0022-538X/78/0025-0078$02.00/0
JOURNAL OF VIROLOGY, Jan. 1978, p. 78-85
Vol. 25, No. 1
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In Vitro Translation of the Two RNAs of Nodamura Virus, a Novel Mammalian Virus with a Divided Genome

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Received for publication 15 July 1977

Nodamura virus is a small ribovirus containing two RNA molecules. Both RNAs were found to be active messengers for protein synthesis in cell-free extracts prepared from wheat embryo or HeLa cells. RNA 2 directed synthesis of a 43,000-dalton product, p43, whose tryptic fingerprint was similar to that of the major viral coat protein, vp40 (molecular weight, 40,000). Though p43 appears to be a precursor of vp40, processing did not occur in the cell-free extracts. RNA 1 directed synthesis of a 105,000-dalton protein, p105. Its tryptic fingerprint revealed no evidence of coat protein sequences. Hence, the two RNAs represent genes with few, if any, redundant coding sequences.

Nodamura virus is an unusual, mosquito-borne, nonenveloped, RNA-containing virus first detected by its ability to kill inoculated suckling mice. It is named after the village in Japan where it was first isolated (18). An epidemiological survey of that area, based on neutralizing antibody, suggests that the virus multiplies naturally in swine but not in humans (19). It also multiplies in a number of insects. It is without apparent effect in mosquitoes, ticks, and larvae of the moth *Plodia interpunctella* (18) but kills honey bees and wax moth larvae (1). Nodamura virus resembles picornaviruses in morphology, stability at pH 3, and buoyant density in cesium chloride, and its pathology in mice is reminiscent of the group A coxsackieviruses (14). However, unlike picornaviruses, Nodamura virus contains equimolar amounts of two RNA species, RNA 1, a 22S species with a molecular weight of 1.1×10^6, and RNA 2, a 15S species with a molecular weight of 0.46×10^6 (15); both RNAs lack poly(A) (16).

Both RNAs are present in the same virion, both are required for infectivity (J. F. E. Newman and F. Brown, personal communication), and replicative forms of both exist in infected baby hamster kidney cells (15). These facts suggest that the two RNAs have independent genetic functions. The finding reported here, that each of the two RNAs functions as a messenger in cell-free extracts, supports this idea. It also strengthens the case that Nodamura virus is a novel ribovirus, distinctly different from picornaviruses and calicivirus.

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MATERIALS AND METHODS

Propagation and purification of Nodamura virus. Methods used for propagation and purification of Nodamura virus were similar to those described previously (15). The stock virus suspension consisted of a 10% mouse muscle extract (one limb per 10 ml) in 0.05 M phosphate, pH 7.2, made 50% with glycerol and stored at −70°C. Four- to seven-day-old mice (noninbred Swiss albino-ARS/Sprague-Dawley) were inoculated intraperitoneally with 0.05 ml of a 10^{-2} dilution (about 10^3 50% lethal doses). When paralysis became severe, usually after 5 to 6 days, the hindlimb muscles from each mouse were collected and stored (frozen at −70°C).

To obtain radiolabeled virus, each mouse was inoculated with either 25 μCi of L-[*methyl*-^{3}H]methionine (10.5 Ci/mmol) (TRK 209; Amersham/Searle, Arlington Heights, Ill.) or 15 μCi of L-[^{35}S]methionine (640 Ci/mmol) (SJ204; Amersham/Searle) intraperitoneally on days 2, 3, and 4 after infection.

Infected muscle tissue from paralyzed mice was extracted in a mixture of 0.05 M phosphate, pH 7.2, and carbon tetrachloride (2:1, by volume) by homogenization for 1 min in a Sorvall Omni-Mixer at 0°C. The insoluble debris was removed by centrifugation at 12,000 × *g* for 30 min, and virus was then pelleted from the supernatant by centrifugation at 60,000 × *g* for 3.5 h. The pellet was suspended in 0.05 M phosphate, and the insoluble debris was removed at 2,000 × *g*. The supernatant was made 1% with sodium dodecyl sulfate (SDS), and a 2-ml sample was centrifuged for 3.5 h at 60,000 × *g* at 6°C on a 27-ml 15 to 45% sucrose gradient in 0.05 M phosphate, pH 7.2. The gradient was fractionated from the bottom of the tube, and the position of the virus was ascertained by spectrophotometric or radioactivity measurements. Pooled

fractions of purified virus were stored at 4°C. Approximately 500 μg of purified virus was obtained from 40 suckling mice.

Preparation and purification of RNA 1 and RNA 2. RNA was extracted by mixing purified virus with an equal volume of water-saturated phenol containing 1% SDS and shaking the mixture for 15 min at 20°C. The phases were separated by centrifugation, and the aqueous phase was reextracted with fresh phenol-SDS. The RNA was then precipitated from the aqueous phase by the addition of 2 volumes of ethanol at −20°C. After storage overnight at −20°C, the precipitate was collected at 10,000 × *g*, dissolved in 0.1 M acetate (pH 5)-0.1% SDS, and reprecipitated with cold ethanol. After overnight storage at −20°C, the RNA precipitate was collected at 10,000 × *g*, dissolved in acetate-SDS, and centrifuged for 16 h at 40,000 × *g* at 10°C on a 5 to 25% sucrose gradient in 0.1 M acetate-0.1% SDS. The gradient was fractionated from the bottom of the tube, and the positions of the two RNAs were determined by measuring extinctions at 260 nm. The appropriate fractions for the RNA 1 and RNA 2 were pooled and alcohol precipitated as above. In some experiments, the isolated RNAs were further purified by recentrifugation on separate sucrose gradients as described above. The concentration of RNA was measured spectrophotometrically, assuming an absorbance at 260 nm of 22 at a concentration of 1 mg/ml.

RNA used to direct protein synthesis was dissolved at a concentration of 100 μg/ml in 30 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid)-100 mM KCl-6 mM β-mercaptoethanol-2.5 mM magnesium acetate (pH 7.5) and stored at −70°C, or, for short periods, it was stored as an ethanol precipitate at −20°C.

Protein synthesis in wheat embryo extracts. Cell-free extracts of wheat embryo (S23, see below) were prepared from commercial wheat germ (General Mills, Inc., Vallejo, Calif.) by a modification of the procedure of Davies and Kaesberg (3). After flotation and grinding 5 g of dry wheat germ with an equal weight of ground glass, 5 ml of buffer A (95 mM potassium acetate-1 mM magnesium acetate-2 mM calcium acetate) was added to give a smooth paste. Grinding was continued with addition of four more 5-ml amounts of buffer A. The usual addition of DNase was omitted. After centrifugation at 23,000 × *g* for 10 min, the supernatant (S23) was buffered to pH 7.6 with 10 mM Tris-acetate and adjusted to 2 mM magnesium acetate. After an identical spin, the top three-quarters of the supernatant was dialyzed for 24 h, and the resultant S23 (in 10 mM Tris-acetate (pH 7.6)-1 mM magnesium acetate-90 mM potassium acetate-1 mM dithiothreitol) was frozen in small aliquots at −90°C.

In vitro protein synthesis was performed according to Shih and Kaesberg (21). A standard reaction mixture (100 μl) contained 50 μl of wheat embryo extract (S23), 95 mM potassium acetate (except as noted), 5 mM Tris-acetate, 0.5 mM dithiothreitol, 20 mM HEPES (pH 7.6), 2.5 mM ATP, 5 mM magnesium acetate, 0.375 mM GTP, 10 mM creatine phosphate, 4 μg of creatine kinase (EC 2.7.3.2; Worthington Biochemicals Corp., Freehold, N. J.), 0.0375 mM each of the appropriate unlabeled amino acids, and RNA as noted. Acid-insoluble radioactivity was measured on Whatman 3MM filter disks after washing in cold and hot trichloroacetic acid and then in ethanol and ether.

Protein synthesis in HeLa cell extracts. In vitro protein synthesis was carried out essentially as described by Villa-Komaroff et al. (23). About 10^8 H-HeLa cells, grown in suspension (12) and harvested at a density of about 4×10^5 cells per ml, were collected by low-speed centrifugation, washed once with cold phosphate-buffered saline (4) and once with buffer B (120 mM potassium chloride-5 mM magnesium acetate-6 mM mercaptoethanol-20 mM HEPES [pH 7.5]). One volume of packed cells was resuspended in 3 volumes of buffer B at 0°C and immediately disrupted with a tight-glass Dounce homogenizer (no. 885300; Kontes Co., Vineland, N.J.) until 80 to 90% of the cells were broken (about 50 to 70 strokes). Disruption was monitored by light microscopy, using 0.1% nigrosine dye, which stains nuclei but not intact cells. All manipulations were carried out at 0°C. Unbroken cells, nuclei, and other debris were removed by centrifugation at 30,000 × *g* for 20 min. The supernatant (S30) was then incubated with 1 mM ATP-0.2 mM GTP-8 mM creatine phosphate-20 μg of creatine kinase per ml for 30 min at 37°C. The extract, 1.5 ml, was then passed through a Sephadex G-25 column (1 by 20 cm) at 4°C and eluted with 30 mM HEPES, pH 7.5-100 mM KCl-6 mM β-mercaptoethanol-2.5 mM magnesium acetate. The opalescent fractions that emerged at the void volume were pooled, immediately frozen in 0.2-ml portions, and stored at −70°C.

The in vitro protein-synthesizing reaction mixture contained, in a volume of 50 μl: 30 μl of S30 cell extract, 100 mM potassium chloride (except as noted), 2.5 mM magnesium acetate, 30 mM HEPES (pH 7.5), 10 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 5 μg of creatine kinase, 0.2 mM each of 20 amino acids (minus that used for radiolabeling), and RNA as noted. Incorporation into acid-insoluble material was measured as described above.

Electrophoretic analysis. Incorporation mixtures were diluted with an equal volume of distilled water and 2 volumes of 2% SDS-1 M urea-0.2% mercaptoethanol; after heating for 1 min in a boiling-water bath, the samples were dialyzed against 0.1% SDS-0.1% mercaptoethanol-0.5 M urea in 10 mM sodium phosphate, pH 7.2. Dialysis was initially at room temperature until the insoluble precipitate of potassium dodecyl sulfate dissolved (about 2 h), then at 6°C overnight. Dialyzed samples were stored at −70°C.

Sample volumes up to 0.2 or 1 ml were applied, respectively, to tubular gels 6 or 12 mm in diameter. When necessary, volumes were reduced by pressure dialysis in size 8 dialysis membranes (Union Carbide Corp., Chicago, Ill.). Just before electrophoresis, samples were made 1% with respect to SDS and mercaptoethanol and reheated for 2 min in a boiling-water bath. Sucrose was added up to 10%; 50 μg of bromophenol blue was added per ml.

Methods for electrophoresis, fractionation, and counting have been described (7, 12). Gels contained 10% polyacrylamide with 0.3% (vol/vol) ethylene di-

acrylate as cross-linker, 0.1% SDS, 0.5 M urea, and 0.1 M sodium phosphate, pH 7.2. Electrode buffer, 0.1 M sodium 3-mercaptopropionate in 0.1 M sodium phosphate, pH 7.2, was circulated between buffer vessels to neutralize pH changes due to electrode reactions. Electrophoresis, at 8 mA per 6-mm gel (25 mA per 12-mm gel), was continued until the dye marker migrated to the base of the gel (about 20 h for a 20-cm gel).

For molecular weight determination, the sample was mixed with a dialyzed extract of [^{3}H]leucine-labeled rhinovirus 1A-infected HeLa cells; molecular weights were computed from relative electrophoretic mobility on SDS gels (5), using previously assigned values (11) for masses of the rhinovirus proteins.

Tryptic digestion and chromatography of peptides. Radiolabeled proteins, isolated from SDS-polyacrylamide gels, were alkylated with iodoacetamide, digested with trypsin, filtered through a 0.2-μm pore size membrane to remove insoluble material, and chromatographed on a Chromabead P (Technicon Corp., Inc., Tarrytown, N.Y.) column as described (T. Matthews, D. Omilianowski, and R. Rueckert, manuscript in preparation). In each case, recovery of radioactivity was determined after the filtration step by removing duplicate 10-μl amounts; the remaining sample, about 1 ml, was applied to the ion-exchange resin.

Amino acid analysis. Protein was recovered from purified virions by extraction into phenol as described previously (17). The protein was precipitated from the phenol phase (containing 0.1 M ammonium acetate and 1% mercaptoethanol) by adding 6 volumes of ethanol; the precipitate was dissolved in 67% acetic acid and dialyzed for 48 h at 6°C against 0.1% mercaptoethanol, and the single lyophilized sample was divided into three portions and hydrolyzed at 110°C for 28, 50, and 75 h, respectively, in 6 N HCl containing 0.2% phenol to minimize oxidation of tyrosine residues (13). Analysis was carried out on a Durrum D-500 amino acid analyzer. Corrections for loss of threonine and serine were obtained by extrapolation to zero time. The remaining values were averages for duplicate determinations for each hydrolysis period. Isoleucine, leucine, and valine values were already maximal in the first 28-h hydrolysis period.

RESULTS

Translation in wheat embryo extracts. Preliminary experiments showed that maximum amino acid incorporation occurred at 95 mM potassium acetate and 5 mM magnesium acetate. These salt concentrations were used in most experiments.

For RNA 2, incorporation of [^{3}H]- or [^{14}C]-leucine into acid-insoluble material increased linearly with RNA concentration in the range of 0 to 40 μg/ml and then gradually reached a plateau at about 60 μg/ml. Incorporation increased linearly with time for about 1 h and leveled off after 90 min. Typically, incorporation was 10 to 20 times background. With [^{14}C]leucine (specific activity, 330 mCi/mmol), 300 pmol was incorporated per μg of RNA, i.e., about 137 mol of leucine per mol of RNA.

The electrophoretic profile of RNA 2 product exhibited one prominent band (p43), a minor band (p39), and a heterogeneous collection of other minor bands (Fig. 1A). The apparent mass of the major product, 43,000 daltons, is only slightly smaller than the coding capacity (48,500 daltons) of a messenger the size of RNA 2 (molecular weight, 0.46×10^6). The amount of label in peak p39 was rather variable. In different experiments, the label ranged in amount from 5 to 50% of that in p43. Band p39 and the envelope of smaller material moving ahead of it probably represent incomplete chains of p43 (see below).

For RNA 1, incorporation of [^{3}H]- or [^{14}C]-leucine into acid-insoluble material increased linearly with RNA concentration in the range of 20 to 60 μg/ml and then gradually reached a

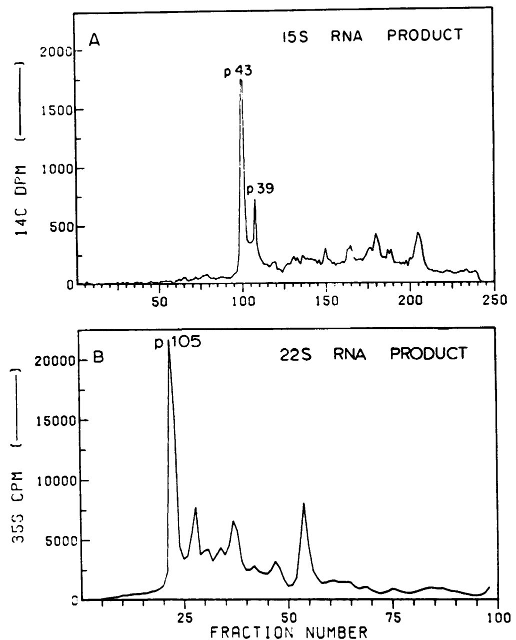

FIG. 1. *Electrophoretic profile of in vitro translation products from Nodamura virus RNA in wheat germ extracts. (A) Product from 1 μg of RNA 2 (15S RNA) and 0.5 μCi of [^{14}C]leucine (specific activity, 330 mCi/mmol) in a final reaction volume of 100 μl; total incorporation was 2×10^5 dpm. Preparation of reaction mixtures and SDS-polyacrylamide gel electrophoresis were as described in the text. Electrophoresis was from left to right. (B) Product from 2 μg of purified RNA 1 (22S RNA) and 25 μCi of [^{35}S]methionine (specific activity, 385 Ci/mmol) in a reaction volume of 100 μl; total incorporation of radioactivity into nondialyzable material was 4×10^6 dpm. Preparation of reaction mixtures was similar to that above except for the use of 120 mM potassium, 2.5 mM magnesium, 800 μM spermidine, and 0.1 mM aurintricarboxylic acid.*

plateau at about 90 μg/ml. Incorporation increased linearly with time for about 1 h and continued to increase for another hour.

Electrophoretic analysis of protein from extracts stimulated by RNA 1 revealed a series of bands of which the most prominent, p105, had an apparent mass of 105,000 daltons relative to rhinoviral marker proteins (Fig. 1B). This value is close to the coding capacity (120,000 daltons) for an mRNA of this size (molecular weight, 1.15×10^6). The proportion of radioactivity in band p105 was larger at RNA concentrations that were low compared with concentrations required for maximum incorporation of [^{14}C]- or [^{3}H]leucine. Moreover, the proportion of p105 was increased by increasing the potassium acetate concentration from 95 to 180 mM after 15 min of incorporation. Addition of spermidine (at zero time) also enhanced the proportion of p105; however, the effects of potassium acetate and spermidine were not additive (data not shown). Since these conditions facilitate chain completion (see below) it seems likely that the bands of apparent molecular weights lower than p105 represent nascent or prematurely terminated chains of p105. A further increase in the proportion of p105 was obtained by the addition of the initiation inhibitor aurintricarboxylic acid after an initial 15 min of incorporation (data not shown).

Translation in HeLa cell extracts. Preliminary experiments showed that maximum amino acid incorporation occurred at about 100 mM potassium chloride and 2.5 mM magnesium acetate. Incorporation increased at a decreasing rate with RNA concentration and reached a plateau at about 20 μg/ml for RNA 2 and 40 μg/ml for RNA 1. Under these conditions, both RNAs stimulated incorporation two- to fourfold over background.

The electrophoretic profile of RNA 2 product showed primarily a single band, p43 (Fig. 2A). There was proportionally less trailing material than was found in the products of synthesis in wheat embryo extracts. Incorporation ceased after about 2 h. Incubation for an additional 2 h revealed no evidence of processing to form smaller proteins.

The electrophoretic profile of the RNA 1 product synthesized during a 2-h incubation period revealed a large peak, p105, with a leading envelope of smaller material. The proportion of p105 was increased when the concentration of potassium chloride was increased to 150 mM after an initial 15 min of incorporation. A typical result is shown in Fig. 2B. The same profile was observed when incubation was continued for an additional 4 h at 30°C; i.e., there was no evidence of processing to form smaller polypeptides (data not shown). These results are consistent with the idea that p105 represents a full-length translation product of RNA 1 and that the smaller material represents incompleted nascent chains.

Thus, a striking difference in translation of RNA 1 between wheat germ embryo and HeLa cell extracts seems to be the ability of the latter to complete nascent chains. (cf. Fig. 1B, 2B.) Although these differences can be minimized by a step-up in potassium acetate concentration or the use of spermidine or aurintricarboxylic acid, the origin of premature termination in the wheat embryo system is not known.

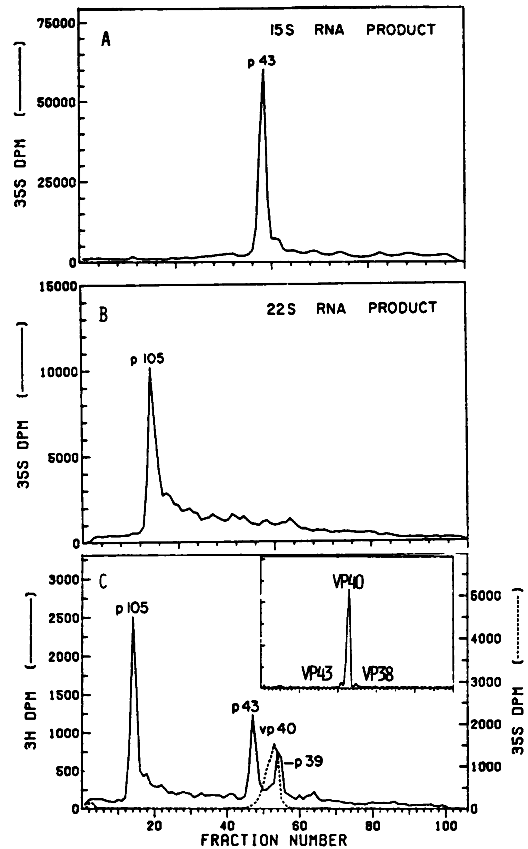

FIG. 2. *In vitro translation products directed by Nodamura virus RNA in HeLa cell extracts. (A) Product from 1 μg of RNA 2 (15S RNA) and 42 μCi of [^{35}S]methionine; total incorporation, 1.3×10^6 dpm. (B) Product from 1 μg of RNA 1 (22S RNA) and 10 μCi of [^{35}S]methionine (specific activity, 545 Ci/mmol) in a reaction volume of 50 μl; total incorporation into nondialyzable material was 6.7×10^6 dpm. (C) [^{3}H]methionine-labeled products from a mixture of RNA 1 and RNA 2 versus [^{35}S]methionine-labeled coat protein; (Inset) electrophoretic profile of [^{3}H]-leucine-labeled Nodamura virus protein, illustrating the presence of minor polypeptides vp43 and vp38 as well as the major protein, vp40.*

Nodamura virions contain a major coat protein, vp40, and two minor proteins, vp43 and vp38 (Fig. 2C, inset). The major protein is unusually rich in alanine residues (Table 1) and poor in histidine residues. The minimum molecular weight of the protein computed from the histidine content was (100/0.54) × 108 = 20,000 daltons. None of the in vitro products p105, p43, or p39 comigrated with the major coat protein from the virions, vp40, on polyacrylamide gels (Fig. 2C). Other experiments indicated that vp43 and p43 comigrated on polyacrylamide gels and might, therefore, be identical but that vp38 and p39 did not comigrate.

Tryptic analysis of RNA 2 products. To further identify the in vitro translation products, methionine-labeled p105 and p43 were isolated by preparative electrophoresis on polyacrylamide gels. Each product was mixed with differentially methionine-labeled coat protein from virions, digested with trypsin, filtered, and chromatographed on the cation exchanger Chromobead P.

The tryptic profile of methionine-labeled p43 from wheat embryo extracts was indistinguishable from that of coat protein (Fig. 3A). Another point of similarity between p43 and coat protein is that both produced a large fraction of insoluble tryptic peptides. In each case, the recovery of radioactivity in the filtered tryptic digest was only 35 to 40% (see legends, Fig. 3 and 4).

In further studies, it was shown that the tryptic patterns of p39 and p43 made in HeLa cell extracts virtually coincided with that of differentially labeled p43 made in wheat germ extracts (data not shown). These results showed that the RNA 2 product synthesized in wheat germ extracts is indistinguishable from that made in HeLa cell extracts, that p39 is probably an incomplete form of p43, and that RNA 2 carries the coat protein gene for Nodamura virus.

Tryptic analysis of the RNA 1 product. In some segmented viruses, the coat gene is repeated in more than one RNA molecule. For example, in brome mosaic virus the coat protein gene is found in RNA 4 (molecular weight, 0.3 × 10^6) and also in the larger RNA 3 (molecular weight, 0.7 × 10^6) (20). To search for evidence of such a repetition of genes in Nodamura virus, the coat protein was mixed with differentially labeled RNA 1 product, p105. The mixture was digested with trypsin, chromatographed on a cation-exchange column, and examined for evidence of coat protein fragments in p105. The profiles revealed no obvious overlap (Fig. 3B). Thus, digests of p105 yielded 12 large peaks, none of which coincided with coat protein peaks.

An important difference not evident from the tryptic profiles was that the bulk of the tryptic peptides from p105 were soluble, whereas some 65% of those from vp40 were not. Thus, the recovery of radioactivity in filtered tryptic digests of p105 in this experiment was 103%; that from coat protein in the same mixture was only 35%.

A similar result was obtained when the p105 profile was compared with that of p43 synthesized in cell-free extracts (Fig. 4). In this case, the chains were labeled with radioactive leucine. Again, the recovery of soluble peptides was high (91%) for p105 and low (40%) for p43. At least four peaks, at fractions 4, 43, 95, and 122, from p43 were lacking in p105. Taking these results together, we conclude that few, if any, coat protein sequences are present in p105.

TABLE 1. *Amino acid composition of Nodamura viral coat protein*

Amino acid	mol/100 mol of amino acid recovered[a]				Residues per chain[b]
	28 h	50 h	75 h	Avg	
Asx	10.1	10.0	10.0	10.2	37
Thr	7.04	6.94	6.79	7.14[c]	26
Ser	6.94	6.71	6.61	7.10[c]	26
Glx	8.96	9.05	9.40	9.08	34
Pro	7.33	7.51	7.34	7.40	27
Gly	6.36	6.30	6.32	6.33	23
Ala	14.6	14.5	14.5	14.5	54
Cys	ND[d]	ND	ND	ND	?
Val	5.75	5.87	5.76	5.80	21
Met	2.51	2.46	2.39	2.47	9
Ile	4.25	4.24	4.16	4.23	16
Leu	6.98	7.01	6.90	6.98	26
Tyr	3.04	3.14	2.92	3.06	11
Phe	4.72	4.77	4.56	4.71	17
His	0.54	0.53	0.55	0.54	2
Lys	3.93	3.94	4.07	3.96	15
Arg	6.95	7.00	7.29	7.04	26
Trp	ND	ND	ND	ND	?

[a] Average, average of five determinations on a single preparation of protein; values for 28- and 50-h hydrolysis periods are the averages of duplicate determinations.

[b] The weighted mass of the average amino acid residue for a protein with the above composition is 108.3 daltons; thus, a protein with a molecular weight of 40,000 would contain about (40,000/108) = 370 residues. From the average the number of serine residues would be (7.10/100) × 370 = 26.2. Other values were calculated in the same way and rounded to the nearest integral value.

[c] Calculated by extrapolation to zero hydrolysis time.

[d] ND, Not done.

DISCUSSION

We have shown that each of the two RNAs in Nodamura virus is an active messenger when added to cell-free extracts of either wheat embryo or HeLa cells. Each directs synthesis of a protein representing about 90% of the theoretical coding information in the RNA (Fig. 5). Thus, RNA 1 generates a protein, p105 (molecular weight, 105,000), whereas RNA 2 produces p43 (molecular weight, 43,000), which we assume to

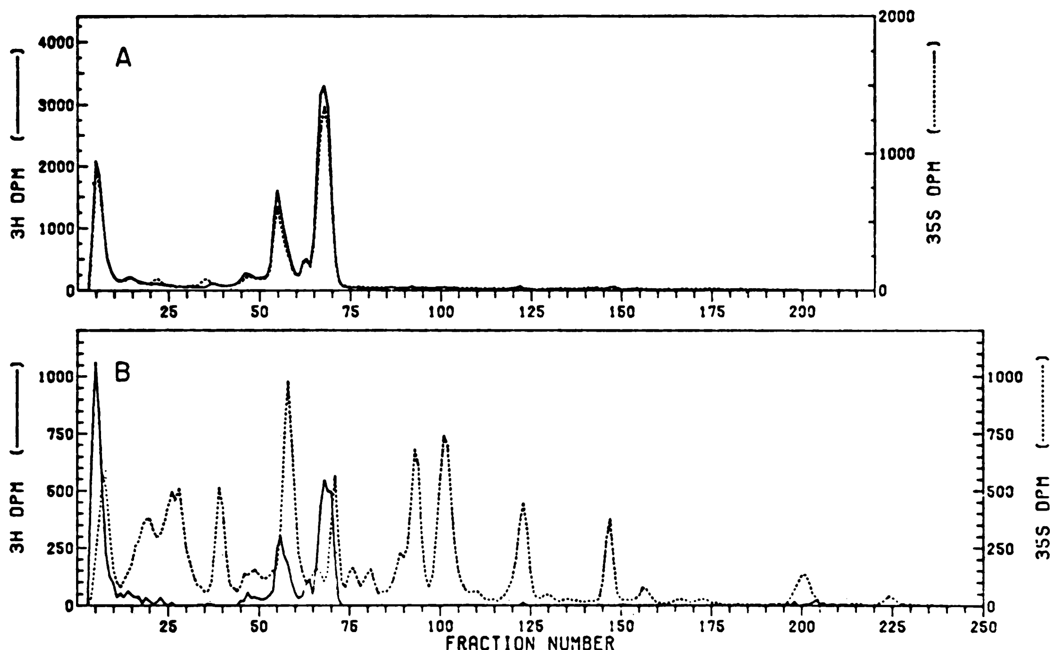

FIG. 3. *Chromatographic profile of tryptic digests from p43 (A) and p105 (B). (A) [^{3}H]methionine-labeled RNA 2 product was synthesized in 100 μl of wheat germ extract, and p43 was purified by gel electrophoresis. Electrophoretically purified p43 (97,000 dpm) was mixed with 60,000 dpm of [^{35}S]methionine-labeled coat protein from Nodamura virus, digested, and filtered to remove insoluble material. The filtrate, containing 44% of the input tritium and 32% of the ^{35}S, was applied to the ion-exchange column for chromatography with a pH gradient. (B) [^{35}S]methionine-labeled RNA 1 product was synthesized in 50 μl of HeLa cell extract; 70,000 dpm of electrophoretically purified p105 was mixed with 22,000 dpm of [^{3}H]methionine-labeled coat protein, digested with trypsin, and chromatographed as described in the text. The filtrate applied to the column contained 103% of the initial ^{35}S and 35% of the initial tritium.*

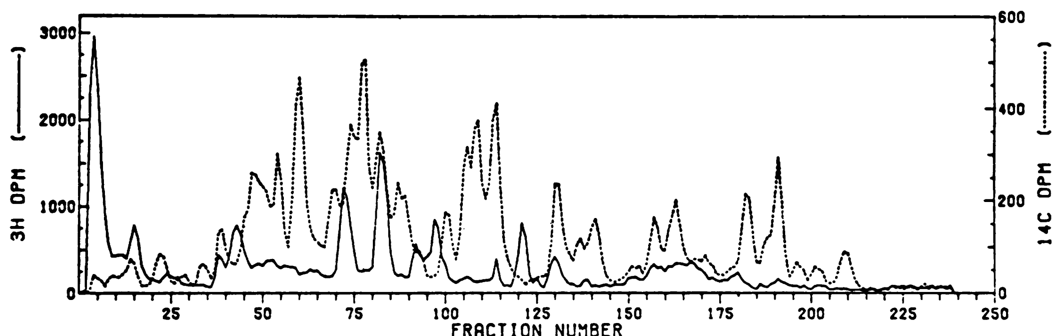

FIG. 4. *Tryptic pattern of p43 (——) and of p105 (-----) made in HeLa cell extracts. A mixture of 185,000 dpm of [^{3}H]leucine-labeled RNA 2 product, p43, and 32,000 dpm of [^{14}C]leucine-labeled RNA 1 product, p105, was digested with trypsin. The digest was filtered as described in the text, and the filtrate, containing 74,000 dpm of tritium and 29,000 dpm of ^{14}C, was chromatographed on an ion-exchange column as described in the text. Both products were synthesized in HeLa cell extracts and purified by preparative SDS gels. Forty percent of the label in p43 and 91% of the label in p105 were recovered as soluble tryptic peptides.*

be a precursor of mature coat protein. The failure of either of the two Nodamura virus RNA products, p43 or p105, to be processed after synthesis in cell-free extracts differs from the behavior of picornavirus proteins, which are reported to form many smaller products when synthesized in cell-free extracts (2, 6, 9, 23).

It would be desirable to document the precursor nature of p43 and to seek evidence of processing of both gene products by studying viral protein made in cells infected in culture. All attempts to produce cytopathic effects with No-

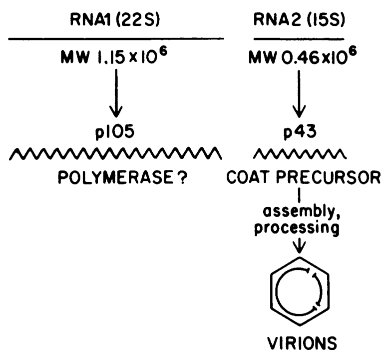

FIG. 5. *Model for synthesis and processing of Nodamura virus protein. The virion contains about 20% RNA (J. F. E. Newman and F. Brown, manuscript in preparation).*

damura virus in a wide variety of cultured vertebrate cells have been negative (14, 19); nevertheless, the virus does multiply in cultured mosquito and baby hamster kidney cells (1). Preliminary attempts in this laboratory to detect synthesis of virus-specific protein by electrophoretic analysis of infected baby hamster kidney and mosquito cells fed radiolabeled amino acids have been unfruitful, since the virus evidently fails to shut off host protein synthesis. It may be possible to overcome this obstacle by immunoprecipitation with neutralizing antiserum against the virion.

Mature Nodamura virions contain, in addition to the major coat protein (vp40), two minor proteins, vp43 and vp38 (Fig. 2C, inset). The observation that p43 and virion protein have virtually identical tryptic profiles (Fig. 3A) indicates that vp40 is a single polypeptide species and not an equimolar mixture of two or three comigrating species, which would be expected of a polio-like virus lacking its smallest chain, vp4. This conclusion erases any residual suspicion that Nodamura virus might actually represent a polio-like virus that had been damaged, for example, by specific nucleolytic cleavage and partial dissociation of specific RNA and protein fragments from the virion during its residence in mouse muscle tissue or during purification.

The identities of the two minor virion proteins are unresolved. They always appear in approximately equimolar proportions (J. Newman, unpublished observations) and might represent some kind of core protein or an enzyme; however, a virion-associated polymerase seems unnecessary because both RNAs are messengers. A more likely explanation is that vp43 represents precursor chain p43, which remained unprocessed during viral morphogenesis; vp38 might then represent a misprocessed precursor. The origin of these minor virion proteins, whether p43, p105, or a host protein, should be easily resolved by tryptic mapping, but the small amount of radiolabeled protein afforded from virions grown in mice has, so far, provided insufficient material for a satisfactory experiment. Each of the minor chains contains about 3% of the leucine in virion protein; assuming 180 chains (15), there are five or six copies of each minor chain per virion.

The function of RNA 1 also remains to be established; an obvious possibility is that it carries a gene for RNA replicase. Optimistic attempts to detect RNA-polymerizing activity in extracts after in vitro protein synthesis were negative; however, such a result was hardly surprising, since interaction with cellular organelles or other missing host components may be needed to generate enzyme activity. Further progress will probably develop through more conventional methods involving extracts of virus-infected cells.

All of the available evidence supports the position that Nodamura virus is the prototype of a new class of nonenveloped riboviruses; a similar virus, isolated from the black beetle, has already been recognized (10). Although their economic importance is not presently known, these viruses are, nevertheless, of interest for several reasons. They have an unusually small genome and are, therefore, favorable for molecular analysis. With only two RNAs, they represent the simplest class of viruses with segmented genomes, and, since both RNAs are packaged in the same virion, Nodamura virus may prove to be of value in studies on mechanisms by which a set of viral RNAs are selected for packaging. They also provide messengers of potential use in dissecting the components of protein-synthesizing machinery. Other viruses with bisegmented genomes include tobacco rattle virus, which has a helical nucleocapsid, and cowpea mosaic virus, an icosahedral virus (for a review, see 8). In each case, the two RNAs are packaged in separate coats. Thus, they differ in this regard from Nodamura virus, which packages both RNAs in a single virion.

ACKNOWLEDGMENTS

This research was supported by grant VC-26 from the American Cancer Society, by Public Health Service (PHS) grants CA 15613, from the National Cancer Institute (NCI), and AI 01466, from the National Institute of Allergy and Infectious Diseases (NIAID), and by Energy Research and Development Administration contract 1633. T.M. was supported by PHS grant T32-CA 09075 from the NCI. R.R. holds a faculty research award (PRA-106) from the American Cancer Society. P.K. holds a PHS research career award (AI 21942) from the NIAID.

We thank Jeffrey W. Davies, who participated in some of our initial experiments.

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