VESICULAR STOMATITIS VIRUS RNA: COMPLEMENTARITY BETWEEN INFECTED CELL RNA AND RNA'S FROM INFECTIOUS AND AUTOINTERFERING VIRAL FRACTIONS*

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Preparations of vesicular stomatitis virus (VSV) are separable into two populations of particles by sucrose density gradient sedimentation. Infectivity is associated with long rods, and the property of autointerference is associated with short particles (Huang et al., 1966; Hackett et al., 1967). In our laboratory these particles are designated L and S, respectively. Huang and Wagner (1966) reported sedimentation coefficients of 43S and 23S for single-stranded RNA's from L and S particles, respectively. We (unpublished experiments) confirm these observations of Huang and Wagner; and Brown et al. (1967) recently reported similar sedimentation coefficients. This paper is part of a study directed toward elucidation of the mechanism of autointerference. The sedimentation patterns of labeled RNA from VSV-infected cells showed a major peak at about 15S, another peak corresponding to viral RNA (plus strand) at about 43S, and usually minor peaks at about 6S and 31S. Most of the viral-specific RNA from cells was single-stranded, and annealing experiments indicated a large portion was complementary (minus strand) to viral RNA. RNA from S particles did not anneal with L particle RNA, but did anneal with viral-specific cellular RNA.

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MATERIALS AND METHODS

The Indiana serotype of VSV was employed, except as noted in Table 1. L and S fractions were prepared essentially as described by Hackett et al., (1967). Briefly, this included propagation from undiluted passage inocula (L-S stock) in cultured chick embryo cells, clarification by low speed and concentration by high speed centrifugation, separation of L and S bands by sucrose density gradient centrifugation (15-35%, 2 hr at 25,000 rpm in Spinco SW-25.1 rotor), and dilution and recycling of each separated band in a second sucrose gradient. Enzyme and fluorocarbon treatments were omitted from the procedure. Prior to RNA extraction, the L and S fractions were diluted and pelleted (2 hr, 30,000 x g). Labeled L and S bands were obtained by the inclusion of 2 μc/ml H^3 (uridine, 16 c/millimole; or adenosine, 4.2 c/millimole) or P^{32} (carrier-free $H_3P^{32}O_4$) in the medium during virus propagation. Monolayers of VERO cells (Earley et al., 1967) propagated in Eagle's medium with 5% fetal calf serum were employed as the source of infected and uninfected cell RNA. The VERO cells were infected at an input multiplicity of about 0.1 plaque-forming units of L-S stock VSV per cell. After adsorption for 1.5 hr at 37°, Eagle's medium, containing 2 μg/ml actinomycin D (a gift of Merck Institute for Therapeutic Research, West Point, Pa.) was added. Incubation was continued for an additional 17 hr, by which time the cells were degenerating. Those cells remaining on the glass were scraped into the medium with a rubber policeman. Free virus, along with intact cells and degenerated cell particulates, were pelleted by centrifuging 1.5 hr at 30,000 x g. For labeled RNA, 2 μc/ml H^3 or 20 μc/ml P^{32} were added 20 min after the actinomycin. (Actinomycin at 2 μg/ml added at the time of VSV inoculation appreciably reduced virus yield in VERO cells, whereas normal yields were obtained when the drug was added after one hour. Incorporation of uridine-H^3 into cellular RNA was appreciable at low levels, ca. 0.5 μg/ml, of actinomycin. The chick cell-VSV system gave erratic results in the presence of actinomycin D.) RNA was extracted from cell pellets and from L and S pellets by minor modifications

(dextran sulfate in place of polyvinyl sulfate, and only 2 phenol extractions) of the procedure of Scherrer and Darnell (1962). RNA-RNA annealing was accomplished by suspending alcohol-precipitated RNA in 2X SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.7), heating in a boiling water bath for 5 min, followed by slow cooling to room temperature in approximately 5 hours. Each sample was diluted to 1X SSC, then assayed for acid-insoluble and ribonuclease-resistant radioactivity. For ribonuclease resistance, samples were treated with 10 μg/ml pancreatic ribonuclease for 30 min at 37° or room temperature. The ribonuclease-treated and untreated control portions were assayed for acid-insoluble radioactivity on 12 x 75 mm filter paper strips which were immersed in 0.5 M $HClO_4$ for 40 min at 4°, washed twice with 0.5 M $HClO_4$, and twice with 95% ethanol. The strips were dried and placed along the inside circumference of counting vials, to which scintillation fluid was added, for counting in a Packard Tri-Carb scintillation counter.

RESULTS AND DISCUSSION

VERO cells were infected under conditions of autointerference, and RNA was extracted from pellets of cells plus particles. Figure 1 shows the sucrose density gradient sedimentation pattern of labeled RNA from one of these preparations. Numerals I through V indicate fractions removed from similar gradients for annealing studies. Fractions I and III correspond to the RNA's of L and S particles, respectively. Whereas Fraction I showed a well-defined peak, a possible peak of S particle RNA in Fraction III was obscured by the adjacent major peak, Fraction IV. Fractions II and IV no doubt represent viral-induced RNA, since similar peaks have been observed in preliminary pulse-labeling experiments as early as 3 hr postinfection, whereas actinomycin-treated uninfected cells show only low levels of acid-insoluble radioactivity as a smear in the middle and upper portions of the gradient. The sedimentation

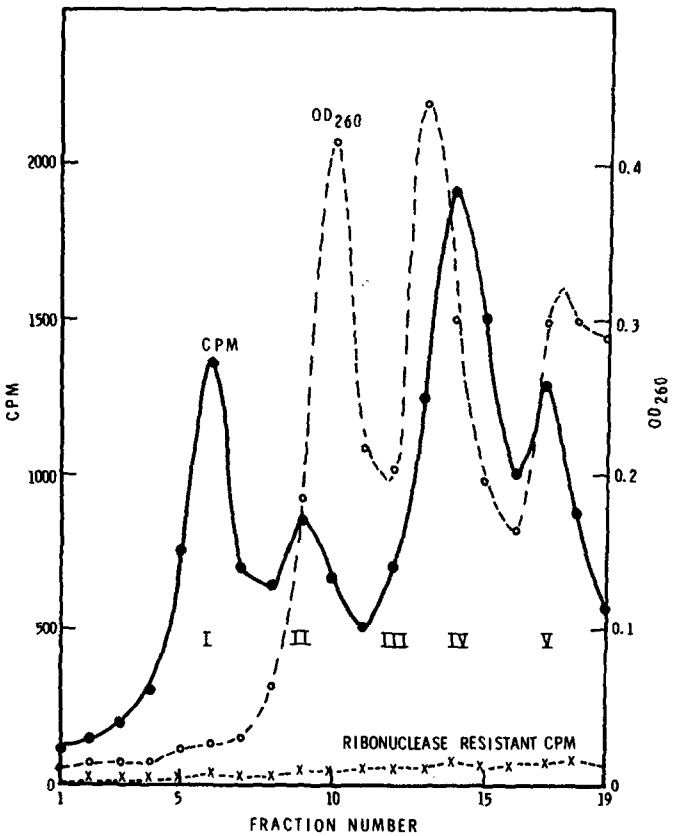

Fig. 1. Sucrose density gradient sedimentation pattern of uridine-H^3-labeled RNA from VSV-infected VERO cells. RNA from monolayers in four 3-oz prescription bottles was layered on a 4.6 ml gradient of 5-20% sucrose (w/w) in pH 5.1 acetate (Scherrer and Darnell, 1962) and centrifuged 96 min in a Spinco SW-50 rotor at 50,000 rpm. Each fraction of 4 drops collected from the bottom of the tube was diluted with 0.4 ml of 1.3X SSC. Optical densities at 260 mμ were measured, and 0.1 ml portions were assayed for acid-insoluble and ribonuclease-resistant radioactivity (CPM).

rates of peaks II, IV, and V, as estimated relative to the ribosomal RNA positions (OD_{260} peaks) were approximately 31, 15, and 6S. Upon sedimentation analysis of VSV-RNA, Huppert and coworkers (1967) found three peaks which correspond to our peaks, I, IV, and V. These authors suggested that the slower sedimenting components resulted from breakage of VSV virion RNA, but they did not present experimental details showing that the RNA was from the mature virion and not from infected cells. As seen in Fig. 1, most of the RNA was sensitive to ribonuclease, indicating absence of appreciable quantities of

double-stranded RNA. Nevertheless, preliminary experiments employing Cs_2SO_4 gradients after sucrose gradient fractionation indicate some double-stranded RNA is present.

Complementary strands of RNA form ribonuclease-resistant, base-paired duplexes upon heating and slow cooling (annealing). Various combinations of VSV- and cell-RNA's were tested by this technique. Tables 1 and 2 present the results of experiments with labeled RNA from L and S particles, and infected cell-RNA fractions, respectively. Limited quantities of RNA were available,

Table 1: Annealing of labeled RNA from L and S particles with unlabeled VERO cell RNA.*

	L particle RNA	S particle RNA
Unheated control	4, --, 19, --	11, --, 17, --
Self annealed	7, 16, 17, 14	12, 19, 28, --
Uninfected cells	6, 10, 13, --	10, 17, 12, --
New Jersey VSV cells	--, --, 18, --	--, --, 17, --
Cocal virus cells	--, --, 14, --	--, --, 15, --
Indiana VSV cells:		
Unfractionated	77, 98, 75, --	50, 88, 55, --
Fraction II	--, --, --, 60	--, --, --, --
Fraction IV	--, --, --, 67	--, --, --, --
Fraction V	--, --, --, 68	--, --, --, --

*Results are expressed as ribonuclease-resistant radioactivity as a percentage of total acid-insoluble radioactivity. Four individual experiments are shown, the first column of figures under "S particle RNA" corresponding to the first column under "L particle RNA", etc; "--" indicates "not done". Cells infected with New Jersey VSV and Cocal virus were prepared as described for Indiana VSV. Reaction mixtures were usually 0.2 ml, and contained approx. 0.3 - 0.6 μg of labeled RNA with 50-350 CPM, and 0.5 - 30 μg of cell RNA.

especially from purified S particles, and as is apparent from Fig. 1, most of the RNA measured by ultraviolet absorbance in cell extracts was ribosomal and 4S cell RNA. Thus it was not feasible to test annealing in all combinations, and at optimal concentrations. Preliminary experiments with labeled cells did show increasing annealing, up to 60%, with increasing amounts of unlabeled crude virus concentrates. Plus strand RNA from L particles annealed efficiently with RNA of cells infected with the homologous virus, but not with RNA from uninfec-

ted cells or cells infected with the antigenically unrelated New Jersey VSV or the related (Federer et al., 1967) Cocal virus. The latter is in contrast to the findings of Kingsbury (1966) with NDV, where viral RNA was complementary to RNA from cells infected with several NDV strains. RNA from S particles also showed complementarity with RNA of Indiana-infected cells, and not with the other RNA's. The apparent lower efficiency of annealing with infected cell RNA and higher self-annealing of S, as compared with L, may reflect a greater possibility of contamination of S with cell RNA. An additional experiment (not shown) revealed that the RNA's from L and S particles are not complementary to each other.

Table 2: Annealing of labeled RNA fractions from VSV-infected VERO cells with unlabeled RNA from L and S particles and from VERO cells.*

	Labeled RNA Fractions				
	I	II	III	IV	V
Unheated control	21, 31	9, --	11, --	10, --	--, 20
Self annealed	15, 47	21, 52	12, --	11, 31	--, 25
L particle RNA	--, 45	44, 70	37, --	45, --	--, 46
S particle RNA	24, 44	31, 64	20, --	--, --	--, 32
Uninfected cells	11, --	--, --	--, --	12, --	--, --
Infected cells:					
Fraction I	--, --	--, 75	--, --	--, 57	--, 46
Fraction II	--, 67	--, --	--, --	--, 34	--, --
Fraction IV	--, 66	--, 56	--, --	--, --	--, 26
Fraction V	--, 71	--, --	--, --	--, 35	--, --

*Results of two experiments expressed as in Table 1. Reaction mixture with approx. 100-3000 CPM contained approx. 0.5 - 7 μg each of labeled and unlabeled RNA, including in the case of Fraction I, yeast RNA added as a carrier.

The results with labeled cell RNA fractions (Table 2), although not as clear-cut as those with labeled viral RNA, provide further evidence for complementarity between viral RNA and virus-specific RNA from infected cells. We believe Fraction I consists mainly of viral plus strands, derived in part from mature virus in the preparations; this is reflected in positive annealing with the slower sedimenting fractions. The experiments do not rule out possible

presence of some minus strands in Fraction I, either as single-stranded RNA of about 4×10^6 Daltons, or as short segments bound in double-stranded form to intact plus strands. Similarly, it is not possible at present to evaluate quantitatively the proportion of plus and minus RNA strands in Fractions II, IV and V, but the evidence (Tables 1 and 2) indicates that minus strands predominate. Fraction III probably includes 23S RNA from S particles and some RNA from the major peak (Fraction IV), and possibly RNA from minor components as yet unresolved. Thus, the low level of annealing observed for Fraction III cannot be interpreted from the present data.

Our results indicate that the S particle RNA is complementary to the infected cell fractions and not to L RNA. Therefore the RNA of the autointerfering particle should also be designated "plus", a designation compatible with the suggestion (Huang and Wagner, 1966) that the S particle contains a portion of the viral genome. The published base analyses of VSV RNA's (Brown et al., 1967; Huppert et al., 1967) show approximate base pairing. This relation is probably fortuitous, making base analyses of little aid in resolving the relationships among the various RNA fractions.

A large portion of the viral-specific RNA's synthesized in cells infected with NDV or Sendai virus are minus strands, which sediment more slowly than plus strands from the virion (Bratt and Robinson, 1967; Kingsbury, 1966,1967; C. Blair and P. Duesberg, personal communication). Such RNA was distributed mainly in a major peak at 18S and a minor peak at 35S. The heights of our peaks II and IV, and their positions relative to the virion RNA, resemble the distribution of the NDV-Sendai systems. Our findings with VSV, namely, complementarity and distribution of RNA, strongly suggest similarities in the modes of replication of VSV and these paramyxoviruses.

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