Expression and Purification of Vesicular Stomatitis Virus N-P Complex from Escherichia coli: Role in Genome RNA Transcription and Replication In Vitro

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Vesicular stomatitis virus (VSV), the prototype rhabdovirus, contains a single-stranded, negative-sense genome RNA consisting of 11,161 nucleotides (27, 28). The genomic RNA is encapsidated by the 49-kDa nucleocapsid protein (N). In association with two proteins, L (241 kDa), the RNA-dependent RNA polymerase, and phosphoprotein P (30 kDa), the subunit of the RNA polymerase, it forms the ribonucleoprotein core (RNP) of the matured virion (12, 13). Two RNA synthetic events, transcription and replication, both initiate on this RNP template in vivo, whereas the former can take place efficiently in vitro in the presence of four ribonucleoside triphosphates and appropriate cations to synthesize a 47-nucleotide leader RNA and five monocistronic, capped and polyadenylated mRNAs (1). The replication event, on the other hand, required ongoing protein synthesis (11, 33). During replication, the transcriptase switches to the replicative mode and transcribes across the gene boundaries, synthesizing the full-length complement of the negative-strand genome RNA. A role of newly synthesized N protein in this switch in which the encapsidated N protein somehow attenuates transcription initiation at the gene boundaries and helps elongate RNA chains into the next gene, and so on, has been proposed (11, 22). The genome-length plus strand is concomitantly encapsidated by the de novo-synthesized N protein. The plus-strand RNP then serves as the template for the synthesis of minus-strand progeny virion RNA encapsidated with the N protein. Although several studies document such synthesis of genome-length RNA and its encapsidation in vitro by using infected cell RNP (9, 16, 18, 20, 24), the process is highly inefficient. Thus, the molecular mechanism underlying the switch from transcription to replication remains unclear. Since the N protein, involved in the crucial encapsidation step in replication, does not seem to exist free in the infected cells, but rather is complexed with the P protein (5), it is generally believed that the N-P complex probably carries out the switch from transcription to replication by its interaction with the nascent RNA chain, leading to the synthesis of plus-strand RNP (18, 23, 25, 26). The role of P seems to keep the N protein in a soluble replication-competent form.

Study of the role of N-P complex in transcription and replication in vitro is hindered by the paucity of purified biologically active complex obtained from either the infected cells (6, 7), expression in eucaryotic cells (29), or translation in vitro from encoded mRNAs (11, 19, 22). Recently, expression of N protein alone in a bacterial system produced a large amount of the protein which required a high salt concentration to keep it in solution and in a biologically active form (10). In this study, we coexpressed the N and P proteins in Escherichia coli, which resulted in efficient complex formation between the proteins, rendering N protein in a soluble form. Using viral RNP as a template, we demonstrate that purified N-P complex has a profound inhibitory effect on transcription in vitro; however, subsequent addition of host cell extract led to the synthesis of genome-length RNA, indicating a requirement of a host factor(s) in the initiation of the replicative process.

MATERIALS AND METHODS

Materials. All enzymes and biochemicals were obtained either from Boehringer Mannheim or from Sigma. Tissue culture reagents and media were purchased from Life Technologies, Inc.

Cell cultures and virus. Monolayers of baby hamster kidney (BHK-21; ATCC CCL 10) cells were grown in Eagle’s minimal medium containing 5% fetal calf serum. The cells were infected with VSV Indiana serotype. Mudd Summers strain, at a multiplicity of infection of 0.05, and the virus was purified as described previously (4).

Cloning, expression, and purification of N-P complex. The recombinant pET-3a vector containing the wild-type P gene was used as a template to amplify the P gene along with the T7 promoter and terminator flanking the insert. The primers used for amplification by the PCR method were 5′-ATTCACAGCTTTGGAAATTATACGACTCA3′ (upstream) and 5′-ATTCACACCGCTCAAAAACCCCTTCAA3′ (downstream); both primers contain a HindIII site along with a hexanucleotide clamp sequence upstream of the restriction site. The PCR-amplified products were then digested with HindIII and subsequently ligated into HindIII-digested pET-3a recombinant vector containing the N gene of VSV. The ligated DNA was transformed into E. coli DH5α. The positive clone was identified by restriction analysis.

For expression, the positive recombinant plasmid containing both N and P genes (pET-3a-N-P) was then introduced into E. coli BL21(DE3). For the production of recombinant N and P proteins, BL21(DE3) containing pET-3a-N-P was grown in LB broth. When the culture reached an A_{600} of 0.2, isopropylthio-
galactopyranoside (IPTG) was added to 0.5 mM, and growth was continued for another 2 h. The cells were then harvested by centrifugation. The cells were lysed by lysozyme-EDTA in the presence of 1% Triton X-100 followed by a centrifugation at 100,000 × g. The soluble supernatant (100) was collected, and the
FIG. 1. Cloning and coexpression of N and P genes in pET-3a. (A) The unique restriction site HindIII was used for ligating the P gene into pET-3a containing the N gene. P<sub>T7</sub> and T<sub>T7</sub> are the promoter and terminator of T7 RNA polymerase present upstream and downstream of NdeI and BamHI sites, respectively. The cloning strategy is described in detail in the text. (B) N and P proteins were coexpressed in E. coli BL21(DE3) and subjected to IPTG induction. Total cellular protein isolated from either the supernatant [N-P (sup)] or the pellet [N-P (pel)] was subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis by the method of Laemmli, and the gel was stained with Coomassie brilliant blue R-250. Lanes marked P and N are the individually expressed and purified bacterial P and N proteins from the pellet fraction; lane M contains molecular weight markers. (C) Western blot analysis of N-P (from both pellet and supernatant), using a polyclonal antibody against total virus. Lanes marked P and N are the control P and N proteins as described for panel B.
pellet was extracted by the Sarkosyl-octylglucoside method (14, 15). The final preparations were dialyzed against buffer A containing 25 mM Tris-Cl (pH 8.0), 50 mM NaCl, 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA, and 5% glycerol.

**Immunoprecipitation and Western blot analysis of N-P complex.** IPTG-induced N-P or N-P5 complex in a total volume of 100 μl of reaction mixture was incubated for 2 h at 30°C. Reactions were terminated by adding 100 mM HEPES (pH 7.0), 150 mM NH₄Cl, 6 mM KCl, 5 mM magnesium acetate, 1 mM DTT, 1 mM each ATP, GTP, and CTP, 100 μM UTP, 25 μCi of [α-32P]UTP, 10 μg of actinomycin D per ml, and 2 U of RNasin per ml. Reaction mixtures were incubated for 2 h at 30°C. Reactions were terminated by extraction with phenol-chloroform and precipitated with ethanol. Viral RNA products were analyzed in a 5% polyacrylamide gel containing 7 M urea.

**Binding of N-P to RNP.** Different concentrations of N-P were incubated with 5 μg of RNP per ml in a 100-μl transcription buffer in the absence of nucleoside triphosphates at 30°C for 30 min. The reaction mixture was then centrifuged through 30% (vol/vol) glycerol at 4°C. The pellet was collected as the RNP bound to exogenously added N-P. Binding of P protein to RNP was carried out similarly.

**In vitro replication of the genome RNA.** Viral RNA (5 μg/ml) was incubated in the presence of absence of uninfected BHK cell extract and bacterially expressed N-P or N-P complex in a total volume of 100 μl of reaction mixture containing 100 mM HEPES (pH 7.0), 150 mM NH₄Cl, 6 mM KCl, 5 mM magnesium acetate, 1 mM DTT, 1 mM each ATP, GTP, and CTP, 100 μM UTP, 25 μCi of [α-32P]UTP, 10 μg of actinomycin D per ml, and 2 U of RNasin per ml. Reaction mixtures were incubated at 30°C for 2 h. The replication products were purified by sedimentation on a 4.5-ml step CsCl gradient prepared by layering the bottom 2 ml of 40% CsCl, 2 ml of 20% CsCl, and 0.5 ml of 50% glycerol in the replication buffer described above. Centrifugation was carried out at 40,000 rpm for 20 h at 16°C in a Beckman SW50.1 rotor. The nucleocapsid band at the 20 to 40% CsCl junction was collected, dialuted with the replication buffer, and precipitated with ethanol. The RNA was then isolated by treatment with proteinase K (200 μg/ml) followed by phenol-chloroform extraction and ethanol precipitation. To check the synthesis of 425 RNA, the preparation was then run in a formaldehyde-agarose gel (21).

**Northern blot and slot blot hybridization of RNA.** In vitro-replicated RNA was either run in a formaldehyde-agarose gel and transferred to a nylon membrane or spotted onto a membrane in a slot configuration. T7 polymerase-transcribed, RNase-free DNA-treated, α-32P-labeled message-sense RNA of either P or N and was used as a probe to score for the genome-sense RNA synthesis. To score for the positive-sense genome-length RNA, an end-labeled oligonucleotide probe was used. Oligonucleotide was synthesized from the 3’ end of the genome-sense RNA, positions 1 to 30.

**RESULTS**

**Cloning and expression of N-P complex in E. coli.** To coexpress the N and P genes, we constructed a recombinant pET-3a expression vector containing both N and P genes but under two separate cassettes, each cassette having its own T7 promoter and terminator so that the two genes are expressed independently of each other (see Materials and Methods) (Fig. 1A). The pET-3a-N-P construct was then introduced into E. coli BL21(DE3), a lysogen containing lambda derivative prophage DE3 in which the T7 RNA polymerase gene is under the control of lacUV5 promoter (30). The transformants were induced with IPTG, and the total cellular proteins were electrophoresed. Figure 1B shows a Coomassie blue-stained protein profile of both the pellet and the supernatant fractions of the induced culture. Both N and P proteins were clearly discernible in both fractions. The authenticity of the expressed polypeptides was confirmed by Western blot analyses (Fig. 1C), which clearly demonstrated that both P and N proteins were expressed in the bacterial cell.

**Coexpression in E. coli results in N-P complex formation.** The rationale for coexpression of N and P in bacteria is to study whether the two proteins can form a complex in each other in the bacterial milieu. To study such complex formation, IPTG induction was carried out in the presence of [35S]methionine, and the labeled proteins were used for immunoprecipitation experiments. By using a monoclonal antibody against P protein, both N and P were precipitated from the pellet as well as from supernatant fractions (Fig. 2A and B). However, by using a monoclonal antibody of N protein, it was not possible to immunoprecipitate the P protein in the complex (data not shown). A possible explanation could be that the epitope against which the N monoclonal antibody was raised is not exposed and thus failed to react with the antibody in the complex. Moreover, the monoclonal antibody also did not precipitate any free N protein from the extract, suggesting that all N protein present in the extract is complexed with the P protein. To further verify the complex formation between N and P and to purify the N-P complex free from any uncomplexed N and P proteins, we performed Sephacryl S-300 gel filtration chro-
matography. As shown in Fig. 2C, both N and P proteins coeluted in the void volume (fractions 1 to 36). It can be seen from the elution profile that the N and the P proteins were present in each fraction in the same ratio, indicating that they eluted as a discrete complex.

**N-P complex inhibits RNP transcription in vitro.** To study the effect of the expressed N-P complex on genome RNA transcription, we purified the N-P complex from the pellet fraction and used it in an in vitro transcription reaction containing RNP purified from the virus. The purified N-P complex did not exhibit any detectable RNase activity in vitro toward exogenously added RNA (data not shown). While the E. coli-expressed P protein alone had no effect on transcription, the purified N protein (2.5 μg/ml) from E. coli inhibited transcription to an appreciable extent (75%) (Fig. 3). Interestingly, addition of N-P complex at a concentration of 1 μg/ml inhibited transcription completely, indicating that the effect of N-P complex on transcription is more drastic than that of the N protein alone. Since N protein is not completely soluble in the physiological salt concentration, it appears that the soluble N-P complex exerts a profound effect on viral transcription at low concentrations. Bacterial extract made from cells transformed with the vector as described above does not have any inhibitory effect on transcription (data not shown).

**Binding of N-P to RNP.** To determine whether the N-P-mediated inhibition of transcription was due to the binding of N-P to RNP, N-P was first incubated in the presence of RNP and used in a transcription reaction in vitro. In control experiments, RNP after binding to N-P without pelleting was directly used in the transcription reaction. As shown in Fig. 6, in both cases, similar patterns of inhibition of transcription, i.e., drastic inhibition of transcription at 2 μg of N-P per ml, were observed. These observations strongly suggest that the N-P complex first binds to the RNP, which then exerts its inhibitory effect on transcription.

**Initiation of replication by N-P complex: requirement of a host factor(s).** The observation that the N-P complex effectively inhibited transcription in vitro led us to investigate whether the transcriptionally arrested RNP requires additional cellular components to allow interaction of nascent RNA chains with the N-P complex for initiation of replication. We carried out a transcription reaction with RNP in the presence of the N-P or N-P5 complex and uninfected BHK cell extract as the source of putative host factor(s). Northern blot analyses were then carried out to detect genome-sense RNA synthesis. As shown in Fig. 7A and B, in the absence of uninfected cell extract, the level of the input genome-sense RNA was discernible when the RNA was hybridized with 32P-labeled message-sense P RNA riboprobe as described in Materials and Methods. On the other hand, when uninfected cell extract was added, the level of hybridization signal in the genome RNA (42S) was increased 3.5- to 5-fold when N-P and N-P5 complexes were used. Since the efficiency of synthesis of 42S genome-sense RNA is not very high, it seemed possible that addition of N-P along with uninfected cell extract somehow increases the efficiency of purification on input RNA rather
than its de novo synthesis. To rule out that possibility, end-labeled genome-sense oligonucleotide probe from the leader region (3' end of the genome) was used in a blot experiment. This probe will detect only the full-length positive-sense genome RNA. As shown in Fig. 7C, in the presence of cell extract 42S positive-strand RNA was detected when N-P or N-P5 was added in the RNP. These results clearly indicate that the in vitro replication system was able to synthesize full-length plus-strand 42S RNA. Thus, it seems that both positive- and negative-sense genome RNAs are synthesized in vitro in the presence of N-P complexes, and a host factor(s) is required to initiate the replication reaction in vitro. It should be noted that we also carried out the replication reaction in the presence of α-32P-labeled ribonucleoside triphosphate and analyzed the labeled RNA products by agarose gel electrophoresis (data not shown). However, a significant level of nonviral RNA, which masked the synthesized virus-specific 42S RNA in the autoradiogram, was synthesized. Although we have not identified this unusual RNA synthetic reaction, it appears that this nonviral RNA (which is synthesized even in the absence of RNP) is possibly made by the contaminant T7 RNA polymerase in the purified N-P complex from bacteria which copies cellular RNAs in the BHK cell extract (8). The Northern blot analyses, as shown in Fig. 7, clearly avoided this problem.

Finally, we studied the concentration dependence of the replication reaction in response to N-P or N-P5 in the presence of uninfected cell extract. Figure 8 shows the slot blot hybridization analyses of the replication products in the presence of cell extract and various concentrations of N-P or N-P5. The 32P-labeled N mRNA was used as the probe in these experiments to detect the synthesis of negative-sense genome RNA. The results clearly show that there is a proportional increase in genome-sense RNA synthesis with increasing concentrations of added N-P or N-P5 complex. Since N-P5 complex was as efficient as N-P in replication, we conclude that phosphorylation in domain I is not required for the replication of genome RNA.
DISCUSSION

The data presented in this report demonstrate that two genes of VSV, the N and P genes, can be coexpressed in bacteria without affecting the expression of each other (Fig. 1). Moreover, coexpression of N and P proteins resulted in efficient N-P complex formation which rendered the otherwise insoluble N protein completely soluble at 50 mM NaCl. This bacterial coexpression system provided the opportunity to obtain soluble N-P complex in large amounts free from eucaryotic cellular proteins for in vitro studies. It is important to note that expression of N protein alone in bacteria makes it completely insoluble and that high-salt (1 M NaCl) extraction of the bacterial inclusion bodies is required to obtain soluble N protein (10). However, coexpression of P protein led to excellent recovery of N protein from the soluble fraction, confirming the notion that the P protein, by complexing with the N protein, directly contributes to the solubilization of the latter protein. The complex formation of N and P in bacteria was confirmed by coimmunoprecipitation of radiolabeled N protein with the polyclonal antibody against P protein (Fig. 2A and B). Moreover, coelution of these two proteins from a gel filtration column at 350,000 molecular weight range further confirms the formation of a complex between the two proteins (Fig. 2C). Since P protein in the N-P complex is not phosphorylated in bacteria, phosphorylation of P protein seems not to be involved in the complex formation, as previously shown in a two-hybrid system (31, 32). This conclusion is further supported by the observation that the phosphorylation-negative P mutant (P5) can efficiently form a complex with N protein in bacteria. At this point, we do not know whether N-P complex formed in bacteria is structurally identical to the corresponding complexes formed in vivo in eucaryotic cells. Nevertheless, the availability of bacterially expressed N-P complex provides an opportunity to study its role in the replication process in vitro.

Here, we demonstrate for the first time that purified bacterially expressed N-P complex drastically inhibits transcription mediated by viral RNP (Fig. 3 and 4), both for the synthesis of leader RNA and for that of mRNA in vitro. A 30-fold molar excess of N-P over genome RNA appears to inhibit the transcription reaction almost completely. The inhibition of transcription seems to be mediated by the direct binding of N-P complex to RNP (Fig. 6) in a dose-dependent manner. The N-P complex exerts its effect on transcription at the level of either initiation or elongation, since addition of N-P after the onset of transcription rapidly inhibits RNA synthesis (data not shown). The precise mechanism by which N-P complex inhibits
transcription by binding to the RNP complex is unclear. It is possible that N-P complex binds directly to the RNA polymerase (L-P) bound to the N-RNA template, thus arresting its function. In fact, we observed that when transcription reconstitution is carried out in the presence of L, P, and N-RNA template, the N-P complex also drastically inhibited transcription (data not shown). These results suggest that N-P complex may interact with any one of the components of the transcription complex, thereby inhibiting transcription. Detailed study along this line would provide insight into the mechanism of transcription inhibition mediated by the N-P complex.

Interestingly, when BHK cell extract was added to the transcription-arrested RNP, both negative- and positive-sense genome RNAs were detected by using appropriate probes and Northern blot analyses (Fig. 8), indicating that a cellular protein(s) is required for the replication reaction. It is important to note that the mutant P5 complexed with N (N-P5) was as active as the wild-type P complex (N-P) but drastically inhibited transcription, thus keeping N protein in a transcriptionally inactive form. It is possible that N-P complex binds directly to the RNA polymerase template, the N-P complex also drastically inhibited transcription, whereas phosphorylation of the P protein is necessary to switch L-P to L-N-P, thereby inhibiting transcription. Detailed study along this line would provide insight into the mechanism of transcription inhibition mediated by the N-P complex.

FIG. 8. In vitro replication of genome RNA with various concentrations of N-P complex. An in vitro replication reaction was carried out in the presence (+) or absence (−) of viral RNP and uninfected cell extract (UCE) at different concentrations of N-P or N-P5. CsCl-banded encapsidated RNA samples were spotted onto a nylon membrane in a slot configuration and hybridized with radiolabeled N message-sense RNA as a probe. The numbers above each slot represent the slot number, and the numbers at the bottom represent the concentrations of N-P and N-P5 used.

The results presented above raise the important issue of whether N-P complex indeed regulates transcription in the infected cells and whether the observed inhibition of transcription in vitro is a prerequisite for the initiation of replication by a putative host protein(s). It is difficult to demonstrate this contention because these two reactions cannot be studied in isolation within the cellular milieu. However, experiments are in progress to measure transcription and replication reactions at early steps of the virus life cycle to address these issues. If indeed N-P complex regulates transcription, it is reasonable to speculate a scenario by which the switch from transcription to replication takes place on the RNP in the infected cells. Since N and P genes are the most proximal to the 3′ end of the transcribing genome, synthesis of N and P proteins results in immediate complex formation, thus keeping N protein in a soluble and replication-competent form. The P protein, once complexed with the N protein, is not required to be phosphorylated in domain I by casein kinase II for its replication function, whereas phosphorylation of the P protein is necessary to interact with the L protein to form the active RNA polymerase complex. As the concentration of N-P complex rises in the cell following infection, it acts on the RNP to down-regulate transcription and in association with a putative host protein(s) switches transcription to replication. The N protein in the complex enwraps the nascent RNA chains of both positive- and negative-sense genome-length RNAs. As the concentration of N-P complex falls below a threshold value, replication ceases and transcription ensues, and this cycle continues during the life cycle of the virus. Development of a completely reconstituted replication system would be necessary to prove this hypothesis.

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