Synthesis of Ribonucleic Acids in KB Cells Infected with Rhinovirus Type 14

By C. J. GAUNTT

Department of Microbiology, Arizona Medical Center, University of Arizona, Tucson, Arizona 85724, U.S.A.

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SUMMARY

KB cells infected with rhinovirus type 14 (RV 14) in the presence of actinomycin D synthesized two major species of virus RNA. One species had properties similar to infectious RNA extracted from virus particles. The other major species of virus RNA exhibited properties that were descriptive of replicative intermediate (RI) structures. Pulse-labelling experiments with $[^3H]$-uridine suggested that the RI structures were involved in synthesis of virus RNA. Two RNA species were isolated in minor amounts from infected cells. One species had properties descriptive of a replicative form of RNA. The second minor species was single-stranded, approximately one-half the size of the virus genome and could also be isolated from purified virus.

INTRODUCTION

Picornaviruses have been classified into four subgroups: enterovirus (polioviruses, Coxsackie viruses, and ECHO viruses), cardioviruses (mengovirus, encephalomyocarditis, and mouse encephalitis viruses), rhinoviruses and foot-and-mouth disease viruses (Fenner, 1968). Particles of the picornaviruses contain single-stranded infectious RNA molecules with mol. wts. ranging from 2.4 to $2.8 \times 10^6$ (Baltimore, 1969; Stott & Killington, 1972).

Cells infected with viruses of the enterovirus-cardiovirus subgroups were found to contain multi-stranded, partially base-paired, RNA structures of various sizes (Baltimore & Girard, 1966; Martin, 1967; Baltimore, 1968; Girard, 1969; Girard & Marty, 1969; Noble, Kass & Levintow, 1969; Oberg & Philipson, 1969; Wall & Taylor, 1970). These RNA structures, termed replicative intermediate RNA (RI), have been implicated in synthesis of progeny virus RNA molecules (Baltimore, 1968, 1969; Oberg & Philipson, 1969; Girard, 1969; Noble & Levintow, 1970). It is proposed that virus RNA is generated via linear displacement from a complimentary RNA template contained in the RI structures (Baltimore, 1968, 1969; Erickson, 1968; Girard, 1969; Oberg & Philipson, 1969; Bishop & Levintow, 1971).

However, in several studies on the replication of foot-and-mouth disease viruses (FMDV) (Brown & Cartwright, 1964; Wild & Brown, 1970) and a bovine enterovirus (Clements & Martin, 1971), single-stranded RNA molecules of a length greater than parental RNA molecules were detected in infected cells. To account for these large RNA molecules, it has been proposed that progeny RNA molecules were synthesized via cyclic displacement from circular intermediate RNA forms (Brown & Martin, 1965).

Few studies have been reported on the replication of the RNA of rhinovirions, which is slightly smaller in size than the RNA of the enteroviruses (Brown, Newman & Stott, 1970;
McGregor & Mayor, 1971; Nair & Lonberg-Holm, 1971; Sethi & Schwerdt, 1972). As the rhinoviruses share properties of acid lability and high buoyant density in CsCl with the FMDV, it was of interest to study the replication of their RNA. It has recently been reported that HeLa cells infected with rhinovirus type 2 (RV2; Yin & Knight, 1972) or rhinovirus type 20 (RV20; Sethi & Schwerdt, 1972) contain a virus RNA species with some properties similar to RI forms found in cells infected with viruses of the enterovirus-cardiovirus subgroups.

In the present report, a more detailed examination of the replication of the RNA of one of the rhinoviruses was performed. KB cells infected with rhinovirus type 14 (RV14) were found to contain RI structures which are involved in some manner with replication of progeny virus RNA. Although some differences were found, the present data on synthesis of RV14 virus RNA is compatible with the model proposed to account for synthesis of RNA by viruses of the enterovirus-cardiovirus subgroups (Baltimore, 1968, 1969; Girard, 1969; Oberg & Philipson, 1969; Noble & Levintow, 1970).

**METHODS**

*Cells and virus.* Methods for the culture of HeLa (rhino-HeLa) and KB cells and preparation of monolayer cell cultures were previously described as well as methods for preparing RV14 stocks and plaque assay of RV14 (Stoker, Kiernat & Gauntt, 1973). All experiments were conducted using an inoculum containing 20 to 30 p.f.u./cell and 5 μg/ml of actinomycin D (act D). Zero time was regarded as the instant of mixing virus and cells. Procedures for challenge of cells with virus and harvest of infected cells for plaque assay of virus on HeLa cell monolayer cultures were previously described (Stoker et al. 1973).

*Assay of infectious RNA (IRNA).* Samples to be assayed for infectious ribonucleic acids (IRNA) were diluted in cold (4 °C) phosphate-buffered saline (Dulbecco & Vogt, 1954) containing 0.1 % bovine serum albumin, fraction V (PBSA) and 500 μg/ml of diethylaminoethyl-dextran (DEAE-D; Pharmacia Fine Chemicals, Piscataway, N.J.). After 20 min at 25 °C for adsorption, the plaque assay method was carried out (Stoker et al. 1973).

*Radioactive labelling of virus RNA in infected cell cultures.* At designated times post-inoculation (p.i.), 1 μCi/ml of [5-3H]-uridine was added directly to the virus growth medium (VGM; Eagle’s minimal essential medium, Eagle, 1959) containing 1 % heat-inactivated foetal calf serum). In some experiments, control uninfected cultures were mock-infected with PBSA containing 5 μg/ml of act D and were manipulated in a manner similar to the infected cultures. Duplicate infected and uninfected cell cultures were harvested by scraping the cells into the VGM with a rubber policeman and centrifuging the mixture at 350 g for 10 min. The cell pellets were washed once with cold PBSA and RNA was immediately extracted.

*Extraction of virus RNA.* RNA was extracted from purified virus or infected cell pellets by use of phenol and SDS according to the method of Watanabe & Graham (1968) for single-stranded RNA, except that Macaloid and deoxyribonuclease were omitted (Figs. 3, 4). The ethanol-precipitated nucleic acids were dissolved in 0.3 M-STE (0.3 M-NaCl, 0.001 M-sodium ethylenediaminetetra-acetate, and 0.01 M-tris, pH 7.6) containing 0.5 % SDS and maintained at 0 °C. In a second method (results of Figs. 1, 2, and 5 to 8), the harvested cells were taken up in 10 vol. of reticulocyte standard buffer, pH 8.5 (RSB; Warner, Knopf & Rich, 1963) and the cells were disrupted by 12 strokes in a Dounce homogenizer. The cytoplasm of these cells was extracted with phenol and SDS as described above.

*Purification of [3H]-uridine-labelled RV14.* The technique of Korant et al. (1972) was used
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to prepare [3H]-uridine-labelled virus in KB cell cultures with the following exceptions. The aqueous phase from freon-extracted cell debris was centrifuged at 30,000 g for 45 min on to a 60 % sucrose cushion (w/v) in TPBT buffer (0.2 % tryptose-phosphate broth and 0.01 M-tris-HCl, pH 8.5). The virus-containing material was passed through a column of G-25 Sephadex which had been equilibrated with 0.3 M-STE and solid CsCl was added to a density of 1.375 g/ml. The mixture was centrifuged for 24 h at 33,000 rev/min in an SW50 rotor at 4 °C. The density of every fourth fraction was immediately determined using a Bausch & Lomb Abbe-3L refractometer. Samples containing RNA were treated with trichloroacetic acid (TCA) according to the method of Bollum (1959). Radioactivity in each sample was determined in a Nuclear Chicago Mark I spectrometer using a toluene-based scintillation cocktail (Watanabe & Graham, 1968). Fractions containing virus were pooled and dialysed against 500 vol. of TPBT buffer for 16 h at 4 °C and virus RNA was extracted using phenol and SDS. Infectious RV 14 banded at a density of 1.408 ± 0.006 g/ml (8 determinations).

Sedimentation of RNA in sucrose gradients. Samples containing RNA were layered on linear 5 ml gradients of 5 to 20 % sucrose in RSB containing 0.5 % SDS. Sedimentation was carried out for 110 min at 47000 rev/min at 20 °C in the SW50.1 rotor in the Beckman Model L2-65B ultracentrifuge. E260 was continuously monitored during fractionation by a Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and recorded with a Honeywell Model 6020 recorder (Honeywell, Fort Washington, Pa.). Gradient fractions were assayed for content of total and ribonuclease-resistant virus RNA as follows. Total TCA-insoluble [3H]-counts in each sample were determined on 0.05 ml (Bollum, 1959). [3H]-counts in ribonuclease-resistant RNA of gradient fractions were determined by adding 0.05 ml to 0.1 ml of 0.3 M-STE containing sufficient pancreatic ribonuclease to give a final concentration of 10 μg/ml. This mixture was incubated for 30 min at 37 °C prior to TCA precipitation (Bollum, 1959). Under these conditions of exposure to ribonuclease, 96 to 98 % of KB cell RNA was degraded to acid-soluble material. Ribonuclease-sensitive RNA in each sample was determined by difference between total and ribonuclease-resistant RNA.

Preparation of [3H]-labelled RI and purification by agarose exclusion chromatography. The RV 14 form of RI was prepared by challenging 10 cultures of KB cells in Blake bottles with 50 p.f.u./cell of RV 14 in the presence of 5 μg/ml of act D. At 7 h p.i., 10 μCi/ml of [3H]-uridine was added and incubation was continued for 5 min. At termination of the pulse period, the cells were quickly chilled at 4 °C, washed once in cold medium containing 100-fold excess of unlabelled uridine and RNA was extracted from the cytoplasmic preparation with phenol and SDS. After precipitation with ethanol, the RNA was taken up in 0.15 M-STE (0.15 M-NaCl), mixed with an equal vol. of 2 M-LiCl and held at 0 °C for 6 h. The insoluble RNA fraction containing the RI forms and single-stranded RNA (Franklin, 1966) was again taken up in 0.15 M-STE and precipitated by addition of 2 M-LiCl. To effect separation of RI forms from single-stranded RNA, the second RNA precipitate was taken up in 0.15 M-STE and applied to a column of 2 % agarose beads (Pharmacia Fine Chemicals; 2.6 cm diam. column packed to 70 cm). The column was prepared and elution of RNA was carried out as described by Erickson & Gordon (1966).

Electrophoresis and polyacrylamide gels. The method of Bishop, Claybrook & Spiegelman (1967) was used to prepare 2-8 % polyacrylamide-ethylene diacrylate gels. Gel columns of approximately 5.5 cm were prepared in quartz glass tubes with an internal diam. of 0.6 cm (AmerSil, Inc., Los Angeles, California). The blank gels were run for 30 min prior to application of 0.01 to 0.05 ml samples of RNA in RSB containing 0.5 % SDS and made to 30 to 40 % in sucrose. Running time was 90 min at 8 mA/gel. E260 were made immediately on each
Fig. 1. Electropherogram of RNA extracted from purified virus. RNA was extracted from CsCl-banded virus, mixed with KB cell ribosomal RNA and electrophoresed into polyacrylamide gels as described in Methods. The left and right arrows indicate positions of migration of unlabelled 28 and 18S KB cell ribosomal RNA, respectively, in this and subsequent electropherograms. The anode is to the right.

gel within the quartz tube using a Gilford Instruments gel scanning device attached to the Gilford Model 240 spectrophotometer. Gel slices of approximately 1 mm thickness were placed in 10 ml of a methanol-toluene scintillation cocktail (Watanabe, Millward & Graham, 1968) and radioactivity was determined in a Nuclear Chicago Mark I spectrometer.

Chemicals. [5-3H]-uridine (21·7 to 28·0 Ci/m·mol) was purchased from International Chemical and Nuclear Corporation (Irvine, Calif.) or from New England Nuclear Corporation (San Francisco, California). Act D was a gift from Merck, Sharpe and Dohme (Rahway, N.J.). Phenol (Mallinchrodt Chemical Works, St Louis, Missouri) was recrystallized prior to use. Ribonuclease-free sucrose was purchased from Schwarz/Mann Chemicals (Orangeburg, New York). Pancreatic ribonuclease was purchased from Worthington Biochemical Corporation (Freehold, N.J.). DEAE-D was a gift from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Freon was a gift from R. Z. Lockart, Jun. (E. I. DuPont de Nemours and Co., Wilmington, Delaware). Agarose was purchased as 2% beads from Pharmacia Fine Chemicals, Inc. under the name of Sepharose 2B. Acrylamide (Eastman Organic Chemicals, Rochester, New York) was recrystallized from chloroform prior to use. Ethylene diacrylate was purchased from K and K Labs, Inc., Hollywood, California.
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RESULTS

RNA in RV14 virus particles

RNA was extracted with phenol and SDS from virus particles that had been banded at 1.41 g/ml in CsCl. This RNA was examined by electrophoresis in polyacrylamide gels. An electropherogram of the results is presented in Fig. 1. The major species of RNA (peak at gel slice 10) migrated slower than 28S ribosomal RNA. The mol. wt. of this RNA was calculated to be $2.4 \times 10^6$ (Bishop et al. 1967). A minor species of virus RNA (peak at gel slice 15) was consistently found in RNA extracted from purified virus particles, but the proportion of RNA in minor to major peaks varied with different preparations of virus. The size of the minor RNA species was calculated to 1.2 to 1.4 × 10^6. In addition to the major and minor species of virus RNA, other RNA species of smaller sizes were found in all preparations of RNA that were extracted from virus banded in CsCl. Studies on the resolution of definitive size-classes of these subgenomic RNA species will be presented elsewhere.

The infectivity of RNA extracted from purified virus was measured by assay of each fraction of a sucrose gradient for IRNA content. The results are presented in Fig. 2. Most infectivity was associated with the leading edge of the major peak, as was previously reported for RV14 (McGregor & Mayor, 1971; Nair & Lonberg-Holm, 1971) and RV2 (Nair & Lonberg-Holm, 1971). Infectivity associated with RNA in fractions 2 to 4 was not found in all experiments of this type, suggesting that this infectivity may be due to aggregates of single-stranded virus RNA. IRNA was not reproducibly associated with the minor RNA peak.
Fig. 3. Kinetics of synthesis of ribonuclease-sensitive and ribonuclease-resistant RV14 RNA in act D-treated KB cell cultures. •—•, TCA-insoluble [\(^{3}H\)]-counts in ribonuclease-sensitive virus RNA; ○—○, ribonuclease-resistant virus RNA; ▲—▲, infectious virus.

**Synthesis of RV14 RNA in the infected KB cell cultures**

The synthesis of RNA in KB cells was inhibited by 90% or more within 4 h, after addition of act D at 5 \(\mu\)g/ml. This concentration effected a 20 to 65% reduction in virus yields in different experiments in which KB cells were challenged with an inoculum containing 20 to 25 p.f.u./cell and 5 \(\mu\)g/ml of act D. In presence of act D, the temporal aspects of the growth cycle remained similar to the growth cycle in absence of act D.

The rates of synthesis of ribonuclease-sensitive and ribonuclease-resistant virus RNA were determined by incubation of infected act D-treated KB cell cultures with 1 \(\mu\)Ci/ml of \(^{3}H\)-uridine for 1 h at different times. Aliquots of each sample were assayed for infectious virus and nucleic acids were extracted from the remainder of the sample with phenol and SDS. Ribonuclease-sensitive and ribonuclease-resistant virus RNA were determined as described in Methods. The results are shown in Fig. 3. Synthesis of ribonuclease-sensitive virus RNA was first detected between 5 and 6 h.p.i. and proceeded rapidly between 6 and 9 h.p.i. The reason for this rapid decline in synthesis is unknown, however, it must be pointed out that rounding up and detachment of infected cells began at 7 h.p.i. and by 10 h.p.i., approximately 50% of cells were detached. The synthesis of ribonuclease-resistant virus RNA was first detected at 6 h.p.i. and maximum synthesis occurred between 7 and 8 h.p.i. This class of virus RNA comprised only 12% of the total virus RNA being synthesized. Experiments in which infected cell cultures were continuously exposed to \(^{3}H\)-uridine from 2 to 11 h.p.i. gave similar results.

RNA was extracted from infected act D-treated KB cell cultures harvested at different
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times p.i. and infectious RNA titrations were performed on total and ribonuclease-treated aliquots of each sample. The time course of accumulation of ribonuclease-sensitive IRNA was coincident with the time course of production of infectious virus, as shown in Fig. 3. Yields of this class of IRNA were approximately $10^{-4}$ of the yields of infectious virus at each time of harvest. Approximately 95% of the total infectivity of IRNA was associated with the ribonuclease-sensitive class of IRNA at all times. Ribonuclease-resistant IRNA was detected at 6 h p.i. and the time course of synthesis of ribonuclease-resistant IRNA paralleled the time course of synthesis of ribonuclease-sensitive IRNA from 7 h p.i. These results establish that portions of each of the two classes of RNA that were detected by incorporation of $[\text{H}]$-uridine into TCA-insoluble RNA represented undegraded virus RNA.

Sucrose gradient sedimentation of IRNA synthesized in infected cells

Velocity sedimentation analyses were performed on RNA extracted from the cytoplasm of infected act D-treated cells that had been incubated with $1 \mu\text{Ci/ml}$ of $[\text{H}]$-uridine at 5 to 6, 6 to 7, 7 to 8 and 8 to 9 h p.i. Aliquots of each RNA sample were centrifuged into 5 to 20% sucrose gradients and each fraction was then assayed for content of TCA-insoluble ribonuclease-sensitive or ribonuclease-resistant RNA. The major virus RNA species in all samples was ribonuclease-sensitive and had a sedimentation coefficient of 30 to 33S. A minor ribonuclease-sensitive RNA species of 26S was readily detected in RNA samples taken between 6 to 7 and 7 to 8 h p.i. Ribonuclease-resistant RNA structures of heterogeneous size (33 to 20S) were found in RNA samples taken between 5 and 9 h p.i. Results similar to these were reported for RNA extracted from act D-treated HeLa cells infected with RV20 (Sethi & Schwerdt, 1972).

Infectivity in ribonuclease-sensitive and ribonuclease-resistant virus RNA was analysed by velocity sedimentation in sucrose gradients. RNA was extracted from infected cells that had been exposed to $[\text{H}]$-uridine between 7 and 8 h p.i. and centrifuged into 5 to 20% sucrose gradients. Each fraction was then assayed for infectivity and TCA-insoluble RNA. The results of a typical experiment are presented in Fig. 4. (A) shows the distribution of $[\text{H}]$-counts in total and ribonuclease-resistant RNA. Major species of RNA sedimented at positions of approximately 30 and 26S and the majority of the RNA in these peaks was sensitive to digestion by ribonuclease. Very few $[\text{H}]$-counts in ribonuclease-resistant RNA were detected due to the long period of labelling (see Fig. 7). Several experiments have shown that the latter RNA is heterogeneous in size and is not reproducibly found in two sizes as suggested in this experiment.

The infectivity assays for total and ribonuclease-resistant IRNA in each fraction are shown in Fig. 4 (B). A peak of IRNA was found at a position corresponding to 30S and the majority (95%) of the infectious RNA molecules at this position were degraded by ribonuclease. A peak of ribonuclease-sensitive RNA of similar size and infectivity was found in RNA extracted from purified virus, suggesting that the 30S RNA peak represents genomic RNA. Other IRNA that was predominantly sensitive to digestion by ribonuclease sedimented in a skewed distribution from 28 to the 16S region of the gradient. Ribonuclease-resistant IRNA sedimented in a broad band from approximately 32 to 20S and comprised approximately 10% of the total IRNA.

Characterization of RI structures

The heterodisperse nature of IRNA which sedimented between 32 and 20S and contained RNA which was partially resistant to degradation by ribonuclease, suggested that this material may represent RV14 forms of RI. It was of interest to separate RI forms from
Fig. 4. Sucrose gradient profiles of total and ribonuclease-resistant RNA isolated from infected cell cultures that had been exposed to [3H]-uridine from 7 to 8 h p.i. (A) shows the distribution of TCA-insoluble [3H]-counts in total (●) and ribonuclease-resistant (○) RNA. (B) shows the distribution of IRNA in total (●) and ribonuclease-resistant (○) virus RNA. E_{260} (—).

double- and single-stranded virus RNA for a more detailed study. Infected act D-treated KB cell cultures were incubated with [3H]-uridine for 5 min at 7 h p.i. and nucleic acids were extracted. A LiCl-insoluble RNA fraction containing RI forms and single-stranded RNA was prepared as described in Methods. This RNA fraction was applied to a 2% agarose bead column and separation of RI forms from single-stranded RNA was achieved as shown in Fig. 5, inset. A sharp band of RNA eluted from the column and was partially resistant (56%) to digestion by ribonuclease. This RNA was precipitated with ethanol, taken up in 0.3 M STE and either directly centrifuged into a linear 5 to 20% sucrose gradient containing 0.5% SDS or exposed to 20 μg/ml of ribonuclease for 30 min at 37 °C, prior to centrifuging. The results, presented in Fig. 5, show that TCA-insoluble counts in [3H] were heterogeneously dispersed from approximately 16S to the bottom of the tube, indicating the presence of very large RNA structures. These RNA structures contained both ribonuclease-sensitive and ribonuclease-resistant regions. Very few counts were found associated with material sedimenting at positions lighter than approximately 16S and no counts were at the top of the gradient, indicating little to no breakdown of RNA in the RI forms to TCA-soluble fragments. Incubation of a portion of the RNA obtained from the agarose column with ribonuclease prior to centrifuging, resulted in identification of a ribonuclease-resistant RNA core...
Fig. 5. Sedimentation in a sucrose gradient of LiCl-precipitated RNA prepared by exclusion chromatography in 2% agarose. RNA from infected cells was twice precipitated in M-LiCl and chromatographed on a column of 2% agarose (inset Fig.). RNA from the fractions enclosed by the bracket was mixed with unlabelled KB cell RNA and one sample was centrifuged into a sucrose gradient. Each fraction was assayed for total and ribonuclease-resistant TCA-insoluble RNA. Another sample of the RNA was incubated with ribonuclease prior to centrifuging into a separate gradient. Note that RNA in the latter gradient was not precipitated with TCA after collection. Symbols for inset Fig.: ●–●, total counts in RNA; ○–○, ribonuclease-resistant RNA. Symbols for main Fig.: ●–●, total RNA; ○–○, ribonuclease-resistant RNA in fractions treated with ribonuclease after centrifuging; ■–■, [3H]-counts in RNA treated with ribonuclease before centrifuging. The left and right arrows indicate positions of sedimentation of 28 and 18S KB cell ribosomal RNA, respectively.

with a sedimentation coefficient of approximately 20S. Other [3H]-counts in RNA of a smaller size were distributed across the top one-third of the gradient. These experiments yielded results that are similar to those described for poliovirus RI forms (Bishop & Koch, 1969).

The RI structures were further characterized by electrophoresis on polyacrylamide gels. RNA was extracted with phenol and SDS from the cytoplasm of infected act D-treated cells that were pulse-labelled with [3H]-uridine from 70 to 7.5 h p.i. LiCl-insoluble RNA was prepared as previously described. One-half of this RNA was applied directly to a gel column and the other half was incubated with ribonuclease at 20 μg/ml for 30 min at 37 °C prior to application. The methods for electrophoresis and assay of gel slices for [3H]-counts were carried out as described in Methods. Two major peaks of RNA (I and II) were observed in the electropherogram of the total LiCl-insoluble RNA sample (Fig. 6A). The RNA of peak I
Fig. 6. Electropherograms of (A) LiCl-insoluble RNA and (B) total RNA synthesized in RV 14-infected KB cell cultures. (A) RNA was directly electrophoresed into the gel or treated with ribonuclease prior to electrophoresis. The electropherogram is a composite profile of total RNA (●○●) or RNA treated with ribonuclease prior to electrophoresis (●○●). (B) RNA was extracted from the cytoplasm of infected KB cell cultures that were incubated with [3H]-uridine from 7 to 7.75 h p.i. After electrophoresis, RNA was eluted from each gel slice and each eluate was assayed for total (●○●) and ribonuclease-resistant (●○●) TCA-insoluble RNA.

migrated poorly into the gel. Ribonuclease-treatment prior to electrophoresis completely altered the electrophoretic mobility of the RNA in peak I. These data suggest that the RNA of peak I contained regions of single-stranded RNA that were accessible to ribonuclease. By analogy to the work of others with similar picornavirus RNA structures obtained from infected cells, the RNA in peak I represent RI forms. RNA arising from the RI structures after ribonuclease-treatment migrated as three distinct peaks (gel slices 7, 40 and 50). Only a small portion of the total ribonuclease-resistant RNA was of a high mol. wt. (RNA peak at gel slice 7) and this RNA is of a size that corresponds to the 20S RNA core shown in Fig. 5. Peak II contained RNA that was sensitive to degradation by ribonuclease and is of the same size as the major species of RNA extracted from purified virus (compare with Fig. 1).

The results of Fig. 6B provide further evidence that the RNA of peak I represents RI forms. RNA was eluted from each gel slice and samples remained untreated or were incubated with ribonuclease prior to precipitation of RNA by TCA. The results show that the RNA of peak I contained both ribonuclease-sensitive and ribonuclease-resistant RNA, as expected from the results presented in Fig. 5. The RNA of peak II was completely digested by ribonuclease as shown in Fig. 6A. This result is in agreement with other experiments which show that RNA extracted from purified virus is totally susceptible to digestion by ribonuclease. The faster migrating RNA (gel slices 48 to 53) probably represents cell transfer RNA, as RNA of similar electrophoretic mobility was also extracted from act D-treated uninfected KB cell cultures.
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Fig. 7. Electropherograms of RNA synthesized in infected cells under short and long-term pulse-labelling conditions. Infected KB cells were pulse-labelled at 7 h p.i. for 5, 15 or 60 min with $[^3]$H-uridine. Aliquots of each sample of RNA were subjected to electrophoresis and counts in $[^3]$H were determined for each gel slice. Twice as much sample was applied to the 5 min gel as to the 15 and 60 min gels.

Involvement of RI structures in synthesis of genomic RNA

If the RI structures are intermediates in the synthesis of RV14 genomic RNA, these structures should be labelled with $[^3]$H-uridine prior to labelling of genomic RNA. This prediction was confirmed in the following experiment. Infected act D-treated KB cells were exposed to 20, 10 or 1 $\mu$Ci/ml of $[^3]$H-uridine, at 7 h p.i. for 5, 15 or 60 min, respectively. At the end of a pulse period, the cells were chilled to 0 °C and RNA was extracted from the cytoplasmic fraction of each cell sample and subjected to electrophoresis as previously described. Each gel slice was assayed for counts in $[^3]$H and the results are presented in Fig. 7.
Three definitive peaks of RNA were discernible in all of the electropherograms: RI structures (peak I), genomic RNA (peak II), and a third peak(s) of RNA that migrated rapidly (gel slice positions of 45 to 53). As mentioned previously, RNA of a size in the latter peak was extracted from act D-treated uninfected KB cells and presumably represents cell transfer RNA. The majority of [3H]-counts in RNA synthesized in a 5 min pulse were associated with RI structures (peak I). In the infected cell cultures that were pulsed with [3H]-uridine for 15 or 60 min, the amount of [3H]-uridine incorporated into the RI structures (peak I) remained approximately the same, suggesting that some fraction of RNA in these structures was turning over at a constant rate. At pulse periods of 15 and 60 min, a large proportion of [3H]-counts were associated with the genomic RNA of peak II rather than with RI structures (peak I). The ratios of [3H]-counts in RI (peak I): genomic (peak II) RNA were 1.96, 0.31, and 0.05 for pulse times of 5, 15 and 60 min, respectively. These results were compatible with the hypothesis that the RI structures were involved in generation of RV 14 genomic RNA.

**Replicative form (RF) of RV 14 RNA**

The infection of HeLa cells by poliovirus (Baltimore, 1966; Bishop & Koch, 1969; Girard, 1969; Noble et al. 1969; Oberg & Philipson, 1969) or RV2 (Yin & Knight, 1972) results in the synthesis of a totally double-stranded RNA molecule termed the RF. Similar RNA species were isolated from RV 14-infected KB cell cultures by the following type of experiment. Infected cells were incubated with [3H]-uridine between 7 and 7.5 h p.i. RNA was extracted from the cytoplasm with SDS and phenol and a LiCl-soluble RNA fraction was prepared. One-half of the sample was directly applied to a gel and the other half was incubated with 20 μg/ml of ribonuclease for 30 min prior to application to a second gel. A composite electropherogram of these results (Fig. 8) shows one peak of high mol. wt. RNA (gel slice 7) which was completely resistant to ribonuclease. As RNA species with similar properties were found in poliovirus-infected cells, this RNA will be referred to as the RV 14 form of RF. In other experiments, the RV 14 form of RF exhibited a similar electrophoretic mobility in polyacrylamide gels as an RF form of mengovirus that was prepared from infected HeLa cells using techniques described in Methods. These results suggest that the RV 14 and mengovirus RF species of RNA are similar in size. The RF form of RV 14 was not extensively labelled, as evidenced by a ratio of [3H]-counts in RF to RI forms of <0.04 in several experiments involving pulse-labelling times of 45 min or 1 h. It was found that pulse-labelling periods of at least 30 min were required to affect sufficient labelling of RF for detection in experiments in which RI and genomic RNA were readily detected. A large amount of RNA of small size and of unknown origin was also detected in this experiment (gel slices 35 to 55). A portion of this RNA probably represents cell transfer RNA which is also soluble in LiCl (Baltimore, 1966; Erickson, 1966).

**DISCUSSION**

KB cells infected with RV 14 in the presence of act D subsequently synthesized two major species of virus RNA. One major species of virus RNA was infectious, degraded by ribonuclease, exhibited a sedimentation coefficient of 33 to 30 S in linear sucrose gradients and had a mol. wt. estimated to be $2.4 \times 10^6$. These properties are similar to those described for RNA extracted from purified RV 14 virus (McGregor & Mayor, 1971; Nair & Lonberg-Holm, 1971) and it is concluded that this major RNA species represent nascent virus genomes. Synthesis of virus RNA was rapid between 5 and 8 h p.i. and the temporal aspects of synthesis of this RNA were identical to the time course of synthesis of infectious RV 14 virus.
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A second major species of virus RNA was isolated from infected cells and was identified as RI forms of RV14 RNA by several experiments. The RI structures contained infectious RNA, were heterogeneous in size (33 to 20S), and contained both single- and double-stranded RNA regions, with portions of the latter being of a 20S size. Also, the RI forms failed to migrate into polyacrylamide gels under conditions which permitted migration of single- and double-stranded RNA species. These properties of the RV14 RI structures are similar to those reported for RI structures isolated from cells infected with poliovirus (Baltimore & Girard, 1966; Baltimore, 1968; Bishop & Koch, 1969; Girard, 1969; Girard & Marty, 1969; Noble et al. 1969; Oberg & Philipson, 1969), mengovirus (Wall & Taylor, 1970), and recently RV2 (Yin & Knight, 1972). Pulse-labelling experiments showed that: (1) RI structures incorporated [3H]-uridine prior to virus RNA under conditions of a short (5 min) pulse; and (2) longer pulse times (15 and 60 min) resulted in steady-state labelling of the RI structures, indicating turnover of at least some of the RNA in these structures. These results are consistent with a precursor-product relationship between RI and virus RNA. The experiments reported herein do not provide information on the mechanism of synthesis of nascent virus RNA, however, the data are in general agreement with the model proposed for synthesis of virus RNA of the enterovirus-cardiovirus subgroups (Baltimore, 1968, 1969; Girard, 1969; Oberg & Philipson, 1969; Noble & Levintow, 1970; Bishop & Levintow, 1971). Single-stranded RNA molecules that are larger than genomic RNA were not consistently isolated from infected cells. The existence of this RNA is suggested by the slight shoulder present on the trailing edge of peak II in Fig. 6A (gel slices
6, 7 and 8), Fig. 6B (gel slices 9 and 10) and in the 5 min pulse Fig. of Fig. 7 (gel slices 9 and 10). Other experiments (Fig. 1, 7 and the 15 and 60 min pulse Fig. of Fig. 7) showed no evidence of this RNA. It will be reported elsewhere that RNA extracted from some purified virus preparations contains large single-stranded RNA molecules suggesting that the large molecules may represent aggregates of RNA. Therefore, although the data do not definitively rule out the model proposed to account for synthesis of RNA by a strain of FMDV or a bovine enterovirus (Brown & Martin, 1965; Wild & Brown, 1970), there is little evidence to favour this model.

Two minor definitive species of virus RNA were consistently isolated in varying quantities from infected cells. One species was determined to be the RF form of RV 14 RNA, on the basis of similarity in size to the RF form of mengovirus RNA (Wall & Taylor, 1970) and complete insensitivity to degradation by ribonuclease. The RF form of RV14 RNA was found only in small amounts in infected cells and could not be detected under short (<30 min) pulse-labelling conditions. The results of this study were unable to demonstrate any role for the RF form in replication of RV14 virus RNA. The second minor species of virus RNA is single-stranded and has a mol. wt. of 1.2 to 1.4 x 10^6. In addition to isolation from infected cells, RNA extracted from purified virus also contained this species of RNA. Studies to be reported elsewhere will show that RNA species of this size and smaller can be generated in vitro by incubation of purified virus at 34.5 °C for at least 8 h, or by incubation of purified 31S genomic RNA at 60 °C for at least 1 min (C. J. Gauntt, unpublished data). The size of this minor species of RNA suggests that it could arise by breakage of the genome at a weak point near the midpoint of its length. RNA species of smaller sizes could similarly be generated from the minor RNA species and this fragility of the RNA genome may account for difficulties reported in isolation of rhinovirus RNA (Stott & Killington, 1972).

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REFERENCES
**Rhinovirus type 14 ribonucleic acids**


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