. Sucrose gradient-purified virus was oxidized as previously noted and sedimented to equilibrium in a CsCl gradient preformed over the narrow

density range of 1.36 to 1.45 g/cm³. In addition to the major viral component which equilibrates at 1.435 ± 0.005 g/cm³, satellite bands of lighter density (Fig. 2, dashed line) were observed by light scattering. Although such satellite bands were usually present after CsCl sedimentation of untreated virus (Fig. 2, solid line and absorbancy at 260 nm), the oxidation procedure enhanced the total amount of these lighter components. The virus band and the satellite shoulder were

FIG. 2. Sedimentation patterns of unoxidized and oxidized virions of cytoplasmic polyhedrosis virus. Sedimentation was on a preformed gradient of CsCl prepared in cacodylate buffer (4), pH 7.4, 0.001 M EDTA for 10 hr in the SW50 rotor at 36,000 rev/min and 6°C. Before collection, the gradients were photographed for their light-scattering patterns. The gradient containing untreated virions was further analyzed for absorbancy at 260 nm (Δ) and buoyant density. The density positions of the intact virus band and the shoulder of lighter satellite components are marked by arrows. This figure includes the superimposed Joyce-Loebl microdensitometer tracings from the negatives of the gradients containing unoxidized (solid line) and oxidized (dashed line) virus.

collected separately and desalted, and the RNA was extracted from both. These RNA species were reduced with ³H-borohydride and analyzed on parallel acrylamide gels. The electropherograms of RNA from intact virus and from the satellite pool are shown in Fig. 3a and b, respectively. Clearly, the smaller components in the RNA preparation from the less dense satellite bands were not present in the same equimolar ratios as in RNA extracted from the more dense viral band. Thus, after oxidation and sedimentation through CsCl, a population of virions of CPV could be isolated missing some of the smaller RNA constituents, missing them in such a fashion (Fig. 3b) as to suggest a possible gradient of loss, with the smallest subunit present in least amount, the second smallest subunit in the second least amount, and so on for the five smaller components of the genome.

DISCUSSION

The above experiments demonstrate that it is possible to oxidize in situ the same number of 3'-terminals present in double-stranded RNA

FIG. 3. Electropherogram of dsRNA extracted from the oxidized virus and satellite pools separated on CsCl followed by reduction with ³H-sodium borohydride. The oxidation and reduction conditions are listed in the legend to Fig. 1, as are the conditions for electrophoresis. (A) RNA from intact virus; (b) RNA from pooled satellite components.

contained in whole CPV as present in free viral RNA. The genome of CPV appears, therefore, to exist in the structurally intact virion in 10 distinct polynucleotide segments present in equimolar amounts.

This conclusion raises the question of whether and how the RNA segments might be physically linked inside the virion. It may be, for example, that the RNA genome of the CPV population examined in this study may have already existed in pieces before the oxidation step, perhaps as a result of the incubation at pH 10.6 required to release the virus from its protective polyhedral protein matrix. Virus released in this fashion, however, retains infectivity for silkworm larvae (3). Due mainly to the lack of any tissue culture plaque assay, there are, unfortunately, no reliable data on the number of physical particles per infectious unit of CPV prepared in this fashion. The key question becomes, therefore, whether biologically active viral RNA, that is, infectious RNA, exists in the structurally intact virus in the physically distinct segments observed in the present study. In the earlier report showing that virus remains infectious after purification, it was noted that RNA extracted from purified virus no longer retains this property. This lack of infectivity of extracted RNA could be due to physical separation of the RNA subunits brought on by phenol extraction or to the loss of some protein required for infectivity, for example, the transcriptase known to be a part of the virion (3).

The problems inherent in replicating and packaging a viral genome composed of 10 distinct subunits in the observed equimolar amount would, however, seem to imply the presence of some type of linkage relationship between the individual RNA components—at least at some stage of viral morphogenesis. Perhaps the RNA subunits exist in the virus joined together by a noncovalent linkage of RNA to RNA or RNA to protein which is particularly susceptible to breakage as a result of any of the laboratory manipulations noted above. If this were true, the small size of newly unlinked subunits might allow them to diffuse out of the virus superstructure with the smallest diffusing most easily

and so on, giving the pattern of subunit loss observed in Fig. 3b. On the other hand, the release of what appear to be strands of viral nucleic acid is frequently observed in electron micrographs of CPV (Lewandowski, *unpublished data*), and it may be that the high salt conditions of the CsCl gradients act to dissociate the subunit junctions of any such extruded portions of the genome. The pattern of label seen in the electropherogram of Fig. 3b would suggest, however, that if a linkage relationship of the subunits does exist, it would consist of the smallest subunit at the extruding end followed by the second smallest and so on for at least the five smaller components.

Studies designed to evaluate the existence of a physical relationship between the RNA subunits inside the structurally intact virus are currently in progress.

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