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Transcriptase Activity Associated with Rabies Virion

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Rabies virion-associated transcriptase activity was investigated in vitro and compared with that of the New Jersey serotype of vesicular stomatitis virus. The concentration of detergent that affected [^{3}H]GMP incorporation into acid-insoluble material was significantly different for both viruses. Vesicular stomatitis virus New Jersey required 0.05 to 0.1% nonionic detergent, whereas rabies virion could not be fully activated unless 4 to 5% detergent was used. Other optimal conditions were as follows: 40 mM NaCl, 5 mM Mg^{2+}, 40 mM Tris-hydrochloride (pH 7.4), 5 mM dithiothreitol, and 30°C. The reaction required four nucleoside triphosphates. The initial rate of RNA synthesis by rabies virion enzyme was 140 pmol of GMP incorporated/mg of viral protein per h and linearly increased until about 8 h, with a slight initial lag phase. The enzyme activity that correlated with the content of L protein was highest when rabies virions were grown at 33°C. The product was single-stranded RNA, which was complementary in base sequences to rabies viral RNA. Most of the RNA synthesized sedimented at 6-16S.

Rabies virus is a representative animal virus belonging to the rhabdovirus group (11). The single- and negative-stranded genome RNA with a molecular weight of 4.4×10^6 to 4.6×10^6, synthesized in the cytoplasm, forms a helical structure of nucleocapsid in association with a protein and is covered with the envelope by the process of budding at the cellular membrane (19, 21). It has been proposed that the synthesis of mRNA at the early step of infection (primary transcription) of negative-strand RNA viruses, such as rhabdo-, orthomyxo-, and paramyxoviruses, is carried out by the virion-associated transcriptase (2). This concept has been confirmed for many viruses having a negative-stranded genome RNA (5).

Attempts to demonstrate this enzyme activity in the rabies virion in vitro have apparently been unsuccessful (5, 22, 24). Bishop reported that the highest activity observed incorporated only 5 to 10 pmol of UMP into the product per h per mg of protein (Abstr. Symp. Adv. Rabies Res., Atlanta, p. 9, 1976). This low activity, calculated to be about 1/2,000 that of the Indiana serotype of vesicular stomatitis virus (VSV) (6), seemed to be paradoxical, because a polypeptide molecular weight that corresponds to that of an L polypeptide of VSV required for transcription has been observed in rabies virus preparations analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The present paper is a description of the optimal conditions required to demonstrate rabies virion-associated transcriptase activity in vitro.

MATERIALS AND METHODS

Viruses and cells. The HEP-Flury strain of rabies virus, obtained from A. Kondo (National Institute of Health, Tokyo, Japan), was passaged more than 40 times through BHK-21 cells and cloned five times by the isolation of a large plaque formed on BHK cell plates containing less than two plaques per plate (10), and clone 2026/20 was used throughout this experiment. An egg-adapted strain of VSV New Jersey was obtained from the National Institute of Animal Health (Tokyo, Japan) and cloned twice in our laboratory, and clone NJ3013 was used in this experiment. Both viruses were grown in BHK-21 cells. To prepare the rabies virus inoculum free of defective interfering particles, 50 to 100 whole plaques were isolated, pooled in 10 ml, sonically treated for 15 s with a 40-W Sonifier, and then used as the inoculum. The suspensions usually contained about 10^5 PFU/ml. Both viruses were purified from infected culture fluids by a slight modification of a procedure described elsewhere (10). After culture fluids were clarified by centrifugation at 4,000 rpm for 30 min at 4°C, viruses were precipitated by the polyethylene glycol method (14) and suspended in 24 ml of NTE buffer. This virus suspension was layered on 6 ml of a 20% (wt/vol) sucrose cushion and spun at 64,000 × *g* for 120 min at 4°C. The pellet was resuspended in 2 ml of NTE buffer and sonically treated for 2 min in ice by a 40-W Sonifier (20 kc). The virus suspension was layered on 28 ml of a 10 to 45% (wt/vol) linear sucrose gradient in NTE buffer and then centrifuged at 33,000 × *g* for 90 min at 4°C. The gradient was fractionated into 30 tubes and monitored for optical density at 260 nm. Two or three tubes, which corresponded to the band of infective bullet-shaped (B) particles, were pooled and concentrated by centrifugation at 64,000 × *g* for 120 min at 4°C. The pellet, resuspended in NT buffer at an optical

density of 2.0 at 260 nm, was estimated to contain 250 to 255 μg of protein per ml and was stored at −70°C until use (13). Under these conditions of virus purification, B and defective interfering particles form individual bands at a distance in the sucrose gradient, and both bands could be separately isolated. Electron microscopic studies revealed that more than 90% of the particles in B-particle preparations were bullet shaped (10).

Infectivity assay. Infective rabies virus was titrated by plaque formation in suspended BHK-21 cells (20). VSV New Jersey was titrated by plaque formation on monolayers of BHK cells.

Estimation of protein content. Concentration of protein in purified virus stocks was determined by the method of Lowry et al. (13), using bovine serum albumin as the standard.

PAGE. Slab gel electrophoresis with SDS was performed by using Laemmli's discontinuous buffer system (12). Separating gels were 10% acrylamide containing 0.267% *N,N'*-methylene-bisacrylamide and 0.12% SDS, and stacking gels were 4.5% acrylamide containing 0.12% *N,N'*-methylene-bisacrylamide and 0.1% SDS. Purified virus suspension was dissolved in the same volume of sample buffer, which was composed of 0.125 M Tris-hydrochloride (pH 6.8), 4.6% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.005% bromophenol blue, and was heated at 100°C for 2 min before electrophoresis. Electrophoresis was carried out at a constant current of 4 to 5 mA until the dye front had reached the bottom of the gel; then gels were stained with 0.25% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid for 2 h at 37°C and destained in 50% methanol and 7% acetic acid. The gels were then either dried under vacuum onto Whatman 3MM filter paper or scanned by a microdensitometer (model MKIII, Joyce, Loebl & Co. Ltd., Gateshead, England) before drying.

RNA polymerase assay. The standard 0.25-ml reaction mixture for rabies virus contained, unless otherwise mentioned, 10 μmol of NaCl, 1.25 μmol of Mg^{2+}, 10 μmol of Tris-hydrochloride (pH 7.4 at 30°C), 0.25 μmol of ATP, CTP, and UTP, 0.05 μmol of GTP and 10 μCi of [8-^{3}H]GTP (10.5 Ci/mmol), 1.25 μmol of dithiothreitol (DTT), 12.5 mg of Triton X-100 or Nonidet P-40 (NP-40), and various amounts of rabies virions as indicated in the text. The standard reaction mixture for VSV-associated transcriptase followed the formula of Naito and Ishihama (16). Reaction mixtures were incubated at 30°C for varying periods as described in the text.

Radioactivity incorporated into the acid-insoluble fraction was determined by a modification of the method of Baltimore et al. (3). Reaction mixtures were cooled in an icebox and made up to 5% (wt/vol) with trichloroacetic acid and 100 mM with sodium pyrophosphate, after which 200 μg of yeast RNA was added. After 30 min at 4°C, samples were filtered through a membrane filter (type HA; Millipore Corp.), and each filter was washed in 5% trichloroacetic acid for at least 6 h (the 5% trichloroacetic acid solution was changed every hour) and finally in 99% ethanol for 30 min, after which they were dried. Samples were then assayed for radioactivity in a liquid scintillation spectrometer (Nuclear-Chicago Mark II) at 15 to 20% efficiency, using a toluene scintillator. Background counts were determined, unless otherwise mentioned, for the zero-time samples and usually ranged from 50 to 70 cpm.

Extraction of RNA. The extraction of rabies virion RNA was carried out by the SDS–hot-phenol method (18). Fifteen milliliters of purified virus sample, containing about 3.6 mg of protein in NTE buffer, was mixed with an equal volume of liquified hot-phenol solution (preheated at 65°C) immediately after addition of SDS to a final concentration of 0.5% and was vigorously shaken for 3 min at 65°C. The solution was then chilled, and the aqueous phase was obtained after high-speed centrifugation. The hot-phenol extraction was repeated once. RNA was obtained from the aqueous phase by two cycles of ethanol precipitation and finally dissolved in 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate) to a final concentration of 15 μg/ml.

Annealing experiment of virion polymerase product. ^{3}H-labeled products were prepared as follows. After incubation for 15 h at 30°C, 0.5 ml of reaction mixture was made 0.2% with SDS and 0.1 M with NaCl, and 200 μg of purified yeast RNA was added as a carrier; the fraction containing RNA was then extracted by cold ethanol precipitation. The precipitate was dissolved in 0.3 ml of 2× SSC containing 5 μg of polyvinyl sulfate per ml. Annealing was done as follows. A 0.11-ml amount of ^{3}H-labeled product, 0.25 ml of virion RNA (15 μg/ml in 2× SSC), and 0.04 ml of 1% SDS in 2× SSC were mixed, boiled for 8 min, and then incubated for 2 h at 70°C. The first samples were neither boiled nor annealed, the second samples were boiled but quickly chilled, the third samples were annealed without addition of excess virion RNA, and the fourth samples were done in the complete system, that is, annealed with excess rabies RNA (Table 4). After being chilled, the samples were divided into two equal portions, one of which was treated with 25 U of RNases A and T_1 per ml for 30 min at 25°C. Samples were then chilled and precipitated with cold trichloroacetic acid, and acid-insoluble radioactivity was determined as described above.

Velocity sedimentation analysis of product RNA. ^{3}H-labeled product was extracted from 0.75 ml of reaction mixture, after the addition of 123 μg of rRNA from BHK cells as a carrier, by cold ethanol precipitation and were dissolved in 0.5 ml of SDS-NAE buffer. The solution was boiled for 4 min, layered on 4.5 ml of a 5 to 20% (wt/vol) sucrose gradient in SDS-NAE buffer, and centrifuged at 130,000 × *g* for 4 h at 22°C, using an RPS-40 rotor (Hitachi Co. Ltd., Tokyo). Extractions of the gradient were monitored for optical density at 260 nm, and then each fraction was divided into two equal portions, one of which was treated with RNase as described above at 30°C. All samples were then precipitated with cold trichloroacetic acid, and acid-insoluble radioactivity was assayed.

Chemicals and buffers. Phosphate-buffered saline contained: NaCl, 0.137 M; KCl, 0.0027 M; NaH_2PO_4, 0.008 M; KH_2PO_4, 0.0015 M; $CaCl_2$, 0.009 M; $MgCl_2$, 0.005 M; pH 7.4 at 25°C. NT buffer contained: NaCl, 0.13 M; Tris-hydrochloride, 0.05 M; pH 7.4 at 25°C. NTE buffer contained: NT buffer plus

0.001 M EDTA. NAE buffer contained: NaCl, 0.05 M; sodium acetate, 0.01 M; EDTA, 0.001 M; pH 5.1. SDS-NAE buffer contained: NAE buffer plus 0.2% SDS.

[8-^{3}H]GTP (10 to 11 Ci/mmol) and [8-^{3}H]ATP (29 Ci/mmol) were products of the Radiochemical Centre, Ltd., Amersham, England. ATP, CTP, GTP, UTP, rifampin, α-amanitin, and DNase I (200 U/mg) were purchased from Boehringer-Mannheim-Yamanouchi Co. Ltd., Tokyo, Japan. RNase T_1 (5×10^5 U/mg) was from Sankyo Co. Ltd., Tokyo, Japan. RNase A (3,233 U/mg) was from Worthington Biochemicals Corp., Freehold, N.J. Triton X-100 was from Nakarai Chemical Co., Kyoto, Japan. NP-40 was from BDH Chemicals Ltd., Poole, England. These commercial products were all used without further purification.

RESULTS

Incorporation of ribonucleoside monophosphate by rabies virion. The activity of the rabies virion for incorporation of [^{3}H]GMP was measured by the same procedure as that applied to VSV New Jersey (16). No significant activity could be observed (Table 1, experiment A). To determine whether such negative results under 1/1,000 of VSV New Jersey might be due simply to inappropriate conditions for the rabies virion, the amount of virions was increased to more than 50 μg of protein and the specific activity of ^{3}H-labeled nucleotide was increased to 2,000 to 2,500 mCi/mol. Incubation time was also prolonged to 12 to 15 h. Even with such a simple device, the count of radioactivity incorporated into the acid-insoluble materials was 500 to 1,000 cpm. These were significantly over the background counts, which usually ranged from 50 to 70 cpm, though far below that of VSV New Jersey (Table 1, experiment B).

Optimal conditions for incorporation of [^{3}H]GMP by the rabies virion. The optimal concentration of NaCl was 40 mM, whereas that of VSV New Jersey was 70 mM (16). Optimal pH was tested by using Tris-hydrochloride-buffered solution in the range of pH 6.8 to 8.2, which was determined at the working temperature and the concentration utilized in the experiment, and optimal activity was obtained at pH 7.4 (data not shown). At various concentrations of Tris-hydrochloride (pH 7.4), the maximal activity was obtained at around 40 mM, and higher concentrations of Tris-hydrochloride were inhibitory. Testing of other Good buffer systems [BES: *N,N'*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; HEPES: *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; TAPS: *N*-tris-methyl-3-aminopropanesulfonic acid; TES: *N*-tris-methyl-2-aminoethanesulfonic acid; Tricine: tris-methyl-glycine] at a concentration of 40 mM (pH 7.4) revealed the effectiveness to be in the order of Tris-hydrochloride, BES, Tricine, TES, TAPS; however, HEPES was most inhibitory. Therefore, Tris-hydrochloride was used throughout this experiment.

To determine the optimal concentration of ribonucleoside triphosphates, the following experiments were performed. (i) Reaction mixtures were prepared to include various concentations but the same molar ratios of four nucleotides. Maximal activity required to incorporate GMP into acid-insoluble material was obtained when the nucleotide concentration was about 1 mM. (ii) Reaction mixtures were prepared to

TABLE 1. *Comparisons of [^{3}H]GMP-incorporating activities of rabies virus and VSV New Jersey under various conditions*

Expt	Virus	Amt of viral protein (μg)	Assay conditions[a]			[^{3}H]GMP incorporated	
			Detergent	Sp act of [^{3}H]GMP (mCi/mmol)	Incubation time (h)	cpm	pmol/mg of protein per h
A	Rabies	13.2	Triton X-100 (0.1%)	133	5	30[b]	
	VSV	6.6	Triton X-100 (0.1%)	133	5	12,010	8,290
B	Rabies	62	Triton X-100 (1%)	2,000	12	543	1.1
	VSV	18	Triton X-100 (1%)	2,000	12	277,500	1,947
C	Rabies	20	NP-40 (4%)	200	5	947	140
D	VSV	16.5	Triton X-100 (0.2%)	200	3	101,900	23,400

[a] Assay conditions for [^{3}H]GMP-incorporating activity of experiments A, B, and D were the same as those described for virion transcriptase of VSV, New Jersey serotype (16) except for the modifications indicated in this table. Namely, the reaction mixture contained 70 mM NaCl, 100 mM Tris-hydrochloride (pH 8.0), 5 mM Mg^{2+}, 5 mM DTT, 0.4 mM (A and B) or 1.0 mM (D) ATP, CTP, and UTP, 0.04 mM (A and B) or 0.2 mM (D) GTP, and others as indicated. Assay conditions for experiment C with rabies virus were as described in the text. Reactions were carried out at 30°C, and the acid-insoluble radioactivity was determined.

[b] Background counts determined for the zero-time samples were 94 cpm for experiment A, 70 cpm for B, 65 cpm for C, and 40 cpm for D, and were subtracted.

include various concentrations of cold GTP but a constant concentration of ATP, CTP, and UTP (1 mM) and ^{3}H-labeled GTP (10 μCi per each reaction mixture). As the concentration of cold GTP increased, the radioactivity incorporated into the acid-insoluble material first increased and attained a maximal level when the cold GTP was 0.1 to 0.2 mM. When the cold GTP was added at higher concentrations, the incorporation of radioactivity decreased considerably, although the total GMP incorporated gradually increased. Thus, when the standard reaction mixture contained 0.2 mM GTP and 1 mM ATP, CTP, and UTP, the maximal incorporation of radioactivity could be obtained, and the total GMP incorporation was about two-thirds that obtained when cold GTP was added at 1 mM.

Requirement of the metal ion was investigated in comparison with that of VSV New Jersey. The activity at various concentrations of Mg^{2+} reached the plateau level at around 5 mM. Similar results were also obtained for VSV New Jersey.

The activity of rabies virion was strictly dependent on the presence of DTT in the reaction mixture. Less than 10% of maximal activity was obtained in the absence of DTT. The optimal concentration was 5 mM, but concentrations higher than 10 mM were inhibitory (data not shown). A similar dependence on the sulfhydryl agent was observed with VSV New Jersey, and optimal activity was obtained at 5 mM DTT (S. Naito, personal communication).

The most conspicuous difference was found in the optimal concentration of nonionic detergent. In the case of VSV New Jersey, the transcriptase activity was fully activated at 0.05% Triton X-100, whereas the rabies virion required 4 to 5% for maximum activity of [^{3}H]GMP incorporation (Fig. 1). A similar high concentration was required when a different detergent, NP-40, was used. An anionic detergent such as sodium deoxycholate was inhibitory when added to the reaction mixture at 0.125% (Table 2).

Utilizing these optimal conditions, the specific activity of GMP incorporation by rabies virion increased to 140 pmol (Table 1, experiment C).

Identification of the active entity that catalyzes the incorporation of [^{3}H]GMP. Table 3 shows that the activity of [^{3}H]GMP incorporation by rabies virion requires four nucleoside triphosphates. The activity observed in the reaction mixture lacking UTP was about half that in the complete system and less than 1% when lacking ATP. In the case of VSV New Jersey, the omission of UTP and ATP resulted in activity of 12 and 7.8% that of the control, respectively. Similar results were obtained when

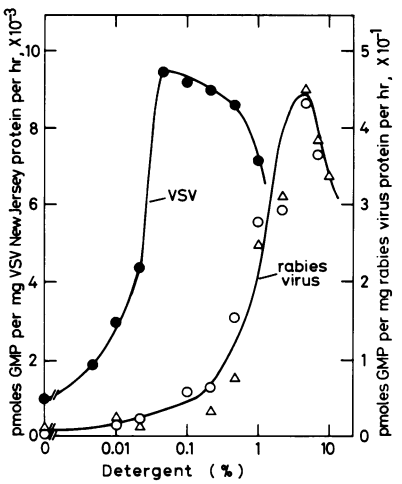

FIG. 1. *Effect of detergent concentration on [^{3}H]GMP-incorporating activity by rabies virus and VSV New Jersey. The reaction mixture for rabies virion was prepared as described in the text and contained the indicated concentrations of nonionic detergent Triton X-100* (○) *or NP-40* (△) *and 16.5 μg of rabies virus protein. The reaction mixture for VSV New Jersey contained 17.5 μmol of NaCl, 25 μmol of Tris-hydrochloride (pH 8.0), 1.25 μmol of Mg^{2+}, 1.25 μmol of DTT, 0.1 μmol of ATP, CTP, and UTP, 0.015 μmol of GTP, 2.0 μCi of [8-^{3}H]GTP (10.5 Ci/mmol), 25 μg of VSV New Jersey protein, and the indicated concentrations of Triton X-100* (●). *After incubation for 10 (rabies virus) or 3 (VSV) h at 30°C, acid-insoluble radioactivity was determined and calculated as shown.*

[8-^{3}H]ATP was used instead of [8-^{3}H]GTP; that is, the residual activity of [^{3}H]AMP incorporation was high in the case of UTP omission. Varieties of residual RNA polymerase activity when each nucleotide was omitted were also reported in many paramyxovirus preparations (4, 17, 23). A plausible explanation is that each nucleotide may be contaminated with others. However, supportive data have not been obtained. The dose response curve in Fig. 2 shows that GMP incorporation is in linear proportion to the amount of virions in the reaction mixture, as tested from 5 to 100 μg of protein. Thus, there appeared to be no inhibitor or cooperative factor that could act at high concentrations. Sequentially, the incorporation commenced with no or only a very short lag phase at the initial step of incubation and linearly increased up to 7 to 8 h (Fig. 3). The maximum activity was observed when the reaction was carried out at around 30°C. The reaction was sensitive to RNase, but resistant to DNase, actinomycin D, rifampin, α-amanitin, and gentamicin (Table 2). The results indicated that the activity was not brought about by DNA-dependent RNA polym-

TABLE 2. *Properties of [^{3}H]GMP-incorporating activity of rabies virion*

Expt	Reaction mixture[a]	pmol of GMP incorporated/mg of protein	Ratio (%)
1	Complete	34.1	100
	+Actinomycin D, 2 μg/ml	29.7	87.1
	+Actinomycin D, 20 μg/ml	28.8	84.5
	+DNase I, 20 μg/ml	37.0	108
	+DNase I, 100 μg/ml	33.8	99.1
	+RNase A, 50 μg/ml	0.92	2.7
	−Virion	<0.44	<1.3
2	Complete	23.4	100
	+Rifampin, 20 μg/ml	21.3	90.9
	+α-Amanitin, 2 μg/ml	23.1	98.8
	+Gentamicin, 100 μg/ml	23.1	98.8
	−Triton X-100	3.5	14.8
	−Triton X-100 + DOC (0.125%)	0.59	2.5
	+DOC (0.125%)	3.2	13.6

[a] The reaction mixture was composed of 40 (experiment 1) or 70 (experiment 2) mM NaCl, 100 mM Tris-hydrochloride (pH 7.4), 5 mM Mg^{2+}, 0.4 mM ATP, CTP, and UTP, 0.04 mM GTP, 25 μCi of [8-^{3}H]GTP (10.5 Ci/mmol), 5 mM DTT, 1% Triton X-100, and 21 μg of viral protein. The reaction was carried out at 30°C for 12 (experiment 1) and 10 (experiment 2) h. Acid-insoluble radioactivity was determined as described in the text and calculated in terms of picomoles of GMP incorporated per milligram of viral protein. DOC, Deoxycholate.

erase derived from host cells or other contaminated microorganisms, which could multiply in the presence of a high concentration of detergent.

To determine whether or not the enzyme that catalyzes [^{3}H]GMP incorporation was associated with the rabies virion, purified virions were layered on a 10 to 45% (wt/vol) sucrose gradient and centrifuged as described in Materials and Methods. Each fraction obtained from the gradient was assayed for infectivity, [^{3}H]GMP-incorporating activity, and protein content. Both these activities corresponded exactly to the virus band (Fig. 4). The results and the requirement of detergent for the reaction suggest that the enzyme concerned with [^{3}H]GMP-incorporating activity is located in the core of the rabies virion.

Analysis of the product. To examine the nature of the acid-insoluble material, the ^{3}H-labeled product was extracted by cold ethanol precipitation after 15 h of incubation. The extracts were examined for sensitivity to RNase and complementarity to the viral genomes. Table 4 shows that a large portion of product was sensitive to RNase but completely resistant to RNase when annealed with the addition of excess viral RNA. Annealing experiments revealed that a large portion of the product self-annealed, and this was interpreted as follows: the amount of ^{3}H-labeled complementary RNA product was relatively small, whereas the amount of template viral RNA in the reaction mixture was so large that the product could hybridize without addition of viral RNA.

Analysis of virion protein by SDS-PAGE. The specific activity of rabies virion-associated transcriptase varied greatly depending on virus stocks of the same strain. The activity was relatively high in stocks in which viruses had been replicated at the lower temperatures of 33 to 33.5°C, but no significant difference was found in thermal inactivation of [^{3}H]GMP-incorporating activity by the purified virion at between 33 and 37°C (data not shown). Furthermore, viruses that were propagated as the first passage of plaque isolates showed higher activity than those that replicated after passages through BHK cells. Although defective interfering particles were produced after several passages, purified B-particle preparations, which were practically free from defective interfering particles, could be obtained by velocity sedimentation centrifugation in the sucrose density gradient, as described in Materials and Methods. In addition, no inhibitory effect was observed on the transcriptase activity of rabies virion when defective interfering particles were added to the reaction mixture (unpublished observations). Thus, it was assumed that a quantitative or qualitative

TABLE 3. *Properties of [^{3}H]GMP-incorporating activity of rabies and VS virions*

Reaction mixture[a]	[^{3}H]GMP incorporated[b]			
	Rabies virus		VSV New Jersey	
	pmol/mg of protein per h	Ratio (%)	pmol/mg of protein per h	Ratio (%)
Complete	80.8	100	23,400	100
−ATP	<0.8	<1	1,830	7.8
−CTP	0.68	0.85	170	0.72
−UTP	37.8	46.7	2,800	12
−ATP, CTP, UTP	<0.8	<1	190	0.83

[a] The complete reaction mixture (0.25 ml) for rabies virion contained 40 mM NaCl, 40 mM Tris-hydrochloride (pH 7.4), 5 mM Mg^{2+}, 5 mM DTT, 1.0 mM ATP, CTP, and UTP, 0.2 mM GTP, 10 μCi of [8-^{3}H]GTP (10.5 Ci/mmol), 4% Triton X-100, and 16.5 μg of viral protein. The complete reaction mixture for VSV New Jersey was as follows: 70 mM NaCl, 100 mM Tris-hydrochloride (pH 8.0), 5 mM Mg^{2+}, 5 mM DTT, 1.0 mM ATP, CTP, and UTP, 0.2 mM GTP and 10 μCi of [^{3}H]GTP, 0.2% Triton X-100, and 16.5 μg of VSV New Jersey protein. The same nucleoside triphosphate solutions were used to prepare the reaction mixture for both viruses.

[b] The reaction was carried out for 5 (rabies virus) and 3 (VSV) h at 30°C, and [^{3}H]GMP incorporated into acid-insoluble material was determined and calculated in terms of picomoles of GMP incorporated per milligram of viral protein per hour.

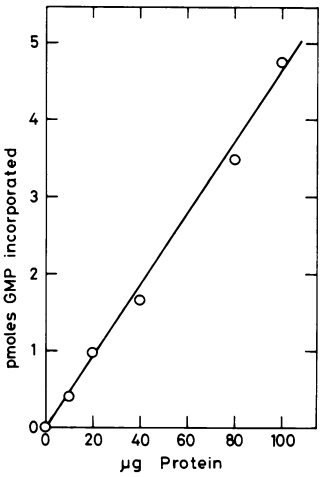

FIG. 2. *Incorporation of [³H]GMP by different amounts of rabies virions. Reaction conditions were as described in Table 2 (experiment 1), except for the amount of virion. The incorporation of [³H]GMP was calculated and expressed in terms of picomoles of GMP incorporated per 10 h.*

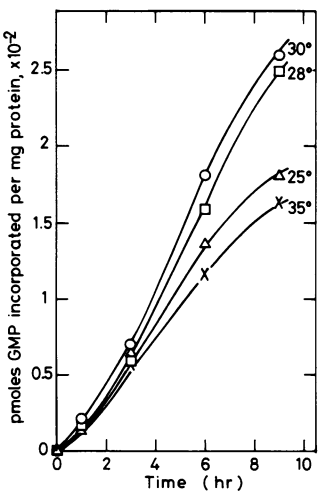

FIG. 3. *[³H]GMP-incorporating activity of rabies virions for different times and at different temperatures of incubation. Standard reaction mixtures, which contained 19 μg of rabies virion protein, were incubated at 25 (△), 28 (□), 30 (○), and 35°C (×), and at indicated times samples were removed and assayed for acid-insoluble radioactivity. The incorporation of [³H]GMP was calculated as shown.*

change might take place in the RNA polymerase of the virion grown under different conditions, such as different temperatures and viral passage levels.

Figure 5 shows the comparison of polypeptide composition of rabies virus and VSV New Jersey. In addition to four major proteins (G, N, M1, and M2), rabies virus contains several minor components, such as P40 (molecular weight, 40,000), P43 (molecular weight, 43,000), and L protein, a large-molecular-weight polypeptide that corresponds exactly to that of VSV New Jersey (25). It was noted that the ratio of four major proteins was constant but amounts of minor components varied greatly. The relative ratio of these polypeptides was examined for several virus stocks, which were propagated under different conditions by a microdensitometer and compared with the RNA polymerase activity (Table 5). The results indicate that the polymerase activity was in a fairly linear proportion to the amount of L protein. On the other hand, no significant difference was found in the contents of four major proteins. Concerning other minor components, no clear correlation was detectable. Thus, it was assumed that there was a parallel relationship between the activity of virion polymerase and the content of L pro-

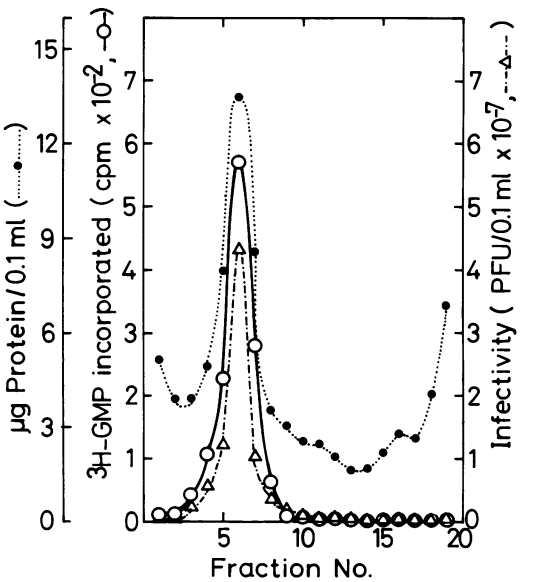

FIG. 4. *Location of [³H]GMP-incorporating activity of rabies virion by velocity sedimentation on a sucrose density gradient. Purified rabies virions were loaded on a 10 to 45% (wt/vol) sucrose gradient and centrifuged at 65,000 × g for 90 min in an RPS-40 rotor (Hitachi Co. Ltd., Tokyo). Nineteen fractions obtained were submitted to the determinations of protein content, infectivity, and activity of [³H]GMP incorporation. Protein content (●) was determined by the method of Lowry et al. (13), infectivity (△) was determined by plaque formation as described in the text, and [³H]GMP-incorporating activity (○) was assayed under the reaction conditions described in the text for 12 h at 30°C.*

TABLE 4. *Annealing experiment with ^{3}H-labeled products of rabies virion*[a]

Treatment	Annealed with	Acid-insoluble cpm		RNase resistance (%)
		RNase(−)	RNase(+)	
None	None	703[b]	102[b]	14.5
Boiled, quickly chilled	None	683	121	17.7
Boiled, self-annealed	Endogenous RNA	690	530	76.8
Boiled, annealed	Excess rabies virion RNA	680	688	100

[a] ^{3}H-labeled products were prepared by the procedures described in the text, using 140 μg of rabies virus protein. Annealing procedures were also as described in the text.

[b] Background counts (20 cpm) were subtracted.

tein, which had the same function as an intrinsic subunit of VSV polymerase.

Analysis of the low specific activity of rabies virion polymerase. The highest activity of rabies virion RNA polymerase remained at approximately 1/100 to 1/200 of that of VSV New Jersey (Table 1). Three possibilities were proposed to explain this: (i) rapid digestion of product RNA into acid-soluble material by RNase, which could not be eliminated during virus purification; (ii) very low speed of chain elongation of RNA synthesis; and (iii) very low frequency of RNA synthesis initiation.

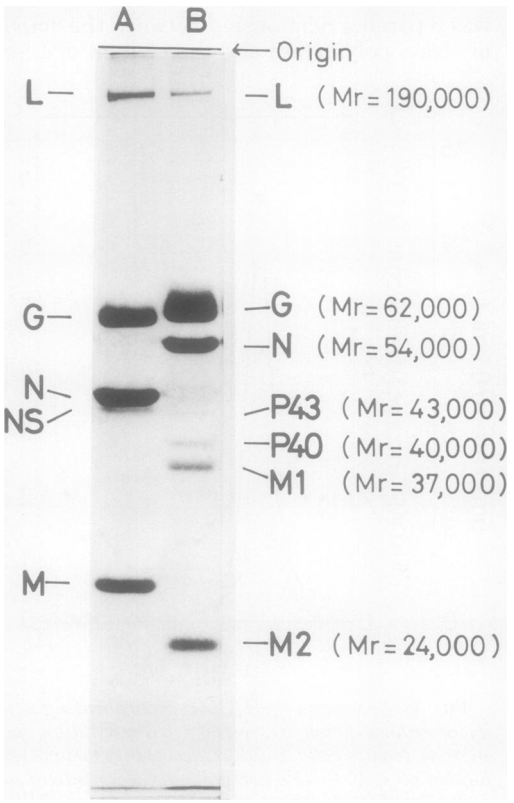

FIG. 5. *Electrophoretic pattern of virion polypeptides of rabies virus and VSV. The SDS-solubilized 16.5 μg of viral proteins in 50 μl was separated by PAGE. (A) VSV New Jersey; (B) rabies virus. The molecular weight of each virion polypeptide was estimated by comparison with several co-electrophoresed marker proteins, such as β-galactosidase (13,500), bovine serum albumin (67,000), ovalbumin (43,000), and chymotrypsinogen (25,000).*

Detection of RNase activity in purified virions was carried out with VSV New Jersey, because a large amount of ^{3}H-labeled complementary RNA could be easily obtained by VSV transcriptase in vitro. This ^{3}H-labeled complementary RNA was mixed with various amounts of purified rabies virions and incubated for 30 min at 30°C in the presence of Triton X-100. Activity to convert ^{3}H-labeled complementary RNA into acid-soluble material was not observed. Support was also obtained in another experiment in which the velocity sedimentation pattern of product RNA was analyzed. The ^{3}H-labeled product from a 14.5-h enzyme reaction was characterized by 5 to 20% (wt/vol) sucrose gradient centrifugation. Each fraction of the gradient was divided into two equal portions, one of which was treated with RNase, as described in Materials and Methods. Results shown in Fig. 6 indicate that large-sized RNA was clearly evident and only a small amount of small-sized RNA had accumulated. Main peaks were present from 6 to 16S, and a faint component sedimented faster than 20S. A small amount of an RNase-resistant fraction was found in the 3–4S peak. The sedimentation pattern of product RNA from a 5-h incubation was quite similar to that seen in Fig. 6. These results indicate that it is unlikely that product RNA was rapidly digested by RNase activity present in the rabies virion preparation. The other two possibilities are now being quantitatively examined.

DISCUSSION

Virion-bound transcriptase activity was demonstrated herein in rabies virions and was 100- to 200-fold lower than that of VSV New Jersey. The activity was ranked at the same level as that seen in Newcastle disease virus, Sendai

TABLE 5. *Comparison of polymerase activity and polypeptide composition of rabies virions produced under various conditions*

Conditions for virus production[a]		Polymerase activity (pmol of GMP/mg of protein per h)[b]	Relative content of virion polypeptide[c]					
Passage no.	Temp (°C)		L	G	N	P40	M1	M2
1	33	140	3.5	41.2	33.1	3.1	6.8	11.8
1	33	125	2.8	37.4	36.2	2.4	5.6	13.5
1	37	79	2.0	48.7	30.6	2.2	5.7	10.4
2	37	46	1.3	37.3	34.8	2.9	6.7	13.2
5	37	16	0.8	49.0	31.4	1.4	5.2	11.3

[a] BHK cells were infected with plaque isolates prepared as described in the text (passage 1) or undiluted culture fluids of infected BHK monolayers (passages 2 and 5).

[b] Polymerase activity was assayed with the standard reaction mixture, as described in the text.

[c] Relative contents of each polypeptide were determined from the electrophoretic pattern of SDS-PAGE by scanning with a microdensitometer (model MKIII, Joyce, Loebl & Co. Ltd.) and weighing each band cut out from tracing papers. Percentages of four major and two minor components are listed; when contents of several minor components were less than 1%, they were omitted from the table.

virus, and mumps virus (4, 9, 17). At the present time, we are not officially permitted to use any strain of VSV Indiana in laboratories for biological research in Japan. For this reason, VSV Indiana, the prototype of VSV, could not be used as the reference virus. The specific enzyme activity of VSV New Jersey, which was used in this experiment, was 23,400 pmol of GMP incorporated/mg of viral protein per h, and this value was considerably higher than that reported for the same serotype of VSV, but it does correspond to that of VSV Indiana (6). The specific activity of virion transcriptase of the HEP-Flury strain of rabies virus was thus estimated to be around 1/150 that of VSV Indiana.

Based on data that (i) [^{3}H]GMP-incorporating activity was associated with the viral core, (ii) the product was sensitive to RNase, and (iii) the product RNA contained sequences complementary to genome RNA of the rabies virus, it was concluded that the rabies virus contained transcriptase.

In a search for the optimal detergent and divalent cation concentrations, ionic strength, pH, and temperature, certain differences between the rabies virus and VSV New Jersey were evident. The concentration of nonionic detergent required was much higher for rabies virus than for VSV. At higher concentrations, the enzyme reaction showed a sharp decrease, presumably due to the viscous condition of the reaction mixture. This raised the question of whether the disruption of virions was insufficient or the viral envelope inhibited the enzyme reaction, as reported with Sendai virus (14). Analysis by SDS-PAGE of virions treated with various concentrations of NP-40 in conditions similar to those used for the RNA polymerase assay showed that a considerable amount of envelope proteins (G, M1, and M2) remained bound in virions treated even with a concentration as high as 5%, and no significant difference was observed in the grade of removal of envelope proteins

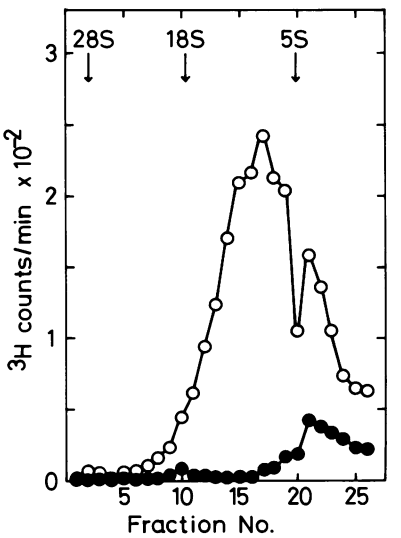

FIG. 6. *Velocity sedimentation analysis of ^{3}H-labeled products. The reaction mixture, which contained 100 μg of rabies virus protein, was incubated for 14.5 h at 30°C, and ^{3}H-labeled product RNA was extracted. The precipitate was resolved in SDS-NAE buffer, boiled for 4 min, loaded on a 5 to 20% sucrose density gradient, and centrifuged at 130,000 × g for 4 h at 22°C. Each of 26 fractions obtained from the centrifuged gradient was divided into two equal portions, one of which was treated with 25 U of RNases A and T_1 per ml for 30 min at 30°C, and then acid-insoluble radioactivity for all fractions was determined. Symbols:* (○) *untreated;* (●) *RNase treated.*

between the treatment with 0.2 and 5% NP-40 (Fig. 7C, D, and E). In another case, even though two-thirds of the envelope proteins were removed and most of the N and L proteins were left behind (Fig. 7B), the polymerase activity was not stimulated, but rather this preparation had one-third the polymerase activity of unfractionated disrupted virions (data not shown). Thus, the inhibitory effect of envelope proteins was not demonstrated, and a reconstitution experiment should be done to fully rule out this possibility. This type of experiment has heretofore not been feasible, since a complete removal of the envelope proteins from rabies virions by treatment with nonionic detergent while preserving the original level of RNA polymerase activity has not been successful. Furthermore, 5% deoxycholate treatment almost completely removed the envelope proteins, but neither kept back L protein nor preserved RNA polymerase activity (Fig. 7A). Therefore, the requirement of a high concentration of a detergent is not fully understood. Concerning this point, an experiment concerning primary transcription when virus-infected cells have been pretreated with actinomycin D and cycloheximide (5, 24) is of interest. These workers showed that purified rabies virion could carry out primary transcription in infected BHK cells, even though the activity was low. A certain intracellular process of infection in the early stage may provide a favorable condition that is lacking in the in vitro transcriptase assay.

The RNA polymerase activity of rabies virus and VSV New Jersey was strictly dependent on the presence of DTT in the reaction mixture. This strict dependence on a sulfhydryl compound is in contrast with findings with VSV Indiana but corresponds to findings with Kern Canyon virus (1). The enzyme of the latter was also essentially inactive when assayed in a reaction mixture lacking a sulfhydryl agent, and DTT was most effective.

Maximal yield of released viruses was obtained at 33°C in BHK cells infected with our HEP-Flury strain and other fixed viruses (7). A similar temperature dependence was also evident in the RNA polymerase activity of rabies viruses grown at 33 and 37°C. Although the reason for such correlation is unknown, the enzyme activity was in parallel with the amount of L protein obtained by SDS-PAGE. Although it has not been established that L protein of the rabies virion is the transcriptase, nevertheless it is possible that L protein may be the main intrinsic subunit, such as is seen in VSV transcriptase (8, 16). Our findings herein show that the rabies virion contains RNA polymerase, even though the amount is low compared with

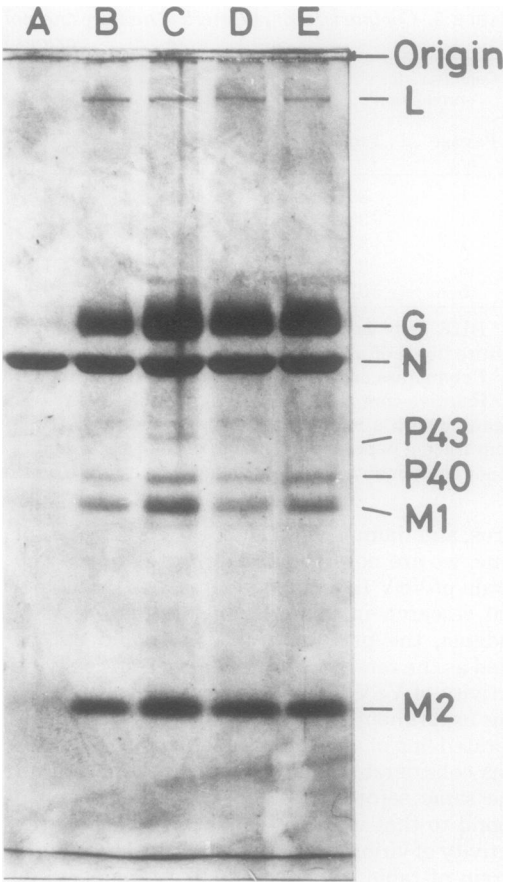

FIG. 7. *SDS-PAGE of polypeptides of rabies virion treated with detergents under various conditions. The same lot of purified rabies virus samples was treated with: (A) 5% deoxycholate (viral protein, 100 μg/ml) in the buffer composed of 40 mM NaCl, 5 mM Mg^{2+}, and 40 mM Tris-hydrochloride (pH 7.4) for 15 min at 20°C; (B) 5% NP-40 (viral protein, 40 μg/ml) in the buffer composed of 16 mM NaCl, 5 mM DTT, 40 mM Tris-hydrochloride (pH 7.4), and 2 mM EDTA for 30 min at 20°C; (C [control], D, and E) 0, 0.2, and 5% NP-40 (viral protein, 100 μg/ml) in the buffer composed of 40 mM NaCl, 5 mM Mg^{2+}, 1 mM ATP, CTP, and UTP, 0.2 mM GTP, 5 mM DTT, and 40 mM Tris-hydrochloride (pH 7.4) for 30 min at 30°C. Each detergent-treated virus sample was layered on a cushion of 1 ml of 15% sucrose solution containing 5 mM DTT and 40 mM Tris-hydrochloride (pH 7.4) and centrifuged at 130,000 × g for 120 min at 4°C. The pellet was resuspended in the original volume of NT buffer containing 5 mM DTT and then analyzed by SDS-PAGE and polymerase assay. The relative content of each viral protein was estimated from the electrophoretic pattern with a microdensitometer, as described in Table 5. Polymerase activity was assayed under the standard conditions described in the text. After incubation for 5 h at 30°C, acid-insoluble radioactivity was determined.*

that of another member of the rhabdovirus group, VSV New Jersey.

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