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Complexes of Sendai Virus NP-P and P-L Proteins Are Required for Defective Interfering Particle Genome Replication In Vitro

SANDRA M. HORIKAMI,[1] JOSEPH CURRAN,[2] DANIEL KOLAKOFSKY,[2] AND SUE A. MOYER[1]*

Departments of Immunology and Medical Microbiology and Pediatrics, University of Florida, Gainesville, Florida 32610-0266,[1] and Department of Genetics and Microbiology, University of Geneva School of Medicine, CH1211 Geneva, Switzerland[2]

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We present evidence that the formation of NP-P and P-L protein complexes is essential for replication of the genome of Sendai defective interfering (DI-H) virus in vitro, using extracts of cells expressing these viral proteins from plasmids. Optimal replication of DI-H nucleocapsid RNA required extracts of cells transfected with critical amounts and ratios of each of the plasmids and was three- to fivefold better than replication with a control extract prepared from a natural virus infection. Extracts in which NP and P proteins were coexpressed supported replication of the genome of purified DI-H virus which contained endogenous polymerase proteins, but extracts in which NP and P were expressed separately and then mixed were inactive. Similarly, the P and L proteins must be coexpressed for biological activity. The replication data thus suggest that two protein complexes, NP-P and P-L, are required for nucleocapsid RNA replication and that these complexes must form during or soon after synthesis of the proteins. Biochemical evidence in support of the formation of each complex includes coimmunoprecipitation of both proteins of each complex with an antibody specific for one component and cosedimentation of the subunits of each complex. We propose that the P-L complex serves as the RNA polymerase and NP-P is required for encapsidation of newly synthesized RNA.

Sendai virus, a paramyxovirus, contains a 15-kb negative-stranded RNA genome which is found both in the virion and in infected cells as an RNase-resistant nucleocapsid by virtue of its tight association with the abundant nucleocapsid protein NP (60 kDa) (8). Two other viral proteins, P (62 kDa) and L (~250 kDa), are associated with the nucleocapsid and function as the RNA-dependent RNA polymerase (10, 11, 25). Three additional Sendai virus structural proteins, HN, F, and M, are associated with the envelope of the virion. For negative-stranded RNA viruses, it is the nucleocapsid and not naked RNA which serves as the template for RNA synthesis by the viral polymerase. Transcription initiates at the 3′ end of the nucleocapsid, sequentially yielding leader RNA and the viral mRNAs (9, 17, 20). RNA replication requires the simultaneous synthesis and encapsidation of genome RNA which occurs via the synthesis of a full-length, plus-stranded, encapsidated RNA intermediate (27).

We have used the Sendai virus defective interfering particle DI-H as the template for the study of genome replication (1, 3, 22). The DI-H genome RNA is 1,411 nucleotides long (about 9% the size of the nondefective genome), contains sequences from the 5′ end of the genome RNA, including a portion of the L cistron, and has copy-back termini, with 110 nucleotides of the 3′ terminus complementary to the 5′ terminus (2, 19). In previous work we showed that purified NP protein would support the replication of intracellular DI-H nucleocapsids, but not of purified virus, which required the addition of infected cell extracts for nucleocapsid RNA replication (1, 22). These data suggested that NP protein alone was sufficient for the elongation and encapsidation of preinitiated replicating RNA present in intracellular nucleocapsids but that additional viral proteins may be required specifically for the initiation of replication and encapsidation needed with purified virus.

A mammalian expression system for Sendai virus proteins was recently described (4), for which expression of transfected plasmids containing the Sendai virus NP, P/C, or L gene cloned downstream of the T7 promoter is provided by infection of cells with a recombinant vaccinia virus expressing T7 RNA polymerase (7). All three viral proteins were shown to be required for DI-H nucleocapsid RNA replication in vivo (4). The limitation of this system is that genome replication in the cell requires the coexpression of all three viral genes, so that one cannot distinguish the individual steps in this process. For this purpose we have extended this methodology to assay specific steps of nucleocapsid RNA synthesis from purified viral templates in extracts of the infected, transfected cells. This system has the significant advantage that individual proteins can be synthesized alone or in combinations and extracts may be mixed to study the possible interactions of the viral proteins. We present both biological and biochemical data suggesting that Sendai virus DI nucleocapsid RNA replication requires two distinct complexes: a P-L complex as the RNA polymerase and an NP-P complex as the substrate for the encapsidation of newly synthesized RNA.

MATERIALS AND METHODS

Cells, viruses, and antibodies. Sendai virus (Harris strain) and the Sendai virus defective interfering particle DI-H were propagated in embryonated chicken eggs and purified as described previously (3). Recombinant vaccinia virus containing the gene for the phage T7 RNA polymerase (VVT7) (7) was obtained from Edward Niles (SUNY, Buffalo, N.Y.) and grown in Vero cells. Transfections and infections for RNA replication assays were performed in human A549

* Corresponding author.

cells. Immunoprecipitation of viral proteins utilized a rabbit anti-Sendai virus antibody [α-SV(3)] and monoclonal antibodies to the Sendai virus NP protein α-NP (M8, from A. Portner, Memphis, Tenn.) and P protein α-P (1.180, from D. Kolakofsky, Geneva, Switzerland). A rabbit antibody was generated against a *trpE*-Sendai virus L fusion protein (α-L). The *Eco*RI-*Eco*RV fragment (nucleotides (1953 to 3795) of a Sendai virus L cDNA (clone G36, from D. W. Kingsbury) was cloned downstream of the N-terminal two-thirds of the *trpE* gene of the pATH11 vector (12, 16). The *trpE*-L fusion protein (molecular weight, ca. 95,000) was separated by electrophoresis on a 2% low-melting-point agarose–0.1% sodium dodecyl sulfate (SDS) gel and identified by staining with Coomassie blue. The fusion protein (280 μg) was cut out, melted, emulsified with Freund's complete adjuvant, and used to immunize a rabbit by intramuscular inoculation. The rabbit was given a booster injection with the protein in incomplete adjuvant at 1-month intervals, and sera was removed 2 weeks later. The α-L antibody was specific for Sendai virus L protein by Western immunoblot analysis of purified virus and immunoprecipitation (data not shown).

Infection and transfection. Subconfluent cultures of A549 cells in 60-mm-diameter dishes (approximately 4×10^6 cells) were infected with VVT7 at a multiplicity of infection of 2.5 for 1 h at 37°C and then transfected as indicated in the figure legends, utilizing lipofectin (Bethesda Research Laboratories) with one or more circular plasmids containing the Sendai virus NP, P/C, or L gene cloned individually in pGEM, downstream of the T7 promoter (4). The transfected cells were incubated at 37°C in Opti-MEM medium (GIBCO) containing penicillin and streptomycin. We found that the level of DI-H RNA replication increased linearly with extracts prepared up to 16 h after transfection and then remained constant to 22 h (data not shown). For these experiments, cytoplasmic extracts were prepared 18 h after transfection.

In vitro replication and RNA analysis. Cytoplasmic extracts and the soluble protein fraction of the VVT7-infected plasmid-transfected or Sendai virus-infected control cells were prepared by lysolecithin permeabilization, essentially as described previously (3). After 1 min with lysolecithin, 3 ml of wash solution was added to dilute the lysolecithin and the solution was aspirated. The cells were then drained for 4 min and scraped into a modified reaction mixture containing 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 8.5, 0.05 M NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate, 2 μg of dactinomycin per ml, 1 mM each of dithiothreitol, spermidine, ATP, GTP, and UTP, 10μM CTP, 10% glycerol, and 0.5 U of RNasin (Promega Biotec) per μl. The cells were lysed by pipetting, and the nuclei and cell debris were pelleted in a microcentrifuge at 4°C. The supernatant was removed, and one of the following templates was added: purified detergent-disrupted (0.1% Triton X-100) DI-H virus (3 μg) or polymerase-free DI-H RNA NP template (1 μg) prepared from virus stripped of associated proteins by detergent-high salt treatment and then banded twice on CsCl gradients. Samples (100 μl, from 4×10^6 cells) were incubated with [α-^{32}P]CTP (500 μCi/ml; New England Nuclear) at 30°C for 2 h and then treated with micrococcal nuclease (1 μg/ml) for 1 h at 37°C to digest any nonencapsidated RNA. The replication products were purified by sedimentation on a 5-ml step CsCl gradient prepared by layering from the bottom the following ingredients: 2 ml of 40% CsCl, 2 ml of 20% CsCl, 0.9 ml of 30% glycerol, in buffer containing 25 mM HEPES, pH 7.5, 2 mM EDTA, and 50 mM NaCl. Centrifugation was at 36,000 rpm for 16 h at 4°C in a Beckman SW55 rotor. The nucleocapsid band at the 20 to 40% CsCl interface was collected and pelleted at 50,000 rpm for 90 min at 4°C in the SW55 rotor. The nucleocapsid RNA was isolated by treatment with proteinase K (250 μg/ml) for 30 min at 37°C, by phenol-chloroform extraction, and then by ethanol precipitation. The purified RNA was analyzed by electrophoresis on 1.5% agarose-acid-urea gels (3) which were dried and exposed to Kodak X-Omat film. The replication products were cut from the gel and quantitated by Cerenkov counting.

Protein analysis. For immunoprecipitation, A549 cells (2×10^7) were infected with VVT7 and transfected with pGEM-NP, pGEM-P/C, or pGEM-L as described in the figure legends. At 4 h posttransfection, the medium was removed and the proteins were labeled with Trans-^{35}S-label (ICN Pharmaceuticals, Inc.; 50 to 75 μCi per ml) in cysteine- and methionine-free minimum essential medium (ICN) plus 10% Dulbecco's minimum essential medium (GIBCO-BRL) for 18 h at 37°C. Cell extracts were prepared in 200 μl of reaction mix salts (RM salts; 0.1 M HEPES [pH 8.5], 0.05 M NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate, 1 mM dithiothreitol, 1 mM spermidine, 10% glycerol), using the lysolecithin procedure. Separate extracts were mixed as indicated in the figure legends and incubated at 30°C for 1 h. The soluble protein fraction from each was prepared as described previously (1). Samples (100 μl) were incubated with 2 μl of the appropriate antibody (see figure legends), collected with inactivated *Staphylococcus aureus* Cowan strain, and analyzed on 10% polyacrylamide-SDS gels as described previously (22). ^{35}S-labeled soluble protein fractions of cell extracts were also fractionated by centrifugation through a gradient of 5 to 20% (vol/vol) glycerol in RM salts at 29,000 rpm for 46 h at 4°C in an SW41 rotor. Aliquots (1 ml) were collected and 0.5 ml was immunoprecipitated by using a mixture of 2 μl each of α-SV and α-L antibodies or 2 μl of the monoclonal antibodies and analyzed as above.

RESULTS

Optimization of conditions for in vitro genome replication. To determine whether an extract from VVT7-infected, plasmid-transfected cells would support replication of DI-H virus nucleocapsid RNA in vitro, we initially transfected the NP, P/C, and L plasmids at 2, 1, and 0.5 μg per dish, respectively. A cytoplasmic extract of these cells did support limited replication of the genome of detergent-disrupted DI virus. Since the level of replication was poor, a series of titrations were performed to optimize the system. When the amounts of P/C and L plasmids were fixed at different levels, DI-H replication first increased with increasing NP plasmid but then was inhibited at higher levels relative to the positive control, which was replication with an extract of Sendai virus-infected cells (Fig. 1A to C). There was no replication in the absence of NP plasmid, showing the requirement for this protein. The data show in each case about a 1:2 ratio of transfected NP-P/C plasmids for optimal nucleocapsid RNA replication, suggesting that a fixed ratio of these proteins was important.

When pGEM-P/C was titrated in the replication assay, keeping NP and L constant, 5 μg of pGEM-P/C gave the greatest level of nucleocapsid RNA replication, which was fourfold better than that of the control Sendai virus extract (Fig. 2A). No replication occurred in the absence of P/C and, furthermore, excess P/C plasmid inhibited replication, as had excess NP plasmid. In a similar titration of pGEM-L, 0.5 μg gave maximal replication, although a fairly broad range of

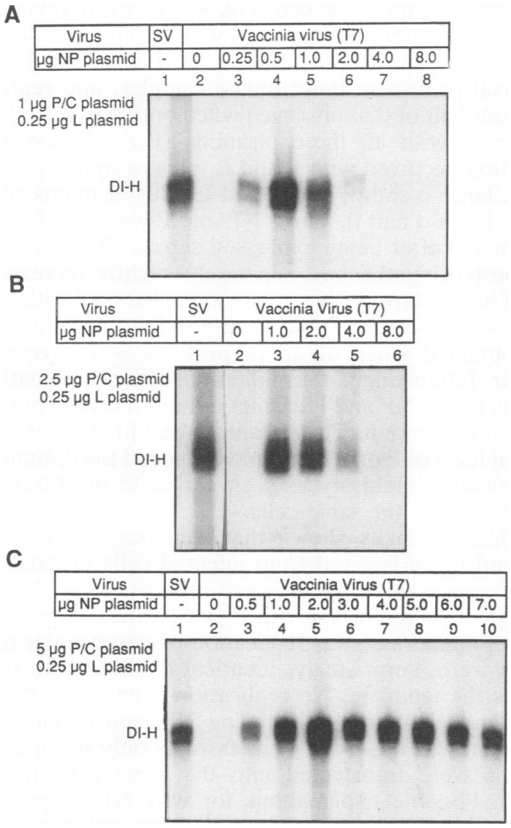

FIG. 1. Titration of pGEM-NP for optimal in vitro replication of the genome of purified DI-H virus. Subconfluent A549 cells were infected with Sendai virus (SV) or VVT7 as indicated, and the VVT7-infected cells were transfected with constant amounts of pGEM-L and pGEM-P/C and increasing amounts of pGEM-NP as indicated in each panel. Cytoplasmic extracts were prepared 18 h after transfection and incubated with detergent-disrupted, purified DI-H virus in the presence of [α-^{32}P]CTP. The samples were treated with micrococcal nuclease, and product nucleocapsids were purified by banding on CsCl gradients. The nucleocapsid RNA was isolated and analyzed as described in Materials and Methods. The position of the DI-H RNAs is indicated on the left.

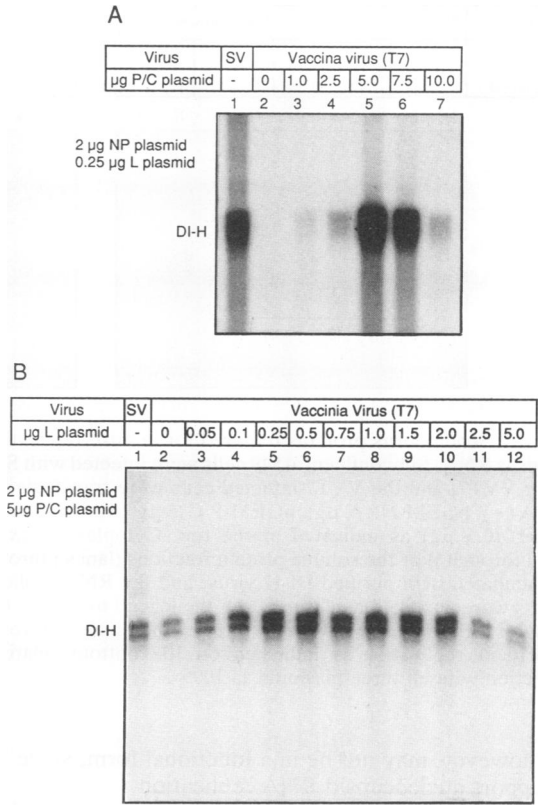

FIG. 2. Titration of pGEM-P/C and pGEM-L for optimal in vitro replication of purified DI-H virus RNA. Subconfluent A549 cells were infected with Sendai virus (SV) or VVT7 as indicated above each panel, and the VVT7-infected cells were transfected with constant amounts of pGEM-NP and pGEM-L and increasing amounts of pGEM-P/C (panel A) or with constant amounts of pGEM-NP and pGEM-P/C and increasing levels of pGEM-L (panel B) as indicated above each gel. Cytoplasmic extracts were incubated with purified DI-H virus, and the RNA replication products were analyzed as described in the legend to Fig. 1. The positions of the DI-H RNAs are indicated.

L (0.25 to 1.5 μg) showed relatively little change (Fig. 2B). Much higher levels of L plasmid (2.5 to 5.0 μg), however, did inhibit the reaction. In contrast to the absolute requirement for both NP and P, limited DI-H replication (25%) did occur in the absence of pGEM-L (Fig. 2B, lane 2). Thus, critical concentrations of all three plasmids and presumably the viral protein products were required for in vitro DI-H nucleocapsid replication. For most of the subsequent experiments 2, 5, and 0.5 μg of NP, P/C, and L plasmids, respectively, were used to transfect cells.

Coexpression of Sendai virus proteins required for genome replication. To determine which proteins were required for in vitro replication, cells were transfected with various combinations of plasmids and extracts were tested with purified DI-H virus as template. As indicated for Fig. 1 and 2, cotransfection of the L, P, and NP plasmids gave greater than threefold better in vitro replication than did an extract of Sendai virus-infected cells (Fig. 3, lanes h and a). No nucleocapsid RNA replication occurred in extracts of cells transfected with any of the individual plasmids alone (Fig. 3, lanes b-d) or with the combination of NP and L or P and L (Fig. 3, lanes f and g). Some replication occurred with only the NP and P proteins (Fig. 3, lane e) as noted earlier (Fig. 2); however, it varied from 15 to 40% in different experiments, with an average value of 25%. These data suggested that the polymerase already present in virus was sufficient for some nucleocapsid RNA replication with NP and P but not with NP alone; however, the level was stimulated by additional polymerase.

We also tested the activity of the soluble protein fraction of cells transfected with various plasmids. Again there was good replication with an extract with P, L, and NP, a lesser amount with NP and P, and none with NP alone (Fig. 3, lanes l, k, and j). The soluble protein fractions that replicated the DI nucleocapsid RNA were threefold less active than the corresponding total cell extract upon quantitation (note the difference in exposure times), which was probably due to partial inactivation during the processing step. Immunoprecipitation and gel analysis of ^{35}S-labeled Sendai virus proteins from these soluble protein fractions showed that coexpression of P with NP or all three proteins yielded 2.5-fold more soluble NP than when NP was expressed alone (data not shown). The limited amount of NP protein in the latter

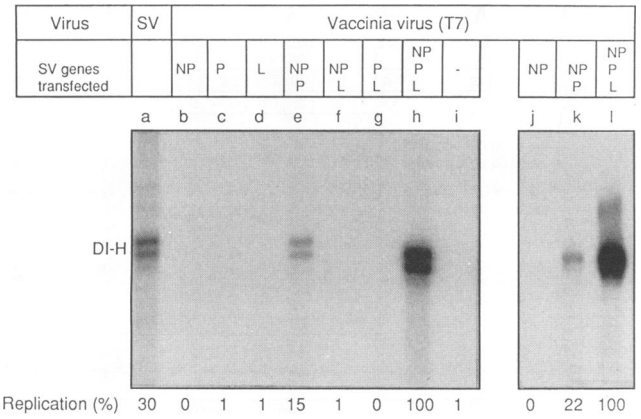

FIG. 3. Sendai virus proteins required for in vitro replication of DI-H virus RNA. Subconfluent A549 cells were infected with Sendai virus or VVT7, and the VVT7-infected cells were transfected with no DNA (−), pGEM-NP (2 μg), pGEM-P/C (5 μg; indicated as P), or pGEM-L (0.5 μg) as indicated at the top. Cytoplasmic extracts (lanes a through i) or the soluble protein fractions (lanes j through l) were incubated with purified DI-H virus, and the RNA replication products were analyzed as described in the legend to Fig. 1. Lanes j through l were exposed three times longer than lanes a through i. The percent replication is indicated on the bottom relative to transfection with all three plasmids as 100%.

case, however, may not be in a functional form, since it did not support nucleocapsid RNA replication.

With the establishment of the in vitro system we could test whether the NP, P, and L proteins must be coexpressed or could be synthesized individually and then mixed to restore activity. We found that mixing extracts from sets of cells in which each of the three proteins was expressed separately did not support DI-H RNA replication (data not shown). Maximal replication occurred when all three proteins were coexpressed in the same cell (Fig. 4, lane b). In this sample, an extract of mock-infected cells was mixed with that of the triply transfected cells as a control for any effect of additional cell protein in these mixed samples, and replication was about half of that observed when both sets of cells were transfected with all three plasmids (Fig. 4, lane a). No replication occurred with P and L, plus or minus added NP (Fig. 4, lanes c and j), or NP and L, plus or minus added P (Fig. 4, lanes d and i). Thus, NP and P were not functional when mixed after being expressed separately. The addition of L protein synthesized separately slightly increased the level of nucleocapsid RNA replication observed with NP and P (Fig. 4, lanes e and f) but clearly did not restore it to the level obtained when all three proteins were synthesized together. Interestingly, the addition of other combinations of proteins to the NP and P extract did not increase but rather inhibited replication (Fig. 4, lanes g and h). In these cases, the total level of P or NP plasmid exceeded the optimum and was probably inhibitory as seen earlier in the titrations of each plasmid in the same cells.

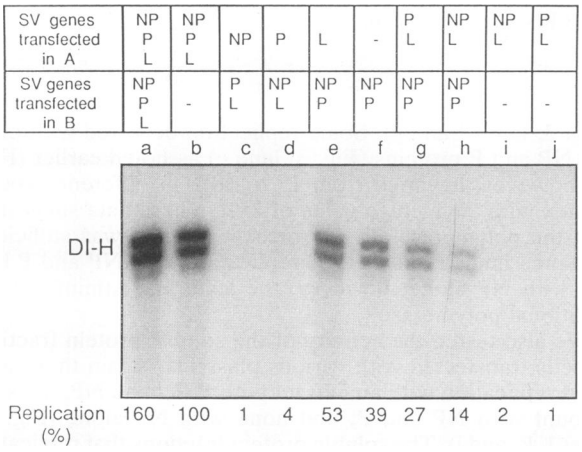

FIG. 4. Coexpression of Sendai virus proteins is required for in vitro replication of DI-H virus RNA. Subconfluent A549 cells were infected with VVT7, and then groups A and B were transfected separately with no DNA (−) or with the indicated combinations of pGEM-NP (2 μg), pGEM-P/C (5 μg; indicated as P), or pGEM-L (0.5 μg). Cytoplasmic extracts from ~2 × 10^6 cells in group A were combined with a similar amount of extract indicated below from group B. The replication of DI-H virus RNA was assayed as described in the legend to Fig. 1, and the percent replication is indicated at the bottom relative to transfection with all three plasmids as 100%.

We had originally shown that nucleocapsids of purified virus and those isolated from infected cells differed in the protein requirements for genome replication (1). When we tested the plasmid-driven expression system, however, the protein requirements for replication of intracellular nucleocapsids were, surprisingly, identical to those with purified virus as the template. No replication occurred with any of the individual plasmids, including NP, and maximal RNA replication was observed from extracts only when all three plasmids were transfected into the same cells (data not shown). Possible explanations for why NP prepared biochemically can support some replication, whereas NP expressed alone cannot, are discussed below.

Complexes of proteins required for in vitro DI-H RNA NP template replication. To study the requirements for expression of the RNA polymerase, we next utilized as template the DI-H RNA NP which lacked endogenous polymerase after its purification on CsCl gradients. All replication should now be dependent on the addition of exogenous viral proteins. With the coexpression of all three proteins, the RNA NP template was replicated at about 40% of the level observed with purified virus (Fig. 5A, lanes b and a, respectively), although approximately equivalent amounts of each template were added. That the RNA NP template was, in fact, free of endogenous polymerase was shown by the absence of replication with a cell extract containing only NP and P proteins compared with that of the control (Fig. 5A, lanes g and f, respectively). Interestingly, the addition of an extract with the L protein to the NP and P extract did not restore RNA replication (Fig. 5A, lane h). In other mixing experiments, the addition of various combinations of the polymerase subunits to an NP and P extract inhibited replication (Fig. 5A, lanes c to e). The inhibition again seemed to be due to altering the ratios of the proteins.

These data suggested that, in addition to an NP-P complex, the polymerase subunits might also be required as a complex. We tested this hypothesis by first lowering the P plasmid by half (P/2), because P is the protein in common between the two complexes, and the total level of each protein could not exceed fixed levels. Various combinations of plasmids were transfected, and cell extracts were assayed with the RNA NP template (Fig. 5B). Compared with nucleocapsid RNA replication with all three proteins (lane a), NP and P/2 gave no replication either alone (lane b) or with P/2 or L added separately (lanes c and d, respectively). However, the addition of P/2 and L that were coexpressed

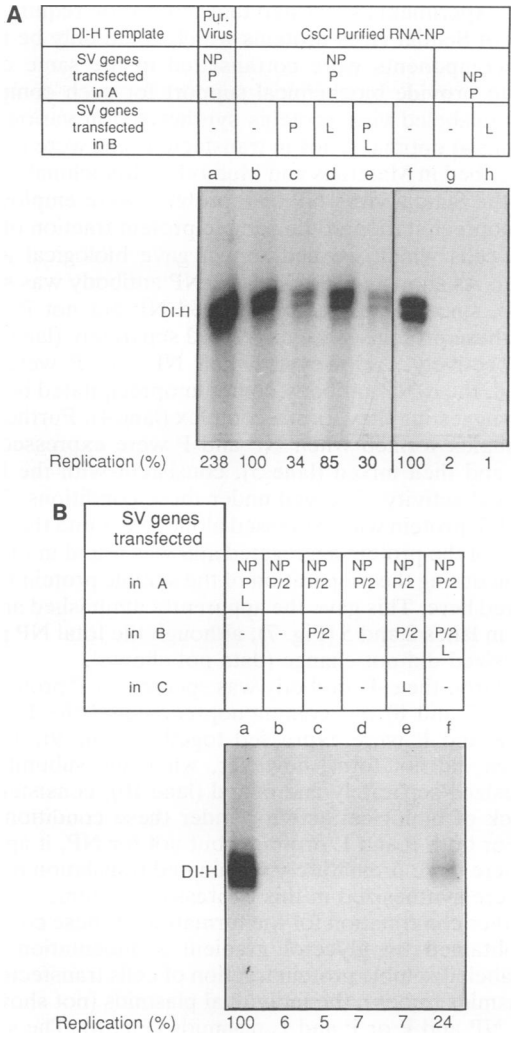

FIG. 5. Coexpression of Sendai virus proteins is required for in vitro replication of CsCl-purified DI-H RNA NP template. Subconfluent A549 cells were infected with VVT7, and then group A, B, or C was transfected with no DNA (−) or various combinations of pGEM-NP (2 μg), pGEM-P/C (5 μg; indicated as P), P/2 (2.5 μg), or pGEM-L (0.5 μg) as indicated above the gels. The cytoplasmic extracts from ~2 × 10^6 cells in group A were combined with a similar amount of extract indicated below from group B (panel A), and the cytoplasmic extracts from ~1.3 × 10^6 cells in group A were combined with similar amounts of extracts indicated below from groups B and C (panel B). The replication of either DI-H virus RNA (panel A, lane a) or CsCl-purified RNA NP template (panel A, lanes b through h, and panel B) in the mixtures of extracts was assayed as described in the legend to Fig. 1. The percent replication is indicated at the bottom of each panel relative to transfection with all three plasmids as 100%.

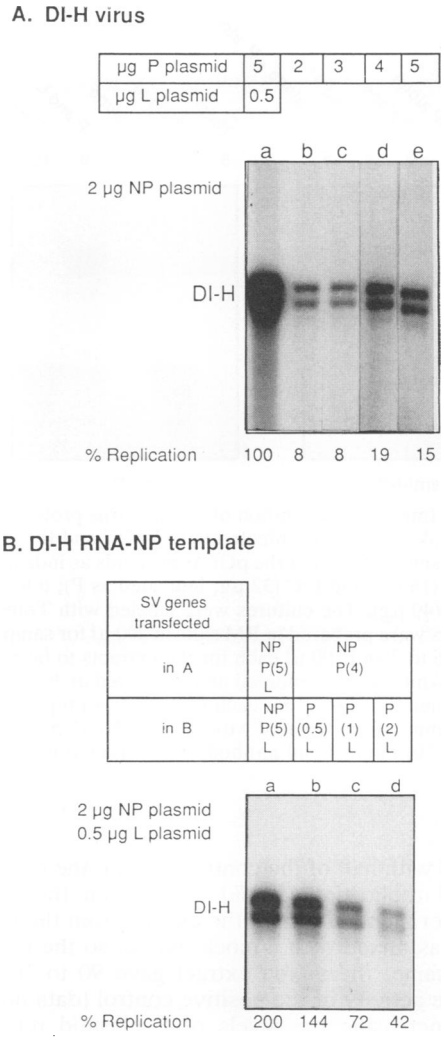

FIG. 6. Optimization of protein complexes required for DI-H RNA replication. Sets of A549 cells (group A or B) were infected with VVT7 and then transfected with constant amounts (in micrograms) of pGEM-NP or pGEM-L and increasing pGEM-P/C (designated P) as indicated above each panel. Cell extracts were prepared and assayed for replication of either DI-H virus RNA (panel A) or RNA-NP template (panel B) as described in Materials and Methods. The percent replication is indicated at the bottom relative to transfection with all three plasmids as 100%.

gave 24% of optimal replication (Fig. 5B, lane f), although mixing extracts of cells in which P/2 and L were synthesized separately gave no replication (lane e). This result suggested that P and L needed to be cotranslated for proper assembly into the polymerase complex.

Since only 24% of optimal RNA replication was achieved at P/2 in Fig. 5B, it appeared that either the proper ratios of components were not provided in one or both complexes or perhaps that a complex in which all three components were coassembled was required. To address this question, a series of reactions were set up to titrate the individual components of each putative complex. Using purified virus with endogenous polymerase as the template, extracts from cells transfected with a constant amount of NP (2 μg) and increasing amounts P were tested for replication. The optimum for P/C plasmid was at 4 μg (Fig. 6A, lane d) instead of the 5 μg needed in the complete system (Fig. 2). Then with the optimal NP-P/C plasmids transfected in one dish (Fig. 6A), P/C was titrated by cotransfection with constant L in another dish, utilizing the replication assay on RNA NP template lacking endogenous polymerase (Fig. 6B). The results showed that P/C and L were each optimal at 0.5 μg, and this mixture of extracts restored the replicative activity observed when all three proteins are coexpressed (Fig. 6B, lanes a and b). In this experiment, the control had twice (200%) the level of viral proteins used in the experimental reactions so,

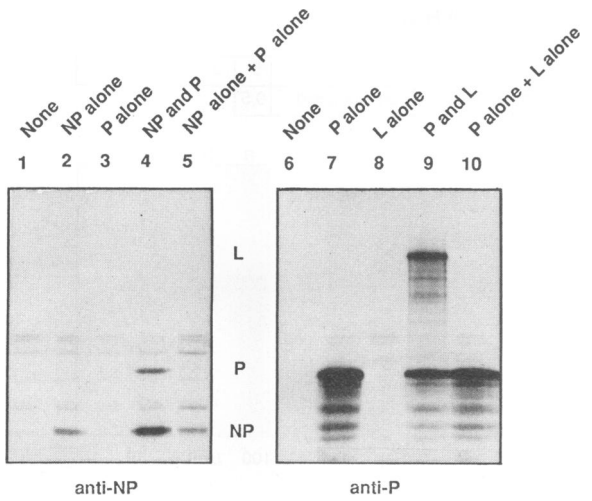

FIG. 7. Immunoprecipitation of Sendai virus protein complexes. A549 cells (4×10^7) were infected with VVT7 and then transfected with no plasmids (None) or the pGEM plasmids as indicated. Lanes: 1 to 5, NP (16 μg) and P/C (32 μg; indicated as P); 6 to 10, P/C (40 μg) and L (40 μg). The cultures were labeled with Trans-^{35}S-label, cell extracts were prepared in RM salts in 200 μl for samples in lanes 1 to 4 and 6 to 9 and 100 μl each for the extracts to be mixed (lanes 5 and 10), which were combined and incubated at 30°C for 1 h. The soluble protein fraction from each extract was prepared, and samples were immunoprecipitated with either α-NP (lanes 1 to 5) or α-P (lanes 6 to 10) monoclonal antibody as described in Materials and Methods.

compared with half of the control (100%), the mixed extract supported replication (144%) better than the control. In other experiments in which the extract from the transfected control was mixed with a mock extract so the total protein was the same, the mixed extract gave 90 to 100% of the replicative activity of the positive control (data not shown). Further increasing the levels of P plasmid relative to L inhibited replication (lanes c and d). Overall, the data suggested that two complexes, NP-P and P-L, synthesized separately under optimal conditions, can be mixed to restore the full activity seen with cosynthesis of all three proteins.

Biochemical analysis of viral protein complexes. The biological experiments suggested that replication required complexes of Sendai virus proteins which could only be formed if the components were cotranslated in the same cell. In order to provide biochemical support for such complexes, the radiolabeled viral proteins synthesized in various combinations at optimal ratios in transfected cells were analyzed as described in Materials and Methods. Monoclonal antibodies to the Sendai virus NP or P proteins were employed for immunoprecipitation of the soluble protein fraction of transfected cells which we had shown gave biological activity (Fig. 3). As shown in Fig. 7, the α-NP antibody was specific for NP, since it immunoprecipitated NP but not P protein when these proteins were expressed separately (lanes 2 and 3, respectively.). However, when NP and P were coexpressed, the α-NP antibody coimmunoprecipitated both proteins, suggesting they form a complex (lane 4). Furthermore, no complex formed when NP and P were expressed separately and then mixed (lane 5), consistent with the lack of biological activity observed under these conditions (Fig. 4). When NP protein was expressed alone, we found that a large portion of the protein aggregated and was found in the pellet fraction during the preparation of the soluble protein fraction analyzed here. This gave the apparently diminished amounts of NP in lanes 2 and 5 (Fig. 7), although the total NP protein synthesized did not change (data not shown).

Similarly, the α-P antibody was specific for P protein (Fig. 7, lanes 7 and 8) but coimmunoprecipitated the L protein when P and L were expressed together (lane 9). The P-L complex did not form, however, when the subunits were synthesized separately and mixed (lane 10), consistent with the lack of biological activity under these conditions (Fig. 5B). For both P and L proteins, but not for NP, it appeared that there were prematurely terminated translation products that were synthesized in this expression system.

Further confirmation for the formation of these complexes was obtained by glycerol gradient sedimentation of the radiolabeled soluble protein fraction of cells transfected with no plasmids (mock), the individual plasmids (not shown), or either NP and P or P and L plasmids (Fig. 8). The samples from each gradient were first immunoprecipitated with a mixture of α-SV and α-L antibodies to visualize all the viral proteins. In the mock-transfected cells (lanes c), there was a low background of vaccinia virus proteins. NP alone sedimented in fractions 2 and 3 (not shown), but when NP and P

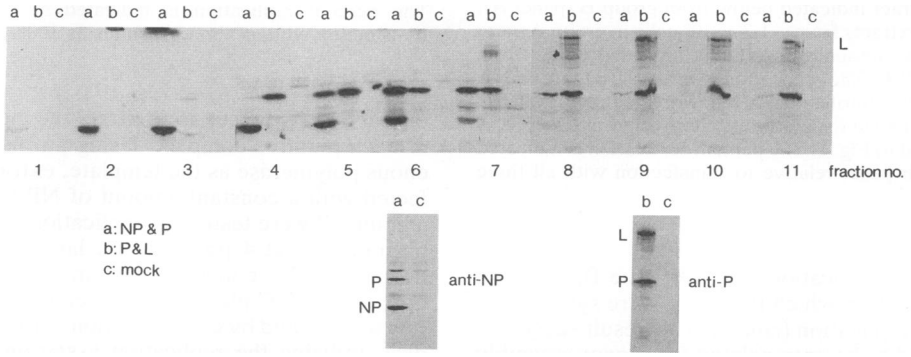

FIG. 8. Glycerol gradient centrifugation of Sendai virus NP-P and P-L complexes. A549 cells (4×10^7) were infected with VVT7; transfected with 20 μg of pGEM-NP and 40 μg of pGEM-P/C (lanes a), 40 μg of pGEM-P/C and 40 μg of pGEM-L (lanes b), or no plasmids (lanes c); and labeled with Trans-^{35}S-label. The soluble protein fraction from each cytoplasmic extract was separated on a 5 to 20% (vol/vol) glycerol gradient as described in Materials and Methods. Samples (1 ml) were collected from the top (fraction 1), and 0.5 ml was immunoprecipitated with a mixture of α-SV and α-L antibodies. Fraction 6 (0.5 ml; lanes a and c) was also immunoprecipitated with α-NP monoclonal antibody, and fraction 9 (lanes b and c) was immunoprecipitated with α-P monoclonal antibody (bottom panels). Sedimentation was from left to right. The locations of the viral NP, P, and L proteins are indicated.

were cosynthesized, NP now sedimented from fractions 2 to 7, that is, NP migrated with P protein sedimenting from fractions 5 to 8 (lanes a). P alone sedimented in fractions 5 to 8 (not shown). Immunoprecipitation of a sample with the α-NP monoclonal antibody in the area of overlap (fraction 6) showed that P coprecipitated (bottom panels) as the NP-P complex, as was also observed in the unfractionated sample (Fig. 7). When P and L were cosynthesized, P protein now sedimented beyond fraction 8 with the L protein in fractions 8 to 11 (lanes b). Immunoprecipitation of lanes b, fractions 9 to 11, with the α-P antibody coprecipitated the P and L proteins as a complex (shown for fraction 9; Fig. 8, bottom panels). The P-L complex had a rather broad sedimentation profile, suggesting that different multimers of the complex were forming.

DISCUSSION

We have developed an in vitro system for the replication of DI templates with extracts of cells expressing proteins from plasmids containing individual virus genes. Nucleocapsid RNA replication in these extracts was not only three- to fivefold better than that obtained with naturally infected cell extracts but also allowed the independent manipulation of each viral gene to identify essential protein-protein interactions. Optimal replication required all three nucleocapsid-associated proteins, NP, P, and L, even though some endogenous polymerase was present in purified virus. The critical parameter appeared to be the ratio of the plasmids used in the transfection, which presumably defines the relative amounts of each protein expressed in the cells. When either too little or too much of one protein relative to the other was present, the level of DI-H genome replication was suboptimal (Fig. 1 and 2). Similar critical ratios of proteins were previously observed in vivo for both Sendai virus (4) and vesicular stomatitis virus (21, 23) replication. The individual proteins alone or in any combination except NP and P could not support RNA replication from any template. The polymerase associated with virus did catalyze limited replication with just NP and P as the encapsidation substrate (Fig. 2B); however, coexpression of NP and P in the same cell was required, since mixing extracts expressing each protein separately gave no replication (Fig. 4). These data suggested that NP and P must form the functional complex concomitant with, or shortly after, their synthesis. Direct biochemical confirmation of the NP-P complex was shown by coimmunoprecipitation of both proteins by an antibody specific for one component either directly from a total extract or after fractionation on a gradient (Fig. 7 and 8). Furthermore, no protein complex or biological activity was found by mixing the individually expressed proteins.

When CsCl-purified RNA NP free of polymerase was used as template, replication was now absolutely dependent on exogenous NP, P, and L proteins. The NP-P complex gave no product, and, interestingly, addition of L protein synthesized separately could not rescue replication (Fig. 5A). These results suggested that the polymerase subunits might also form a complex. This was confirmed in the biological and biochemical assays in which coexpression of P and L gave both nucleocapsid RNA replication as well as coimmunoprecipitation and cosedimentation of P and L, whereas mixing of extracts with the subunits expressed separately did not (Fig. 5B, 7, and 8). These data are still formally compatible with two possible replication models--one in which two complexes, NP-P and P-L, are required or one with a single complex of all three proteins. Our indirect evidence favors the first model, since we found that by optimizing the ratios of NP and P expressed in one cell, and L and P in another, the two complexes can be mixed to restore the level of replication observed with all three proteins together (Fig. 6B).

From these data we propose that two separate complexes, NP-P and P-L, must be formed prior to their association with the nucleocapsid template for replication. Direct support for an NP-P complex comes from the immunoprecipitation and sedimentation analyses shown here (Fig. 7 and 8) as well as from previous work for several negative-strand RNA viruses. For Sendai virus, an NP-P interaction was also demonstrated by a protein-protein blotting technique and the binding site on NP was partially characterized (13). Conversely, Sendai virus P protein was shown to bind to holo-nucleocapsids (26) and to CsCl-purified RNA NP (17). In measles virus, the NP and P proteins were shown to interact specifically to keep the NP protein in the cytoplasm (15). Likewise, for vesicular stomatitis virus, an N-P complex has been shown to be necessary for nucleocapsid RNA replication and encapsidation in vitro (14, 24) and in vivo (18). Interestingly, in this study cotranslational assembly of the polymerase complex (P-L) also seemed to be required for functional replication activity, although in vitro reassembly of the purified polymerase subunits supported transcription for vesicular stomatitis virus (6) and Newcastle disease virus (11). Superimposed on this general model of assembly is the apparent negative regulatory role of the Sendai virus V and C nonstructural proteins (4, 5) which have been postulated to affect the assembly and/or function of the polymerase complex.

We had previously found that NP protein purified biochemically (from nucleocapsids) could support replication from intracellular nucleocapsids but not from those present in virus particles (1). Since intracellular nucleocapsids, but not virion nucleocapsids, are likely to contain templates with preinitiated RNA chains, this suggested that there might be viral proteins in addition to NP, which are required for the initiation of replication and encapsidation as opposed to elongation, which might require NP alone. In contrast to those data, we now show that when the viral proteins are expressed in vivo (by transfection) and used as crude cell extracts, the protein requirements for encapsidation were identical, namely an NP-P complex was required for replication of both DI-H virus and intracellular nucleocapsids. From the characterization of the putative complexes described here, there are probably several reasons for this apparent contradiction. First, in the plasmid expression system it appears that NP expressed alone was mostly aggregated and was not functional with any template. P must be coexpressed with NP, presumably forming a soluble complex that was the natural substrate for the encapsidation step with all templates. The purified NP protein utilized previously was required in much larger amounts (microgram levels), perhaps because only a small proportion of the purified protein was actually functional in comparison to the physiological levels of active NP-P complex formed in transfected cells. Nevertheless, the previous observation of the absence of replication with DI-H virus with purified NP still suggests that it is the NP-P complex which is essential for the initiation of encapsidation and that initiation could not be forced even with large amounts of purified NP, whereas in the elongation reaction it could. Apparently, the amount of soluble NP alone that can be achieved in transfected cells was simply not sufficient to support replication from intracellular nucleocapsids.

In summary, we present evidence that the cotranslational expression of the Sendai virus proteins in the complexes NP-P and P-L is essential for their biological activity in nucleocapsid RNA replication in vitro. The flexibility of this expression and assay system should facilitate further study of the interactions and domains of these essential viral proteins by directed mutagenesis of the cloned genes.

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REFERENCES

1. **Baker, S. C., and S. A. Moyer.** 1988. Encapsidation of Sendai virus genome RNAs by purified NP protein during in vitro replication. J. Virol. **62:**834–838.
2. **Calain, P., J. Curran, D. Kolakofsky, and L. Roux.** Submitted for publication.
3. **Carlsen, S. R., R. W. Peluso, and S. A. Moyer.** 1985. In vitro replication of Sendai virus wild-type and defective interfering particle genome RNAs. J. Virol. **54:**493–500.
4. **Curran, J., R. Boeck, and D. Kolakofsky.** 1991. The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. EMBO J. **10:**3079–3085.
5. **Curran, J., J.-B. Marq, and D. Kolakofsky.** The Sendai virus nonstructural C proteins specifically inhibit viral mRNA synthesis. Virology, in press.
6. **Emerson, S. U.** 1987. Transcription of vesicular stomatitis virus, p. 245–269. *In* R. R. Wagner (ed.), The rhabdoviruses. Plenum Press, New York.
7. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
8. **Galinski, M. S., and S. L. Wechsler.** 1991. The molecular biology of the paramyxovirus genus, p. 41–82. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York.
9. **Glazier, K., R. Raghow, and D. W. Kingsbury.** 1977. Regulation of Sendai virus transcription: evidence for a single promoter in vivo. J. Virol. **21:**863–871.
10. **Gotoh, H., T. Shioda, Y. Sakai, K. Mizumoto, and H. Shibuta.** 1989. Rescue of Sendai virus from viral ribonucleoprotein-transfected cells by infection with recombinant vaccinia viruses carrying Sendai virus L and P/C genes. Virology **171:**434–443.
11. **Hamaguchi, M., T. Yoshida, K. Nishikawa, H. Naruse, and Y. Nagai.** 1983. Transcriptive complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. Virology **128:**105–117.
12. **Hardy, W. R., and J. H. Strauss.** 1988. Processing the nonstructural polyproteins of Sindbis virus: study of the kinetics in vivo by using monospecific antibodies. J. Virol. **62:**998–1007.
13. **Homann, H. E., W. Willenbrink, C. J. Buchholz, and W. J. Neubert.** 1991. Sendai virus protein-protein interactions studied by a protein-blotting protein-overlay technique: mapping of domains on NP protein required for binding to P protein. J. Virol. **65:**1304–1309.
14. **Howard, M., and G. Wertz.** 1989. Vesicular stomatitis virus RNA replication: a role for the NS protein. J. Gen. Virol. **70:**2683–2694.
15. **Huber, M., R. Cattaneo, P. Spielhofer, C. Oervell, E. Norrby, M. Messerli, J.-C. Perriard, and M. A. Billeter.** 1991. Measles virus phosphoprotein retains the nucleocapsid protein in the cytoplasm. Virology **185:**299–308.
16. **Jenison, S. A., J. M. Firzloff, A. Langenberg, and D. A. Galloway.** 1988. Identification of immunoreactive antigens of human papillomavirus type 6b by using *Escherichia coli*-expressed fusion proteins. J. Virol. **62:**2115–2123.
17. **Kolakofsky, D., S. Vidal, and J. Curran.** 1991. Paramyxovirus RNA synthesis and P gene expression, p. 215–233. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York.
18. **La Ferla, F. M., and R. W. Peluso.** 1989. The 1:1 N-NS protein complex of vesicular stomatitis virus is essential for efficient genome replication. J. Virol. **63:**3852–3857.
19. **Leppert, M., L. Kort, and D. Kolakofsky.** 1977. Further characterization of Sendai virus DI RNAs; a model for their generation. Cell **12:**539–552.
20. **Leppert, M., L. Rittenhouse, J. Perrault, D. Summers, and D. Kolakofsky.** 1979. Plus and minus strand leader RNAs in negative strand virus-infected cells. Cell **18:**735–747.
21. **Meier, E., G. G. Harmison, and M. Schubert.** 1987. Homotypic and heterotypic exclusion of vesicular stomatitis virus replication by high levels of recombinant polymerase protein L. J. Virol. **61:**3133–3142.
22. **Moyer, S. A., and S. M. Horikami.** 1991. The role of viral and host cell proteins in paramyxovirus transcription and replication, p. 249–274. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York.
23. **Pattnaik, A. K., and G. W. Wertz.** 1990. Replication and amplification of defective interfering particle RNAs of vesicular stomatitis virus in cells expressing viral proteins from vectors containing cloned cDNAs. J. Virol. **64:**2948–2957.
24. **Peluso, R. W., and S. A. Moyer.** 1988. Viral proteins required for the *in vitro* replication of vesicular stomatitis virus defective interfering particle genome RNA. Virology **162:**369–376.
25. **Portner, A., K. G. Murti, E. M. Morgan, and D. W. Kingsbury.** 1988. Antibodies against Sendai virus L protein: distribution of the protein in nucleocapsids revealed by immunoelectron microscopy. Virology **163:**236–239.
26. **Ryan, K. W., and D. W. Kingsbury.** 1988. Carboxy-terminal region of Sendai virus P protein is required for binding to viral nucleocapsids. Virology **167:**106–112.
27. **Vidal, S., and D. Kolakofsky.** 1989. Modified model for the switch from Sendai virus transcription to replication. J. Virol. **63:**1951–1958.