PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 116-121 (1976)

Biochemical Characteristics of Rat C-Type Virus WF-1[1] (39340)

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The discovery and morphological-immunological properties of the rat C-type virus WF-1, infectious for normal rat embryo cell cultures, have been previously described (1, 2). This presumably endogenous C-type virus was spontaneously released by a cell line (WF-1) derived from normal Wistar-Furth rat embryos. The virus was also shown to be antigenically related to two C-type viruses (RMTDV and R-35) produced by cell lines derived from rat mammary tumors (3).

Although all three viruses are similar morphologically and antigenically, only RMTDV had been found to produce some leukemogenic effects when inoculated into rats (4).

It was therefore of interest to determine the pertinent biochemical characteristics of the WF-1 virus, non-oncogenic for rats, and to compare them with those of known oncogenic RNA viruses of other animal species. The results of this study are presented in this report.

Materials and methods. Reagents. TNE buffer (0.01 *M* Tris, 0.1 *M* NaCl, and 0.001 *M* EDTA, pH 7.2), TNM buffer (0.01 *M* Tris, 0.1 *M* NaCl, and 0.001 *M* $MgCl_2$), and sucrose solutions prepared in TNE buffer were sterilized by filtration (0.45-μm porosity, Millipore Corp., Bedford, Mass.). Ribonuclease-free sucrose, [^{3}H]uridine (sp act 25 to 28 Ci/mmole), [^{14}C]uridine (sp act 36.5 mCi/mmole), and [^{3}H]uridine triphosphate (sp act 15 Ci/mmole) were purchased from Schwarz/Mann, Orangeburg, N.Y. [^{3}H]Thymidine triphosphate ([^{3}H]TTP, sp act 18 Ci/mmole) was purchased from New England Nuclear, Boston, Mass. Electrophoretically purified nucleases (RNase, code Rase; and DNase, code DPFF) were purchased from Worthington Biochemical Corp., Freehold, N.J.

Other reagents were: 2-mercaptoethanol (2-ME), dithiothreitol (DTT), dATP, dCTP, dGTP, rATP, rCTP, rGTP (Sigma Chemical Co., St. Louis, Mo.), Nonidet P-40′ (Shell Chemical Co., New York, N.Y.), diethylpyrocarbonate (DEP), diethyl oxydiformate (Eastman Kodak Co., Rochester, N.Y.), and trichloroacetic acid (TCA).

Cell cultures and meda. The origin and propagation techniques of WF-1 cells have been described (1). For the studies reported here, WF-1 cells between the sixteenth and twenty-eighth *in vitro* passages were used. The growth medium was Eagle's minimal essential medium containing 100 units of penicillin G and 100 μg of streptomycin per milliliter and 10% fetal calf serum (MEM-FCS). Rat embryo (RE-2) cells, grown in MEM-FCS, were obtained from Dr. N. Biswal, Baylor College of Medicine. Chicken embryo fibroblasts (SPAFAS, CEF) fully transformed by Rous Sarcoma Virus (Rous-Associated-Virus) [RSV(RAV-1)] were kindly supplied by Dr. P. Meyers.

Virus purification. Culture supernatants were harvested at 10- to 14-hr intervals after the addition of 10 μCi/ml of [^{3}H]uridine to the WF-1 cells or 20 μCi/ml of [^{3}H]uridine to the transformed CEF cells. The fluids were clarified at 3000 *g* for 10 min and concentrated to approx 10 ml by diaflo ultrafiltration at 4° under positive nitrogen pressure. The 402 diaflo unit (Amicon Corp., Lexington, Mass.) was autoclaved before use, and the centrifuge tubes

[1] This work was supported by Contract No. NIH-70-2211 within the Virus Cancer Program of the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

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and the Amicon PM-30 membrane were sterilized with uv light. Concentrates were clarified at 10,000 *g* for 10 min.

Concentrates were layered onto discontinuous gradients of 8 ml of 20% sucrose cushioned by 5 ml of 55% sucrose and then centrifuged at 25,000 rpm for 2 hr in a Spinco SW 25.1 rotor at 4°. The band at the interphase was collected by puncturing the bottom of the tube. It was diluted with TNE buffer, and layered over a 20-ml linear gradient of 20 to 55% sucrose. After 8 to 10 hr of centrifugation as described above, fractions of approx 1 ml were collected from the bottom of the tube. The radioactivity in a 50-μl sample of each fraction was measured in 10 ml of Beckman Ready-Solv VI (Beckman Instruments, Inc., Fullerton, Calif.) or as the acid-insoluble counts retained by Millipore filters (5) in 10 ml of Beckman Ready-Solv IV in a Packard Tri-Carb Model 3320 scintillation spectrometer at 4°. The buoyant density of each fraction was determined by refractometry.

RNA extraction. Appropriate gradient fractions were combined and then diluted with TNE buffer to contain less than 10% sucrose. To each 10-ml vol were added 0.1 ml of 2-ME, 1 ml of 1% DTT in 1% phenol, and 0.2 ml of DEP. After mixing in an ice bath for 10 min, the preparations were warmed to approx 20°, and SDS was added to give a final concentration of 1%. Preparations were deproteinized with PMQN (100 ml of freshly distilled phenol saturated with TNE buffer, 12 ml of freshly distilled *m*-cresol, and 0.1 g of 8-hydroxyquinoline, pH 7.4) as described by Biswal and Benyesh-Melnick (5). The RNA in the aqueous phase was precipitated at −20° for at least 2 hr after adding one-tenth vol of 20% Na acetate (pH 5.2) and 2 vol of iced 95% ethanol. The RNA precipitate was collected by centrifugation at 15,000 *g* for 20 min and was dissolved in TNE buffer.

Alternatively, radioactive virions from the interphase of the discontinuous gradient or from the appropriate gradient fractions were pelleted at 25,000 rpm for 1 hr in the SW 25.1 rotor. The pellet was resuspended in 0.5 ml of TNE buffer containing 2-ME, DTT, and DEP (as described), and the RNA was extracted with 1% sodium dodecyl sulfate (SDS).

Centrifugation of RNA. RNA preparations were layered onto 4.6-ml linear gradients of 5 to 20% sucrose and sedimented at 35,000 rpm for 2 hr at 4° in an SW 39 rotor. Fifteen-drop fractions were collected from the bottom of the tube into tubes containing 0.4 ml of TNE buffer. The radioactivity in each fraction was assayed as the acid-precipitable material (5) in 10 ml of Beckman Ready-Solv IV. Sedimentation coefficients were determined by the method of Martin and Ames (6) using ^{14}C-labeled 28 S ribosomal RNA (rRNA) isolated from RE-2 cells or ^{3}H-labeled high mol wt RNA isolated from RSV(RAV-1).

RNA was recovered from gradients by collecting 15-drop fractions into tubes containing 1 ml of TNE buffer, 50 μg of unlabeled rRNA, and 0.1% (v/v) DEP. The RNA in selected fractions was precipitated with Na acetate and ethanol as described above.

Reverse transcriptase assay. Culture supernatants were harvested, concentrated, and purified as described herein. The virus concentration was based on the amount of protein present in the viral band as determined by the method of Lowry *et al.* (7). The endogenous polymerase assay was modified from the method of Spiegelman *et al.* (8). A standard incubation mixture of 0.1 ml contained: 0.2 mmole each dATP, dCTP, and dGTP; 0.109 μmole [^{3}H]TTP (15–18 Ci/mmole); 2.8 mmole 2-ME; 12 mmole $MgCl_2$; 40 mmole KCl; and 57 mmole Tris–HCl (pH 8.3). Virus suspended in Tris–HCl (pH 8.3) was preincubated for 10 min at 0° with 0.08% Nonidet P-40 and 0.4% 2-ME. The preincubation mixture was added to the incubation mixture and placed immediately at 37°. After incubation for 180 min, each sample was co-precipitated with 50 μg of bovine serum albumin by the addition of 0.3 ml of TCA mixture (equal volumes of 100% TCA, saturated NaH_2PO_4, and saturated $Na_4P_2O_7 \cdot 10$ H_2O). After 10 min, the precipitate was collected on nitrocellulose filters and washed with 5% TCA and then with 2 ml of 95% ethanol. The radioactivity on dried filters was determined as described herein.

RNA-dependent RNA polymerase (RDRP) assay. The incubation mixture consisted of 0.25 ml containing (in micro-

moles): 1.0 each of rATP, rCTP, and rGTP; 2.67×10^{-4} of ^{3}H-rUTP; 3 of $MgCl_2$; 10 of KCl; and 14.25 of Tris-HCl (pH 8.3). The preparation of virus, preincubation mixture, and assay for radioactivity were done as described above.

Results. Cultures of WF-1 cells were labeled with [^{3}H]uridine, and the supernatant fluids were tested for the presence of radioactive virus particles by equilibrium centrifugation in sucrose gradients. The RE-2 cells, in which C-type particles have not been seen in numerous examinations by electron microscopy, were treated in a similar manner. Figure 1 demonstrates that culture fluids from WF-1 cells produced a prominent peak of radioactivity of a buoyant density of 1.15 to 1.16 g/cm^3. Culture fluids from RE-2 cells labeled with [^{3}H]uridine, or from WF-1 cells labeled with [^{3}H]thymidine, failed to yield a similar peak.

Volumes of 300 to 450 ml of freshly harvested fluids from radiolabeled WF-1 cell cultures were concentrated, purified, and used for isolation of viral RNA. The RNA from purified virions was extracted with SDS-PMQN and sedimented in 5 to 20% linear sucrose gradients. Approximately 55 to 65% of the RNA was located in the 60 to 70 S region of the gradient. In some experiments, radiolabeled virions were collected from the interphase of discontinuous gradients and RNA was extracted with 1% SDS. There were no differences in sedimentation coefficients of RNA species obtained by either of these methods. When compared to the position of 28 S rRNA in several gradients, WF-1 RNA sedimented at 62 to 68 S (Fig. 2). Sedimentation of RNA from RSV(RAV-1) and WF-1 virions, either in separate gradients or in the same gradient, indicated that the RNA from RSV(RAV-1) sedimented slightly faster than the RNA from WF-1 virus (Fig. 3). Accepting a sedimentation coefficient of 71 S for the avian virus RNA (9), WF-1 viral RNA sedimented at approx 64 S. It has also been reported that high mol wt RNA from RSV(RAV) sediments slightly faster than the high mol wt RNA from mouse mammary tumor virus (9, 10).

The experiments described above were done with RNA extracted from virions harvested after 10- to 14-hr incubation. However, when WF-1 cells were labeled for 24 to 48 hr, or when multiple harvests were made and each harvest was held at 4 or −20° until the final harvest, the recovery of high mol wt RNA ranged from 0 to 40%, with the remainder of the cpm spread over a range of 4 to 40 S. Virus preparations that were frozen and thawed only once yielded primarily (80 to 100%) 4 to 12 S RNA. In

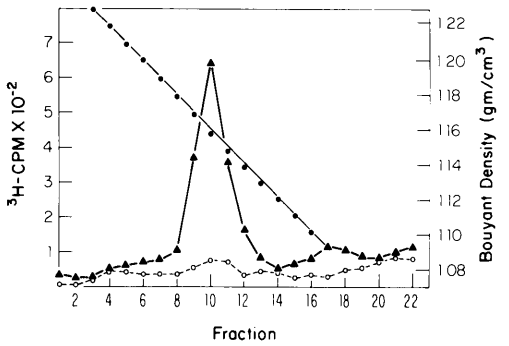

FIG. 1. Density gradient centrifugation of culture fluids from WF-1 cells. Cultures were labeled for 12 hr with 10 μCi/ml of [^{3}H]uridine (▲——▲) or [^{3}H]thymidine (O- - -O). Supernatant fluids were concentrated and purified by centrifugation in sucrose gradients. Fractions of approx 1 ml were collected from the bottom of the tube, and a 50-μl sample of each fraction was assayed for acid-precipitable radioactivity.

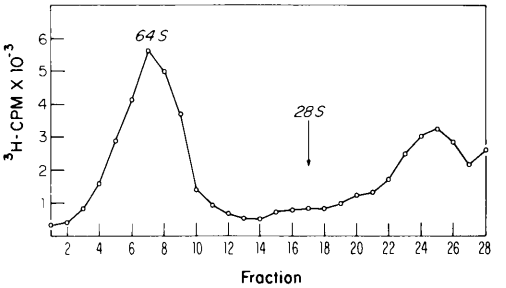

FIG. 2. Velocity sedimentation of viral RNA. Radioactive WF-1 virions were collected from the interphase of a discontinuous 20 to 55% sucrose gradient, pelleted, and lysed as described in the text. The ^{3}H-labeled viral RNA was centrifuged with ^{14}C-labeled 28 S rRNA in a 5 to 20% sucrose gradient at 35,000 rpm for 2 hr in a Spinco SW 39 rotor. Fractions of 15 drops were collected from the bottom of the tube and assayed for acid-precipitable radioactivity. The arrow in this and the following figures shows the position of the peak of ^{14}C-labeled 28 S rRNA from normal rat embryo cells.

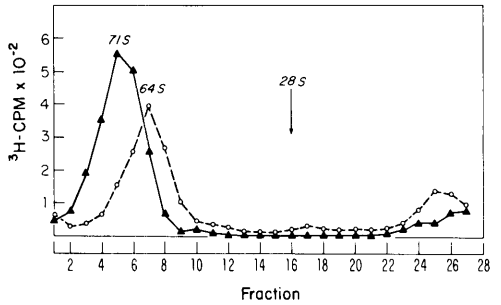

FIG. 3. Velocity sedimentation of RNA. Deproteinized preparations of ^{3}H-labeled RSV(RAV-1) RNA (▲——▲) and WF-1 RNA (O- - -O) were sedimented in separate 5 to 20% sucrose gradients. The viral RNAs had been separated from the more slowly sedimenting RNA components by preparative sucrose gradient centrifugation (see Methods). The procedures for centrifugation, gradient fractionation, and assay for radioactivity are as described in Fig. 2.

some of these preparations, as much as 1 mg of unlabeled 28 S rRNA or 2000 cpm of ^{14}C-labeled 28 S rRNA was added prior to disruption of the virus. Assays of gradient fractions for ^{14}C-radioactivity or optical density indicated good recovery of 28 S rRNA, suggesting that the RNA was not degraded during the disruption and extraction procedures.

These results suggest that long labeling intervals and storage at 4 or −20° may cause degradation of the 60 to 70 S viral RNA into components which sediment more slowly. Similar results have been reported in studies of RNA extracted from other oncornaviruses (9, 11, 12).

The RNA precipitated from the 60 to 70 S region of the gradient was dissolved in 0.2 *M* NaCl for alkaline hydrolysis or in TNM buffer for assay with RNAse. Duplicate samples containing 1850 cpm of acid-precipitable radioactivity retained an average of 30 cpm (1.4%) of acid-insoluble material after treatment with 25 μg of RNase at 37° for 30 min. After treatment with an equal volume of 1 *M* NaOH at 80° for 30 min, duplicate samples containing 3674 cpm each yielded only 0.4% of acid-insoluble radioactivity. These results indicate that the high mol wt RNA is single-stranded and that no more than 0.4% of the nucleic acid could have been DNA.

Treatment of the high mol wt RNA of numerous oncogenic RNA viruses with heat or DMSO converts the 60 to 70 S RNA into more slowly sedimenting components, indicating that the high mol wt RNA consists of subunits. Figure 4 depicts the results obtained upon centrifugation of WF-1 high mol wt RNA after the sample was heated at 100° for 2 min in a sealed ampule. In comparison with unlabeled 28 S rRNA, the heated viral RNA dissociated into components of approx 36 S, 18 to 20 S, and 4 to 12 S. Similar results have been reported for heat-dissociated high mol wt RNA from murine sarcoma-leukemia virus (13).

In reverse transcriptase assays, the incorporation of [^{3}H]TTP into acid-insoluble material by the virion-associated reverse transcriptase was decreased by the omission of $MgCl_2$ or two of the deoxynucleotide triphosphates (Table I). The reaction was only slightly affected by the absence of 2-ME, but disruption of the virions was necessary for maximum polymerase activity. If the disrupted particles were treated with RNase before addition to the incubation mixture, a 47% loss of precipitable counts resulted, indicating dependence upon the presence of RNA for maximum activity. The product of polymerization using this endogenous template was not affected by RNase but was rendered acid-soluble by DNase treatment (Table II), indicating the presence of DNA in the product. However, annealing experi-

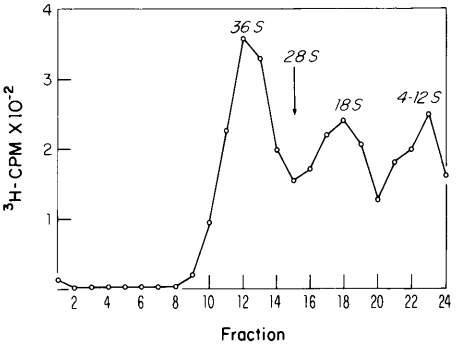

FIG. 4. Velocity sedimentation of heat-dissociated WF-1 viral RNA. ^{3}H-labeled viral RNA was separated from more slowly sedimenting RNA components by preparative sucrose gradient centrifugation and heated at 100° for 2 min in a sealed ampoule. Centrifugation was in a linear 5 to 20% sucrose gradient at 35,000 rpm for 2.5 hr in a Spinco SW 39 rotor. Gradient fractionation and assay for radioactivity were performed as described in Fig. 2.

TABLE I. REQUIREMENTS OF WF-1 VIRUS-ASSOCIATED REVERSE TRANSCRIPTASE.

Condition[a]	^{3}H [TTP] incorporated[b] (cpm)
Complete assay system	1175
Complete, RNase preincubation[c]	627
No virus	276
No dCTP and dGTP	352
No $MgCl_2$	432
No 2-ME	957
No Nonidet P-40	512

[a] Assay conditions using the endogenous template are described in Methods. The preincubation mixture contained 80 μg of protein.

[b] Total cpm in 0.1-ml test volumes, as the average of two tests.

[c] To the preincubation mixture after the initial 10 min of incubation was added 10 μl of RNase (1 mg/ml) and 1.2 μmole $MgCl_2$, which usually was in the standard incubation mixture. The preincubation mixture plus RNase was incubated at 37° for 15 min before addition to the standard incubation mixture.

TABLE II. PROPERTIES OF THE PRODUCT OF VIRION-ASSOCIATED REVERSE TRANSCRIPTASE.[a]

Treatment[b]	cpm[c]	Percentage cpm recovered[d]
None	1462	100
RNase (100 μg/ml)	1551	106.1
DNase (100 μg/ml)	548	37.3

[a] Assay conditions using the endogenous template are described in Methods. The preincubation mixtures contained 80 μg of protein.

[b] After reaction at 37° for 3 hr, 0.1-ml aliquots were treated as follows: No treatment but further incubation at 37° for 90 min; RNase treatment for 90 min at 37°; DNase treatment for 90 min at 37°.

[c] Total cpm in 0.1-ml test volumes, as the average of two tests.

[d] Untreated sample assumed as 100% reaction.

ments to demonstrate complementarity of the product to viral high mol wt RNA were not done.

Assays to detect possible RDRP were also performed. All attempts to detect this enzyme in association with WF-1 virus were unsuccessful.

Discussion. The results of the present study indicate that the virus isolated from WF-1 cells has several biochemical characteristics in common with known oncogenic C-type viruses.

The buoyant density of the WF-1 virus in sucrose gradients is 1.15 to 1.16 g/cm^3. This density is slightly greater than that (1.14 g/cm^3) previously reported for WF-1 virus (2). This may reflect the differences in the time intervals used for labeling the virus (10 to 14 hr as opposed to 48 hr) and the methods employed to process supernatant culture fluids and purify the virus. The C-type RNA tumor viruses contain single-stranded 60 to 70 S RNA which, upon treatment with heat or DMSO, dissociates into components which sediment more slowly, suggesting a subunit structure. As demonstrated here, the WF-1 virus possesses single-stranded RNA with a sedimentation coefficient (approx 64 S) slightly less than that of the RNA from RSV(RAV-1) (71 S). The results obtained upon sedimentation of heat-dissociated high mol wt RNA suggest that the WF-1 viral RNA may be composed of subunits.

The presence of the WF-1 virus reverse transcriptase is indicated by the *in vitro* synthesis of a DNA-like product when disrupted virions are present in the assay. Preincubation of disrupted virus preparations with RNase significantly decreased the incorporation of [^{3}H]TTP, indicating that the template is RNA, and the product of the polymerase assay was sensitive to DNase but not to RNase. The enzyme thus shares some of the characteristics attributed to polymerases from various other C-type viruses (14–16). No RDRP activity could be detected in association with the virus.

To date all attempts to demonstrate a biological activity of the WF-1 virus in rats have been unsuccessful despite of: (a) its ability to infect rat cells *in vitro* (2), and (b) high malignancy of the rat embryo cell line (1) from which the WF-1 virus was isolated. It appears at the present time that the WF-1 virus is a non-oncogenic endogenous virus of rats. However, it may be equally likely that the oncogenicity of this virus is repressed upon inoculation by host-associated factors, a possibility to be carefully considered in future studies with this and other rat C-type viruses in their natural host.

Summary. The non-oncogenic rat C-type virus WF-1, isolated from a Wistar–Furth rat embryo cell line, was characterized biochemically. The purified virus has a buoyant density of 1.15 to 1.16 g/cm^3 in sucrose, RNA-dependent DNA polymerase (reverse transcriptase) activity, and RNA with a sed-

imentation coefficient of 62 to 68 S. The viral RNA is single-stranded, and, upon treatment with heat, yields components with sedimentation coefficients of 36 S, 18 to 20 S, and 4 to 12 S.

We thank Ms. H. Asmussen for technical assistance, and Ms. J. G. Bronson for preparing the figures.

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Received October 6, 1975. P.S.E.B.M. 1976, Vol. 152.