CIRCULAR STRUCTURES IN PREPARATIONS OF THE REPLICATIVE FORM OF ENCEPHALOMYOCARDITIS VIRUS RNA

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1. Introduction

Double-stranded virus-specific, replicative form (RF), RNA can be isolated from cells infected with viruses containing single-stranded RNA. In our previous studies [1, 2] it was shown that RF RNA of encephalomyocarditis (EMC) virus contained a readily renaturable fraction which could be revealed either by its resistance to the action of RNAase after treatment with different denaturing agents (e.g. after heating or treatment with dimethyl sulfoxide) or by the changes of absorbance after heating and rapid cooling. The data were interpreted as indicating that there was no complete separation of complementary strands (despite destruction of the secondary structure) under denaturing conditions, at least in a portion of RF RNA molecules. It was suggested that a fraction of EMC virus RF RNA molecules exists which has either a double-stranded circular structure with both strands closed or denaturation-resistant cross-links between the complementary strands.

The present note reports evidence showing the existence of circular structures in the preparations of EMC virus RF RNA.

2. Isolation of RF RNA

The original samples of EMC virus and Krebs-II ascites carcinoma cells were obtained from Dr. N.V. Kaverin (D.I.Ivanovski Institute of Virology, Moscow).

A suspension of cells in a balanced salt solution [3] (10^8 cell/ml) was infected with about 10 plaque-forming units of virus per cell. After 30 min incubation at room temperature, the suspension was diluted 5-fold with the salt solution and incubation was continued at 37° with constant shaking. Two hours later ^{3}H-uridine (5 Ci/mmole, 2.5 μCi/ml) was added to a portion of the cell suspension. The total duration of incubation at 37° was 5 hr. Cells were harvested by low-speed centrifugation and were stored at −20°.

RNA was extracted from the cells with phenol in the presence of sodium dodecyl sulfate at 60° [4]. The total RNA was precipitated by the addition of two volumes of ethanol, the precipitate was dissolved in a buffer solution (STED: 0.1 M NaCl, 0.01 M tris, 0.001 M EDTA, 0.01% sodium dodecyl sulfate, pH 7.4) and high-molecular-weight single-stranded RNA was precipitated by 2 M LiCl. Double-stranded RNA was precipitated from the supernatant by addition of two volumes of ethanol; the precipitate was dissolved in 0.1 M NaCl, 0.01 M $MgCl_2$, 0.01 M tris, pH 8.0, and treated with DNAase (Worthington Biochemical Co; 10 μg/ml, 5 min, 25°). Then EDTA was added to a final concentration of 0.02 M, and the solution was layered onto a 15–30% sucrose gradient in STED. Centrifugation was performed in a Spinco SW 25 rotor at 24,000 rpm and about 20° for 20 hr. Fractions were collected by puncturing the bottom of the tubes; acid-insoluble radioactivity was determined in small aliquots of the fractions using a Decem NTL^{314} liquid scintillation counter (Turku, Finland). Fractions containing RF RNA were pooled and dialysed against an appropriate buffer solution or RNA was precipitated by addition of two volumes

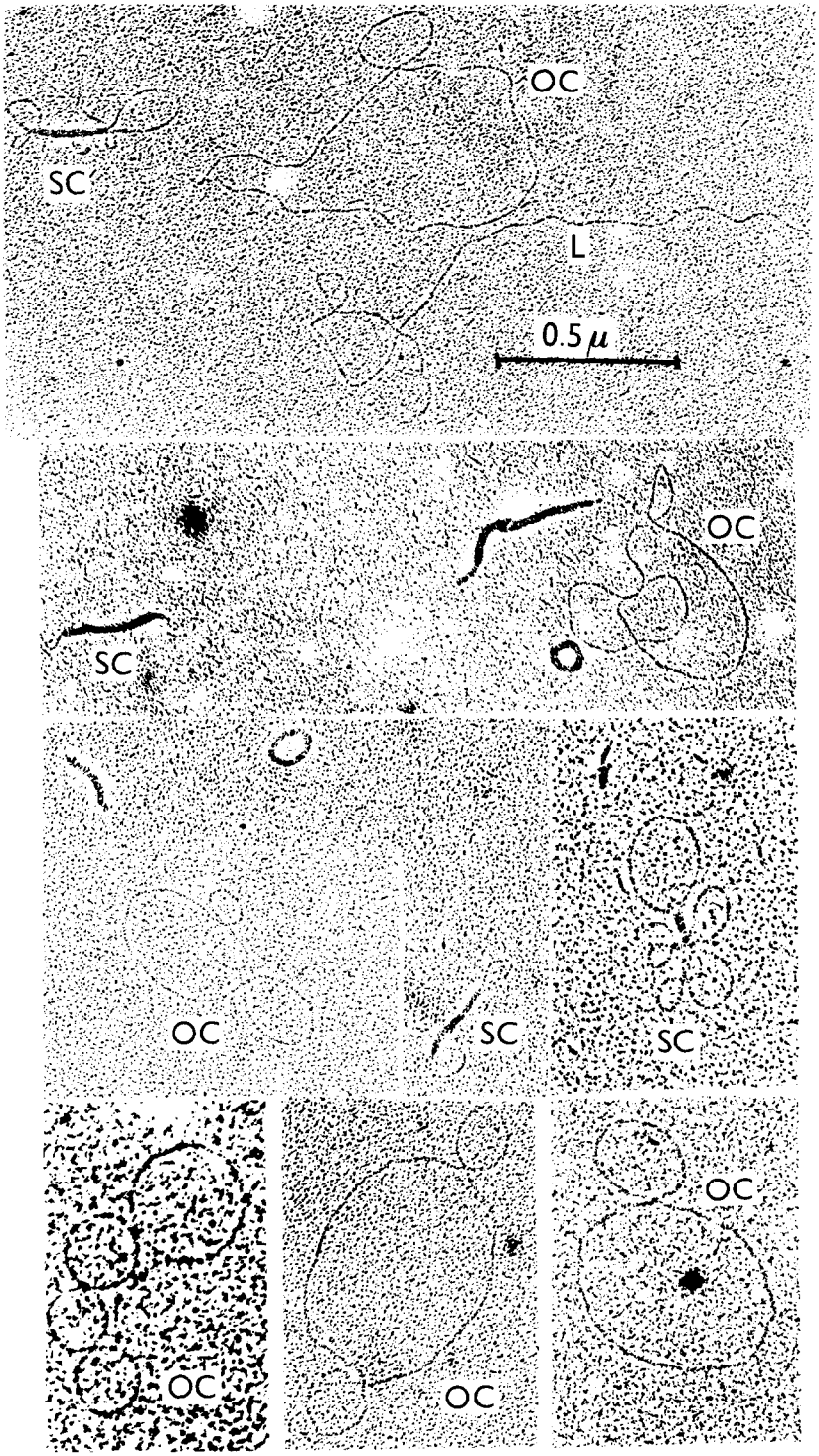

Fig. 2. Electron micrographs of different types of molecules of EMC virus RF RNA treated with ethidium bromide. Linear (L), open circular (OC), and presumably closed circular, supercoiled (SC) forms are visible.

of ethanol. It may be noted that the latter procedure sometimes results in the formation of RNA aggregates after dissolution of the precipitate.

3. Results and discussion

Some properties of EMC virus RF RNA prepared by similar methods, were described earlier [1, 2]. In particular, a certain heterogeneity with respect to denaturation-renaturation characteristics was noted. Nevertheless, in equilibrium sedimentation experiments, labeled preparations of RF RNA band in a rather homogeneous peak with a mean buoyant density of 1.64 g/cm^3 (fig. 1); the buoyant density of single-stranded EMC virus RNA, according to our unpublished experiments, is 1.68 g/cm^3. Thus the possible content of single-stranded polynucleotides in the preparations of RF RNA is low, if any. Other data [1, 2] led to the same conclusion.

Since one of the assumptions made from our previous studies was that of the existence of double-stranded closed circular RF RNA, we attempted to isolate such structures by means of the equilibrium

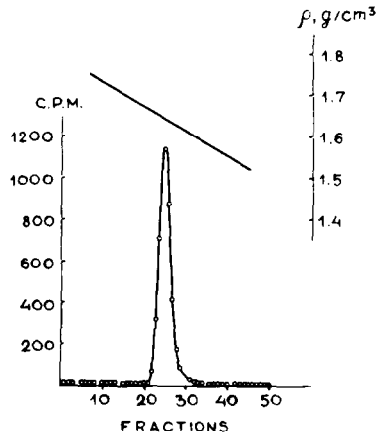

Fig. 1. Equilibrium density centrifugation of a preparation of RF RNA. Onto 1.5 ml of a solution of Cs_2SO_4 in 0.1 M NaCl, 0.01 M tris, pH 8 (ρ = 1.69 g/cm^3) was layered an equal volume of Cs_2SO_4 solution (ρ = 1.505 g/cm^3) containing labeled RF RNA; tubes were filled up with a mineral oil. Centrifugation was performed in a Spinco SW 39 rotor for 10 hr at 38,000 rpm and then for 48 hr at 31,000 rpm.

sedimentation in Cs_2SO_4 in the presence of ethidium bromide, a procedure similar to that employed for fractionation of circular forms of DNA [5]. So far these attempts have failed because of the strong adsorption of the RF RNA-dye complex on the tube walls. Electron microscopy of these complexes, however, yielded some significant results.

Electron microscopy of RNA was performed by the protein monolayer technique [6]. 0.06 ml of RNA solution containing 100 μg/ml of ethidium bromide were mixed with an equal volume of a solution of diisopropylphosphoryl trypsin (1 mg/ml) in 1 M ammonium acetate. Approximately 0.05 ml of this mixture were spread onto the water surface. Areas of the resulting film were picked up on copper grids covered by a carbon-coated collodion film, and dried in methanol or blotted with filter paper and dried in air. Then the rotating preparations were shadowed with palladium at an angle of 7°. All specimens were examined at a nominal magnification between 12,000 and 17,000 in a Hitachi HS-8 microscope.

Three main types of RF RNA molecules can be distinguished (fig. 2). First, there are linear molecules. Second, molecules exist that look like open circles, often having several twists. We are inclined to believe that these molecules are circular double-stranded polynucleotides with at least one "nick" in a strand. Third, there are closely packed entities that look like short thick rods or rings. Such molecules sometimes possess one or several thin loops. These entities may be considered tentatively as formed by supercoiled circular RNA molecules having both strands closed.

Molecules of all the three types are completely absent from preparations treated with RNAase (Nutritional Biochemicals Corporations; 10 μg/ml, 30 min, 30°) at a low ionic strength (about 0.02).

Molecules which might be described as linear and circular appeared to be present also in preparations of EMC virus RF RNA in the absence of ethidium bromide [2]. It may be mentioned that several years ago it was noted in a review [7] that N.Granbouland and L.Montagnier had observed EMC virus RF RNA molecules with "ring-like aspect".

The closed circular RF RNA may account for the existence of readily renaturable fraction of RF RNA described earlier [1, 2]. However, definite conclusions about the structure and physico-chemical properties of the circular RF RNA molecules can be made only after further studies.

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