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In Vitro Synthesis of the Nonstructural C Protein of Sendai Virus

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In vitro translation was used to study the mRNA for the Sendai virus nonstructural C protein. The C protein mRNA was found to be coordinately expressed with the mRNAs for the structural proteins. However, the C protein mRNA appeared to be translated more efficiently in vitro than the other mRNAs. In addition, the 22,000-dalton C protein mRNA cosedimented on sucrose gradients with the 79,000-dalton P protein mRNA. The C protein mRNA thus appears to be much larger than expected.

Sendai virus, a prototype of the parainfluenza virus family, contains all of its genetic information in a single RNA chain of negative polarity (−). The genetic information of this virus appears to be expressed via monocistronic mRNAs transcribed from the (−) genome, since Sendai virus-infected cells contain multiple mRNA species of opposite polarity to the viral genome that correlate roughly with the expected coding capacity of the major virion proteins (1, 8). To date, six virion proteins have been clearly defined: NP (60,000 daltons [60K]), P (79K), and L (~200K), which are associated with the nucleocapsid core, and HN (72K), Fo (65K), and M (34K), which are associated with the viral envelope (4, 6).

In addition to the structural proteins of the virion, Lamb et al. (6) also described a protein that is present in virus-infected cells, but is not found in mature virions. This nonstructural protein (C; 22K) was clearly shown to be virus specific and to contain a peptide map different from the other viral proteins (3, 5). Since the Sendai virus C protein is not a structural component of the virion itself, it seems likely that it functions in a control capacity during intracellular virus replication. For this reason, the synthesis of this nonstructural protein is of particular interest.

In a previous publication, Lamb et al. (6) studied the appearance of Sendai virus C protein throughout infection by pulse-labeling virus-infected chicken embryo fibroblasts with [^{35}S]methionine. They found that the virus structural proteins could be detected by 2 h postinfection (p.i.) and that once established, synthesis continued at a relatively constant rate throughout the infection. The synthesis of the nonstructural C protein, on the other hand, appeared a little later, at 6 to 8 h p.i., and again continued throughout the infection. This delayed synthesis of the C protein relative to the viral structural proteins suggests that its mRNA might be transcribed later in infection than those mRNAs coding for the structural proteins. This raised the possibility that the C protein mRNA was a product of secondary transcription, i.e., was transcribed only after viral genome amplification.

To examine this question more closely, we studied the appearance of the virus-specific mRNAs in vivo by their in vitro translation. BHK cells were infected with 10 PFU of the H (Harris) strain of Sendai virus per cell (7). At various times p.i., actinomycin D (2 μg/ml) was added, and 2.5 h later the infected cells were harvested in phosphate-buffered saline, pelleted, and quickly frozen until the experiment was completed at 27 h p.i. Mock-infected cells were also harvested at 4, 14, and 20 h p.i., and the results at each time were identical; thus, only the 20-h sample is shown. The frozen cells were thawed, and the cytoplasmic RNA was isolated by centrifugation through a 20 to 40% CsCl gradient as previously described (7). The pelleted RNA was dissolved in water, reprecipitated with 3 volumes of ethanol, and then redissolved in water. The RNA concentration of all samples was adjusted with water to a constant optical density at 257 nm of 50 U/ml. Identical amounts of the cytoplasmic RNAs, as judged by optical density, were then translated in a reticulocyte lysate, and the protein products were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 1). Protein bands that comigrated with proteins P, NP, and M from purified virions were clearly synthesized from virus-infected cytoplasmic RNA but not

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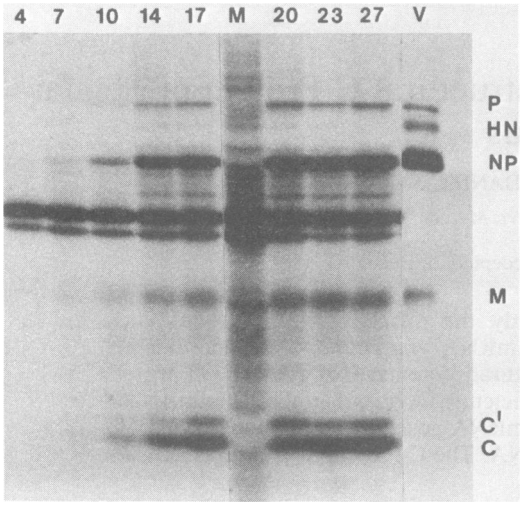

FIG. 1. In vitro translation of mRNA from Sendai virus-infected BHK cells. BHK cells in monolayer were infected with Sendai virus, and cytoplasmic RNA was isolated as previously described (7) (see text). Identical amounts (3 μl) of these RNAs were then used in the reticulocyte lysate translation kit (New England Nuclear Corp.) for in vitro translation. The sample was incubated at 33°C for 60 min in a 13.5-μl reaction mixture containing 25 μCi of [^{35}S]methionine, 2.75 μl of cocktail, and 5 μl of lysate. The final potassium acetate and magnesium acetate concentrations were 0.08 M and 0.65 mM, respectively. After incubation, the samples were treated with 2 μg of pancreatic RNase for 15 min at 37°C and then mixed 1:1 (vol/vol) with 2× sample buffer (10% glycerol, 10% β-mercaptoethanol, 6% SDS, 0.02% BPB, 0.125 M Tris-hydrochloride [pH 6.8]). The samples were boiled for 2 min, loaded onto 10% acrylamide–0.8% bisacrylamide slab gels, and electrophoresed overnight at 12 mA. The gels were fluorographed with 2,5-diphenyloxazole–dimethyl sulfoxide as described previously (2), dried, and exposed to Kodak XS-5 film at −70°C. Numbers, harvest time p.i.; M, mock-infected culture; V, purified virions used as a marker. Virion proteins are shown to the right, except for Fo, which migrated just above NP and was not clearly resolved on this gel.

from mock-infected RNA and were presumed to represent these virus proteins. In addition, a doublet band at the position of the C protein, noted as C and C′ (3), was also synthesized by virus-infected but not by mock-infected cell RNA. The strongest protein band between N and M (Fig. 1) was not dependent on exogenous mRNA. Its intensity correlated with the age of the [^{35}S]methionine and was thought to be the result of a reaction between a lysate component and an oxidation product of the radiolabel. We could detect the C protein and the structural proteins as early as 7 h p.i. As judged by in vitro translation, the level of viral mRNAs increased until approximately 14 to 17 h p.i. and then reached a steady state. Thus, mRNAs coding for the nonstructural protein C as well as the structural proteins appear at the same time after infection, and their steady-state concentrations appear to be coordinately controlled throughout the infection.

The reasons for this difference in the appearance of C relative to the structural proteins in vivo and in vitro are not clear. Possible explanations include a relative delay in the translation of the C protein mRNA in vivo and a higher rate of turnover of the C protein in vivo than in vitro. In addition, this difference may also be related to a somewhat curious finding, the different amounts of C relative to structural protein synthesized in vivo and in vitro. When [^{35}S]methionine was used to estimate the relative amounts of the viral proteins in vivo, the amount of radiolabel that accumulated in the C protein was generally less than that in the P protein (Fig. 2A, lanes 3 and 4) (also see Fig. 5 in reference 6). However, when [^{35}S]methionine was used as a radiolabel in in vitro translation, considerably more label accumulated in the C protein than in the P protein (Fig. 1 and 2A, lane 1) (also see Fig. 2 in reference 3). Although this effect could be due simply to selective degradation of the mRNA used in the in vitro translation, this seems unlikely, because a similar difference was noted with as many as 10 different RNA preparations, some of which were selected by oligodeoxythymidylate-cellulose chromatography, and because of the finding discussed below that the C and P protein mRNAs cosedimented by velocity sucrose centrifugation. Additionally, although this effect could also be due to the selective premature termination of the P protein in vitro, this does not appear to be the case, since the ratio of P to NP proteins was roughly the same whether these proteins were synthesized in vitro or in vivo (Fig. 2A, lanes 1, 3, and 4).

To further examine whether the Sendai virus C protein mRNA was also a product of primary transcription, viral mRNA was synthesized in an in vitro polymerase reaction with detergent-activated virions. The RNAs of the polymerase reaction, which by definition are products of primary transcription, were similarly translated in a reticulocyte lysate (Fig. 2B). Since the C protein (C and C′) and the virus structural proteins were synthesized in similar amounts by either the in vitro-synthesized RNA or the in vivo mRNA, the C protein mRNA was clearly also a product of primary transcription. Also note that [^{35}S]methionine-labeled C protein synthesized in vitro with virion polymerase products as mRNA again represented a major product of the in vitro translation.

To date, the size of the Sendai virus C protein

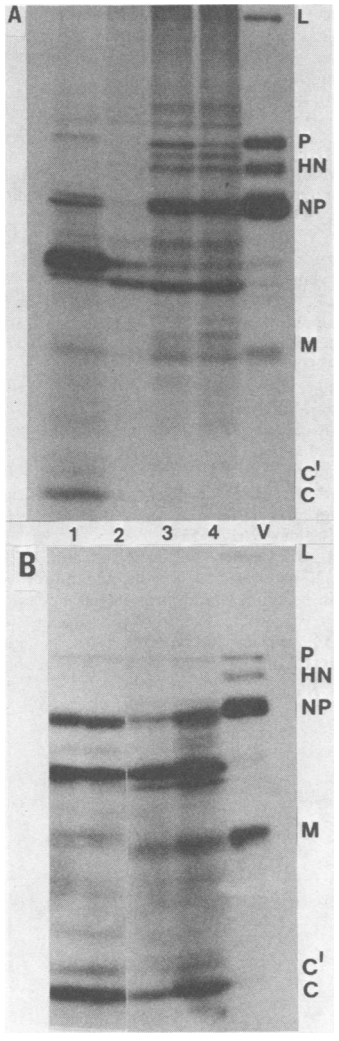

FIG. 2. Comparison of the relative amounts of Sendai P and C proteins. (A) In vivo versus in vitro synthesis. Virus-infected cells were pulse-labeled for 2 h with 10 μCi of [^{35}S]methionine per ml at 15 and 21 h p.i. Cytoplasmic extracts were prepared (see the legend to Fig. 1), and a sample containing 20,000 cpm was precipitated with 10% trichloroacetic acid, washed with acetone, and directly dissolved in sample buffer (lanes 3 and 4). Lane 2, Pulse-labeled proteins from a mock-infected control culture. Cytoplasmic RNA was isolated from the remainder of the virus-infected cells harvested at 15 h p.i. and translated in vitro (lane 1) as described in the legend to Fig. 1. V, Purified virions. (B) In vitro translation of in vivo and in vitro mRNAs. Sendai virus mRNA was synthesized in vitro with purified virions as previously described (7), except that poly-L-glutamic acid at 1 mg/ml was used in place of tRNA. Samples containing 26 or 52 ng of in vitro-synthesized RNA, as judged by [^{32}P]GTP incorporation, were then translated in vitro (lanes 1 and 2, respectively). Lanes 3 and 4, Products of the in vitro translation of 8 or 16 μg of total cytoplasmic RNA, respectively.

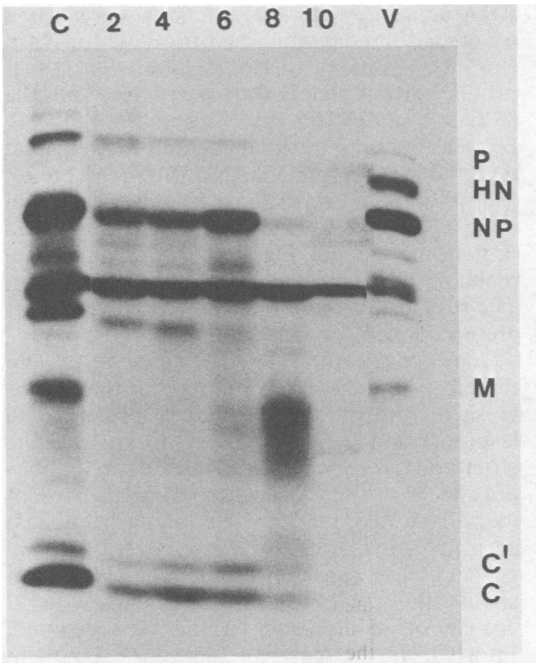

FIG. 3. In vitro translation of in vivo mRNA separated by sucrose gradient centrifugation. Total cytoplasmic RNA (600 μg) was chromatographed on a 2-ml column of oligodeoxythymidylate-cellulose to select polyadenylic acid-containing RNA. This RNA was recovered by two ethanol precipitations and dissolved in 200 μl of water. The RNA sample was then heated to 70°C for 2 min, made 100 mM in NaCl and 0.1% in SDS, and sedimented on a 5 to 23% sucrose gradient containing 0.1 M LiCl, 20 mM Tris-chloride (pH 7.4), 2 mM EDTA, and 0.1% SDS for 165 min at 59,000 rpm in an SW60 rotor. Twenty-two fractions were collected, and the RNA was recovered by ethanol precipitation after the addition of 15 μg of carrier *Escherichia coli* tRNA. The RNA samples were dissolved in 20 μl of water, and 3-μl samples of the even-numbered fractions were translated in vitro. Numbers, Gradient fraction numbers; C, in vitro translation of 4 μg of the cytoplasmic RNA used as starting material; V, purified virions. Odd-numbered fractions were translated in a separate experiment, and the results complemented the results shown here.

mRNA has not been determined. Previous attempts to locate a virus-specific mRNA having the approximate coding capacity for a 22K protein by polyacrylamide gel electrophoresis have not been successful (1) (data not shown). Since the C protein mRNA can be detected easily by in vitro translation, we reexamined this question by performing velocity sedimentation on sucrose gradients. Total cytoplasmic RNA from virus-infected cells was isolated at 21 h p.i., purified by oligodeoxythymidylate-cellulose chromatography, and sedimented on an SDS-sucrose gradient so that 18S rRNA moved three fourths of the length of the tube (fraction 6). The

RNA present in alternate fractions was isolated by ethanol precipitation and translated in vitro (Fig. 3). Contrary to expectation, the 22K C protein mRNA sedimented faster than both the 35K M protein (fraction 8) and the 60K NP protein (fraction 6) and appeared to cosediment with the 79K P protein mRNA (fractions 2 to 6). The M protein bands (lane 8) and the control (lane C) migrated slightly ahead of the virion protein marker (cf. Fig. 2A), and their precise mobility varied from experiment to experiment. The reasons for these relatively small variations are not known.

The nonstructural Sendai virus C protein therefore appears to have properties that distinguish it from the virus structural protein. The level of its synthesis relative to that of the structural proteins in virus-infected cells does not appear to be controlled simply by the level of its mRNA, and the C protein is translated from an mRNA that does not correlate with its expected coding capacity. To date, little is known about either the functions of the Sendai virus C protein or its location on the viral genome. Efforts to further describe this gene are now in progress.

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