JOURNAL OF VIROLOGY, July 1971, p. 81-86
Vol. 8, No. 1

Ribonucleic Acid Polymerase Activity in Sendai Virions and Nucleocapsid

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Received for publication 12 March 1971

After dissociation of purified Sendai virus with the neutral detergent Nonidet P-40 and 2-mercaptoethanol, it catalyzed the incorporation of ribonucleoside triphosphates into an acid-insoluble product. The enzyme activity was associated with viral nucleocapsid as well as whole virions. The reaction product was ribonucleic acid (RNA) which annealed specifically with virion RNA. Sedimentation of the ^{3}H-RNA reaction product revealed two components, a 45*S* component with properties of double-stranded RNA and 4 to 6*S* component which appeared to be mostly single-stranded RNA.

Recent experiments have shown that when cells are infected with Sendai virus in the presence of cycloheximide, which inhibits protein synthesis, virus-specific ribonucleic acid (RNA) species complementary to virion RNA but not the 57*S* virion RNA are synthesized (W. S. Robinson, Virology, *in press*). This indicates that synthesis of the complementary RNA species after infection does not require new protein synthesis and suggested that the enzyme involved in synthesis of these RNA species might be part of the virion.

Very recently, Huang, Baltimore, and Bratt (8) found RNA polymerase activity in Newcastle disease virions, and this report describes a similar activity in Sendai virions and in viral nucleocapsid isolated from virions.

MATERIALS AND METHODS

Materials. Uridine-5'-triphosphate-5-^3H (^{3}H-UTP) and ^{3}H-adenosine-5'-triphosphate (^{3}H-ATP) were purchased from New England Nuclear Corp.; Nonidet P-40 (NP40) was a gift of the Shell Oil Co. Crystalline bovine pancreatic ribonuclease A free from deoxyribonuclease and T_1 ribonuclease were purchased from Worthington Biochemical Corp.; crude yeast RNA and sodium deoxycholate (DOC) were purchased from Sigma Chemical Co.

Virus. The Harris strain of Sendai virus was grown in embryonated eggs as previously described (2), and chorioallantoic (CA) fluid containing 10^9 egg infective units of virus per ml (2) was used for virus purification.

Virus purification. Sendai virus was purified from large volumes of CA fluid by first centrifuging the fluid at 10,000 rev/min in the Sorvall GSA rotor to remove cell debris and then by pelleting the virus in the Spinco 19 rotor at 19,000 rev/min for 4 hr at 4 C. The virus was resuspended in buffer 1 which consisted of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.5), 0.10 M NaCl, and 0.001 M ethylenediaminetetraacetic acid (EDTA) at 0.01 of the original volume and was sonically treated to disperse aggregated virus. The virus was then layered over a stepwise density gradient consisting of 5 ml of 65% sucrose in D_2O with buffer 1 and 15 ml of 20% sucrose in buffer 1 and centrifuged in the Spinco SW 27 rotor for 2 hr at 26,000 rev/min and 4 C. The band of virus floating on the 65% sucrose layer was collected, and the virus was then centrifuged to equilibrium (15 hr) in a linear 20 to 65% (in D_2O) sucrose gradient containing buffer 1 in the Spinco SW 27 rotor at 26,000 rev/min and 4 C.

Hemagglutination. Serial twofold dilutions of virus or dissociated viral fractions were made in phosphate-buffered saline for the chicken erythrocyte agglutination test as previously described (2).

Protein determination. The concentration of protein in virus preparations and dissociated viral fractions was determined by the method of Lowry et al. (13) or estimated by ultraviolet absorbancy (*A*) for protein solutions with 280/260 greater than 1.0 by using the formula: $1.54 \times A_{280} - 0.76 \times A_{260}$ = protein concentration in milligrams per milliliter (18).

RNA extraction. RNA was extracted from purified virus and from enzyme reaction mixtures by adding sodium dodecyl sulfate, extracting with phenol, and precipitating the RNA with alcohol as previously described (5). Chick embryo cell RNA was used as carrier in some experiments.

RNA-RNA annealing. RNA samples were heat-denatured, placed in buffer consisting of 0.1 M Tris-hydrochloride (*p*H 7.5) and 0.1 M NaCl, annealed at 85 C, and incubated in pancreatic ribonuclease and T_1 ribonuclease before determining trichloroacetic acid-precipitable radioactivity as previously described (15).

Assays for radioactivity. Radioactivity in sucrose

density gradient fractions was determined by washing trichloroacetic acid precipitates on glass fiber filters and counting in a scintillation counter as previously described (16). The trichloroacetic acid-precipitable radioactivity in enzyme reaction mixtures was determined by spotting samples on Whatman no. 3 paper discs; washing the discs batchwise in 5% trichloroacetic acid in 0.1 M sodium pyrophosphate three times, in 5% trichloroacetic acid in water one time, and in 95% ethanol two times; drying; and counting in a scintillation counter.

RESULTS

Incorporation of ^{3}H-UTP into acid-insoluble product by purified Sendai virus. After incubation of purified Sendai virus with NP40 detergent and 2-mercaptoethanol, it was shown to convert ^{3}H-UTP into a trichloroacetic acid-insoluble product. Table 1 shows the basic reaction. The enzyme reaction with detergent-treated Sendai virus proceeded without added primer and was dependent

TABLE 1. *Sendai virion RNA polymerase reaction*

Reaction mixture	Counts/min
Complete[a]	310[b]
−$MgCl_2$	23
−CTP, ATP, GTP	70
−ATP	102
−CTP	101
−GTP	93
−NP40	21
+Actinomyin D (10 μg/ml)	285
+Rifampin (100 μg/ml)	305
+Ribonuclease A[c]	45
+Deoxyribonuclease[d]	325

[a] Complete reaction mixture contained 30 μmoles of Tris-hydrochloride (*p*H 7.4); 3 μmoles of $MgCl_2$; 0.10 μmole each of adenosine triphosphate (ATP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP); and 0.5 pmole of ^{3}H-UTP (16 Ci per mmole) in a volume of 200 μliters plus 100 μliters of enzyme consisting of a purified preparation of Sendai virus (14 mg per ml of protein and hemagglutination-positive at 1:100,000) made up to 0.2% with respect to both 2-mercaptoethanol and Nonidet P-40 (NP40) and incubated at room temperature for 5 min before addition to the reaction mixture. The numbers represent the trichloroacetic acid-precipitable radioactivity in 100-μliter samples of the reaction mixtures after 60 min of incubation at 31 C.

[b] Samples were counted for 10 min, and the background of a blank filter (6 counts/min) was subtracted from each value.

[c] Pancreatic ribonuclease was added to make 100 μg/ml in the Sendai virus preparation before addition of the 2-mercaptoethanol and NP40.

[d] Deoxyribonuclease was added to make 100 μg/ml in the Sendai virus preparation before addition of the 2-mercaptoethanol and NP40.

for optimal activity on $MgCl_2$ and all four ribonucleoside triphosphates. No enzyme activity was detected without treating the virus with detergent. Both NP40 and Brij 35 were found to be effective in concentrations from 0.01 to 1.0%, and optimal activity was found with 0.1 to 0.2% final detergent concentration. Sodium dodecyl sulfate- and DOC-treated virus added directly to the reaction mixture resulted in no enzyme activity. Actinomycin D and rifampin had no effect on the reaction. Prior incubation of detergent-treated virus with ribonuclease abolished almost all activity and deoxyribonuclease had no effect. The results with actinomycin D and deoxyribonuclease indicate that the enzyme reaction is not a deoxyribonucleic acid (DNA)-dependent polymerase reaction.

Table 2 shows that the optimum $MgCl_2$ concentration was around 10 μmoles/ml, and almost no enzyme activity was found with $MnCl_2$ or $CoCl_2$ over a concentration range from 0.1 to 10 μmoles/ml. The maximum rate of ^{3}H-UTP incorporation was found over a broad range between 30 and 37 C, with lower activity above and below these values. At 30 or 31 C, the reaction remained linear for at least 2 hr.

The *p*H optimum of the reaction was between 7.0 and 7.5, with decreasing activity above and below this *p*H range. The rate of nucleotide incorporation into trichloroacetic acid-insoluble product was not stimulated by adding Sendai virion RNA or other single-stranded RNA species or several double-stranded DNA species to the reaction mixture. Under optimal reaction conditions, the virion enzyme activity was extremely low as shown in Table 1, in which the enzyme concentration was limiting the reaction rate. ^{3}H-UTP with very high specific activity was needed to detect incorporation, and higher UTP concentrations than that in the experiment in

TABLE 2. *Polymerase reaction[a] dependence on $MgCl_2$*

Amt (μmoles) per ml of $MgCl_2$	Reaction time at		
	0 min	20 min	60 min
0	14[b]	21	27
5	21	52	92
10	19	120	271
15	17	74	142

[a] Complete reaction mixture, as described in Table 1 except for the $MgCl_2$ concentration which was varied as designated in the table, was incubated at 31 C and 100-μliter samples were removed at the designated times.

[b] Results expressed as counts per minute.

Table 1 did not increase the rate of UTP incorporation. Similar low activity was observed for several preparations of virus including freshly prepared virus which was rapidly purified and immediately tested for enzyme activity without freezing or storage.

Properties of the RNA product of the reaction. Table 3 shows some properties of the trichloroacetic acid-insoluble reaction product. After heating to 100 C for 10 min in 0.01 M Tris-hydrochloride (*p*H 7.5), the product was almost completely digested by ribonuclease to a trichloroacetic acid-insoluble form, and without prior heat denaturation 37% was resistant to digestion by using conditions under which double-stranded or base-paired RNA is resistant to ribonuclease digestion (7). After heat denaturation, self annealing the reaction product increased the ribonuclease-resistant fraction to 56% and annealing in the presence of added 57*S* virion RNA increased the fraction to 91%. In other experiments, no annealing was observed with 57*S* Newcastle disease virus RNA or with chick cell ribosomal RNA. These results indicate that the reaction product is RNA and suggest that more than one-third which is not digested by ribonuclease before heating is probably in a base-paired or double-stranded form. The 57*S* virion RNA has been shown to be a mixture of complementary RNA molecules with 5- to 10-fold more of one strand than of its complement (15, 16). Since almost all of the RNA product anneals with a large excess of virion RNA, most of the RNA product is complementary to the major strand of 57*S* virion RNA, although annealing to both virion strands may occur. The complementarity of the product with virion RNA is consistent with the virion RNA serving as the template for the polymerase reaction.

TABLE 3. *Properties of the reaction product*[a]

Determination	Counts/min	Per cent of total
Total acid-precipitable ^{3}H-RNA	1,280	100
After ribonuclease[b]	480	37
After heating, rapid cooling, and ribonuclease[b]	19	1.5
After annealing and then ribonuclease[b]	712	56
After annealing (4 μg of virion 57*S* RNA) and then ribonuclease[b]	1,165	91

[a] A 10-ml reaction mixture with all components in the proportions described for the complete reaction mixture in Table 1, except that adenosine triphosphate (ATP) was replaced with 1 pmole of ^{3}H-ATP (7 Ci per mmole), was incubated at 31 C for 1 hr. EDTA and sodium dodecyl sulfate were then added to make concentrations of 0.005 M and 1%, respectively. Water (10 ml) was added, and the RNA was isolated by phenol extraction and alcohol precipitation. The RNA was redissolved in 2 ml of buffer 1 and 0.1-ml samples were used for each determination in the table.

[b] Pancreatic ribonuclease (10 μg), ribonuclease T_1 (1 μg), and RNA sample in 0.5 ml with 0.10 M Tris-hydrochloride (*p*H 7.5) and 0.10 M NaCl were incubated at 37 C for 1 hr before trichloroacetic acid precipitation.

The RNA product from a 1-hr enzyme reaction was further characterized by sedimentation in a sucrose density gradient, and the results are shown in Fig. 1. The A_{260} tracing shows the position of 57*S* virion RNA (peak in fraction 16) and cellular RNA species sedimenting at 28*S* (peak in fraction 26), 18*S* (peak in fraction 30), and 4*S* (peak in fractions 33 and 34). Two main components of ^{3}H-RNA were present. The faster sedimenting component had a sedimentation

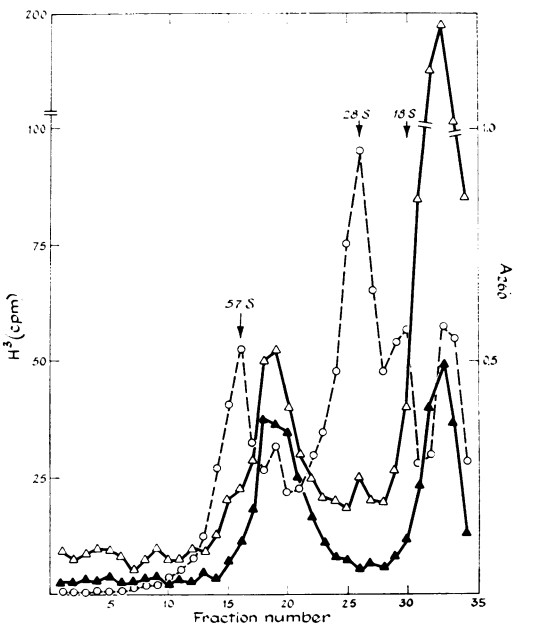

FIG. 1. *Sedimentation of the Sendai virion enzyme reaction product. One-half of the RNA product from the reaction described in Table 3 and unlabeled chick cell RNA were dissolved in 0.2 ml of buffer 1, layered over a 13.5-ml 5 to 20% sucrose density gradient containing buffer 1, and centrifuged at 40,000 rev/min and 4 C for 4.5 hr in the Spinco SW 40 rotor. Fractions collected from the bottom of the tubes were used to determine A_{260} (○), and trichloroacetic acid-precipitable radioactivity before (△) and after (▲) ribonuclease digestion as described in Table 3 was determined for one-half of each fraction.*

coefficient around 45S (peak in fractions 18 and 19), and about 75% of this radioactive RNA was resistant to digestion by ribonuclease, suggesting that it consisted of base-paired or partially double-stranded RNA. The other ^{3}H-RNA component sedimented around 4 to 6S (peak in fractions 31 and 32), and about 20% of this RNA was resistant to ribonuclease digestion. Sedimentation of the product synthesized during a 15-min enzyme reaction revealed that more than 50% of the radioactive RNA was in the 45S component.

RNA polymerase activity associated with viral nucleocapsid. Purified virus treated with DOC in concentrations from 0.01 to 1.0% was found to have no detectable enzyme activity in an assay including detergent in the reaction mixture. However, when virus was dissociated with 1% DOC and the resulting nucleocapsid (3) was separated from the detergent and other virion components by centrifugation, the nucleocapsid was found to possess RNA polymerase activity. Figure 2 shows the results of RNA polymerase assays on fractions of sucrose density gradients after equilibrium centrifugation of (a) intact virus, (b) virus treated with 1% NP40, and (c) virus treated with 1% DOC.

Treatment of the virus with both NP40 and DOC resulted in visible clearing of the turbid virus solution (greater with DOC than with NP40) and much sharper bands of particulate material compared with intact virus after centrifugation. This was demonstrated by the A_{280} tracing in Fig. 2 and was clearly evident by visual inspection of the material in the centrifuge tubes. The buoyant density was 1.200 g per ml for whole virions, 1.235 for the structures resulting from NP40 treatment, and 1.275 for the DOC-treated material. The peak fraction from each gradient was tested for hemagglutinating activity. Fraction

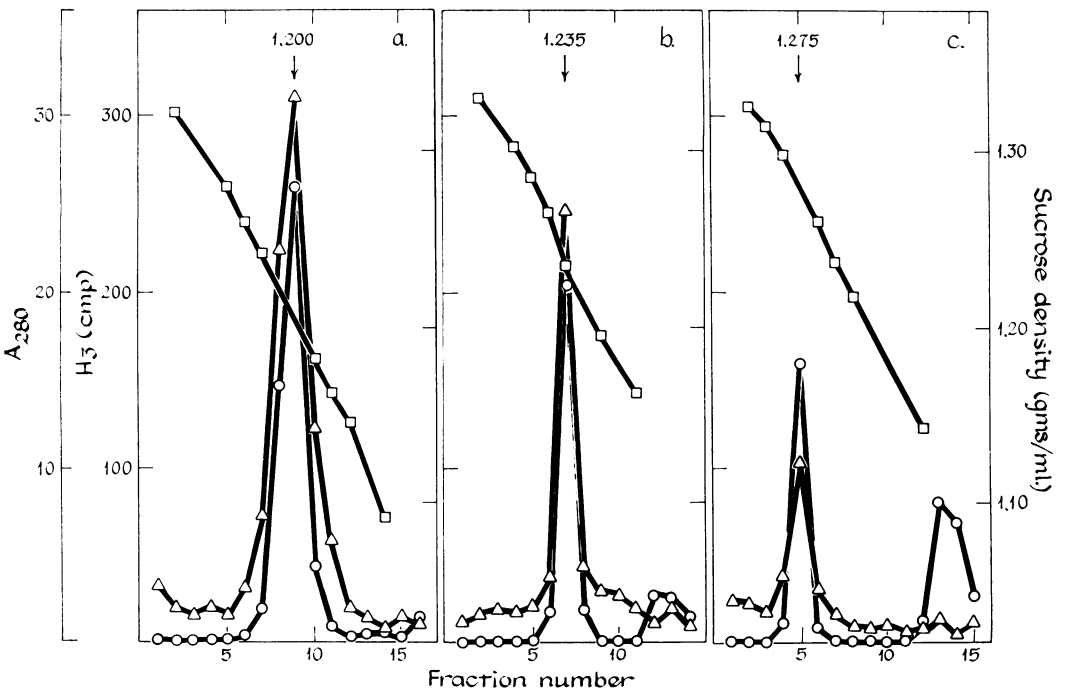

FIG. 2. *Equilibrium density gradient centrifugation of (a) Sendai virus and (b and c) detergent-dissociated virus. (a) A 1-ml amount of purified Sendai virus (14 mg per ml of protein and hemagglutination-positive at 1:100,000), (b) 1 ml of virus after incubation at room temperature for 10 min with 1% Nonidet P-40 (NP40) and 0.01 M 2-mercaptoethanol, and (c) 1 ml of virus incubated with 1% sodium deoxycholate and 0.01 M 2-mercaptoethanol were each layered over a 12.5-ml preformed linear density gradient from 65% sucrose in D_2O to 20% sucrose in water containing buffer 1 and 0.01 M 2-mercaptoethanol and centrifuged at 40,000 rev/min and 2 C for 4 hr in the Spinco SW 40 rotor. Fractions collected from the bottom of each tube were used to determine solution density (□) and A_{280} (○). The fractions from each gradient were made up to 0.1% with respect to NP40, and 0.10-ml samples of each fraction were added to complete enzyme reaction mixtures for incubation at 31 C for 1 hr as described in Table 1 before determining trichloroacetic acid-precipitable radioactivity in the whole reaction mixture* (△).

9 (Fig. 2a; intact virus) was positive at 1:100,000, fraction 7 (Fig. 2b; from NP40-treated virus) was positive at 1:100, and fraction 5 (Fig. 2c; from DOC-treated virus) was negative at 1:10. These results are consistent with previous experiments from this laboratory, showing that after DOC treatment of virus the hemagglutinating activity remains near the top of the tube and the component with a density of about 1.27 is without hemagglutinating activity and has the structure of viral nucleocapsid (3). The intermediate buoyant density of the material resulting from NP40 treatment of virus suggests that it may represent an intermediate stage in virus dissociation between intact virus and free nucleocapsid. The sharpness of the band suggests that it is an homogeneous structure.

In several experiments, RNA polymerase assays consistently showed the presence of substantial enzyme activity associated with the structure resulting from NP40 treatment (Fig. 2b) and significantly less but regularly observed activity associated with nucleocapsid (Fig. 2c).

DISCUSSION

The virus-specific RNA synthesized in parainfluenza virus-infected cells differs in several respects from the RNA in cells infected by small RNA viruses such as poliovirus and RNA phages. Eighty to 90% of the virus-specific RNA synthesized in Newcastle disease virus (5, 12) and Sendai virus (2)-infected cells consists of several single-stranded RNA components which are complementary in base sequence to virion RNA, unlike cells infected with poliovirus and other small RNA viruses in which most of the virus-specific RNA synthesized is identical to virion RNA (6, 9). The complementary RNA species and not the virion RNA appear to be associated with polyribosomes in Newcastle disease virus- (5) and Sendai virus (3)-infected cells in contrast to the small RNA viruses in which the virion RNA is associated with polyribosomes and undoubtedly functions as viral messenger RNA (6, 9). In Sendai virus-infected cells, the virion RNA is rapidly and quantitatively incorporated into viral nucleocapsid (3). The free virion RNA of paramyxoviruses has not been demonstrated to be infectious (11; W. S. Robinson, *unpublished data*) as is the RNA of many small RNA viruses (9). These findings suggest the possibility that, unlike small RNA viruses, the complementary RNA species function as messenger RNA and the virion RNA alone is insufficient to initiate parainfluenza virus infection.

Consistent with this possibility are more recent findings with Sendai virus. When cells were infected at a high multiplicity with Sendai virus in the presence of cycloheximide so that no detectable protein synthesis occurred, early synthesis of the complementary RNA components but not of the 57*S* virion RNA was observed (W. S. Robinson, Virology, *in press*). The Sendai virion enzyme described in the present study may be involved in the synthesis of the complementary RNA components in infected cells in the presence of cycloheximide, and this enzyme, in addition to virion RNA, may be essential for successful infection. Virus-specific RNA synthesis is not initiated in cells infected with small RNA viruses in the absence of protein synthesis (9).

The RNA product of the virion enzyme reaction was complementary in base sequence to virion RNA, although little of the product sedimented in the 35, 22, and 18*S* positions observed for the complementary RNA species synthesized in infected cells (2). Most of the RNA product synthesized during a short reaction time sedimented around 45*S* and had properties of base-paired or partially double-stranded RNA suggesting that the newly synthesized complementary RNA species were probably complexed with virion RNA. This RNA component is similar to the double-stranded RNA found in Sendai virus-infected cells which appears to be an intermediate in virus-specific RNA synthesis (16). Longer enzyme reaction times resulted in formation of smaller pieces of mostly single-stranded RNA sedimenting around 4 to 6*S*. The small size could result from ribonuclease breakdown of larger single-stranded RNA pieces or synthesis of only small pieces by the in vitro enzyme. The purified virus preparation has been found to contain some ribonuclease activity when tested by its ability to degrade radioactive viral RNA to smaller pieces, although none was converted to a trichloroacetic acid-soluble form (W. S. Robinson, *unpublished data*).

The Sendai virion enzyme activity was extremely low under all reaction conditions used. With the optimal detergent and divalent cation concentrations, ionic strength, *p*H, and temperature, very large amounts of virus were required to even detect enzyme activity. Under optimum reaction conditions as in Table 1, in which the enzyme concentration was limiting, only 0.02 pmole of ^{3}H-thymidine monophosphate per mg of virion protein per hr was incorporated into the trichloroacetic acid-insoluble product. This is at least 100-fold lower activity than reported for the vaccinia virus (10, 14), reovirus (4, 17), and vesicular stomatitis virus (1) RNA polymerases. Whether this is an intrinsic property of the Sendai virion enzyme or alternatively the enzyme is unstable or requires still different reaction conditions for greater in vitro enzyme activity is not

clear at this time. The enzyme recently found in Newcastle disease virions (8) has many properties similar to the Sendai virion enzyme and it has a similar low activity. The very low enzyme activity undoubtedly accounts for the failure of earlier attempts to demonstrate virion enzyme for Newcastle disease virus (1) and Sendai virus (W. S. Robinson, *unpublished data*) in which smaller amounts of virus were used. The low enzyme activity is probably not due to the presence of the small amounts of nuclease in the virus preparation. The observation that the reaction is linear for at least 2 hr suggests that the RNA template is not extensively degraded, and none of the RNA product was degraded to a trichloroacetic acid-soluble form in an experiment in which the reaction was stopped with EDTA and incubation was continued for 4 hr (W. S. Robinson, *unpublished data*). In addition, Baltimore et. al. (1) showed that no inhibition of a vesicular stomatitis virus RNA polymerase reaction occurred when Newcastle disease virus enzyme was mixed with it.

Vesicular stomatitis virus (1) is the only virus known outside of the parainfluenza virus group which appears to share the unique features of viral RNA structure and synthesis found for Sendai virus and Newcastle disease virus.

ACKNOWLEDGMENTS

I thank Nona Stone for technical assistance.

This investigation was supported by Public Health Service grant CA 10467 from the National Cancer Institute.

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