

Leader RNA of Rinderpest virus binds specifically with cellular La protein: a possible role in virus replication

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Abstract

Rinderpest virus (RPV) is an important member of the *Morbillivirus* genus in the family Paramyxoviridae and employs a similar strategy for transcription and replication of its genome as that of other negative sense RNA viruses. Cellular proteins have earlier been shown to stimulate viral RNA synthesis by isolated nucleocapsids from purified virus or from virus-infected cells. In the present work, we show that plus sense leader RNA of RPV, transcribed from 3' end of genomic RNA, specifically interacts with cellular La protein employing gel mobility shift assay as well as UV cross-linking of leader RNA with La protein. The leader RNA synthesized in virus-infected cells was shown to interact with La protein by immunoprecipitation of leader RNA bound to La protein and detecting the leader RNA in the immunoprecipitate by Northern hybridization with labeled antisense leader RNA. Employing a minireplicon system, we demonstrate that transiently expressed La protein enhances the replication/transcription of the RPV minigenome in cells. Sub-cellular immunolocalization shows that La protein is redistributed from nucleus to the cytoplasm upon infection. Our results strongly suggest that La protein may be involved in regulation of Rinderpest virus replication.

Keywords: Rinderpest virus; Cellular La protein; Leader RNA

1. Introduction

Rinderpest virus (RPV) belongs to the *Morbillivirus* genus in the family Paramyxoviridae. The 15,882-base-long negative sense RNA genome is encapsidated with the nucleocapsid protein N to form the ribonucleoprotein (RNP) core. The transcriptionally active RNP contains two additional viral proteins, the large protein L and the phosphoprotein P, which together constitute the viral RNA-dependent RNA polymerase (RdRP). Immediately after virus infection, the viral polymerase carries out transcription to produce a 52-nt-long leader RNA (*le*-RNA) and six mRNAs. In replication mode, full-length antigenomic RNA followed by genomic RNA is synthesized by the polymerase complex. For viruses in the Paramyxoviridae family, cellular proteins have been shown to participate in the regulation of gene expression (Moyer et al., 1986, 1990; De et al., 1995; Ghosh

et al., 1996). The involvement of a number of cellular proteins in transcription and replication of RNA genomes, in close association with 3' and 5' *cis*-acting elements of genomic RNA, and their modes of action have been reviewed earlier (De et al., 1997; Lai, 1998). In the review, an attractive possibility has been proposed, where the interaction of cellular proteins at 5' and 3' ends of genomes has been thought to provide a mechanism for the use of only intact RNAs as templates for replication and transcription.

One of the major cellular factors involved in virus life cycle is the host protein kinase. The P protein, which is the major phosphoprotein of RPV, belongs to the family of proteins, which undergo phosphorylation by the host cell kinases as has been observed in Human Para Influenza Virus (HPIV3) (Huntley et al., 1995), Measles Virus (MV) (Das et al., 1995), Canine Distemper Virus (CDV) (Lui et al., 1997), and Sendai Virus (SeV) (Huntley et al., 1997). In RPV, casein kinase II (CKII) has been identified as the host kinase that phosphorylates the P protein (Kaushik and Shaila, 2004). Besides the host kinases, several other cellular factors are believed to play an important role in the activation of viral polymerase.

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It is becoming increasingly clear that certain cellular RNA-binding proteins are specifically associated with viral RNAs, such as Vesicular Stomatitis Virus (VSV) and rabies virus leader RNA (Kurilla and Keene, 1983; Kurilla et al., 1984; Wilusz et al., 1983), MV *cis*-acting RNAs (Leopardi et al., 1995), and HPIV3 *cis*-acting RNAs (De et al., 1996). Cellular La protein has been shown to interact with *cis*-acting RNA of HPIV3 (De et al., 1996). La protein was shown to specifically interact with the viral *cis*-acting RNAs containing the 3' non-coding region of genomic RNA and the plus sense *le*-RNA sequence, in vivo and in vitro. However, the consequences on viral RNA synthesis such interactions would bring about have not been addressed so far. The biologic significance of these interactions between viral RNAs and the cellular proteins is still not understood.

The unique transcription map position of the *le*-RNA indicates that it may play an important role in viral replication by providing the necessary regulatory sequence for encapsidation (Leppert et al., 1979). After infection, *le*-RNA of VSV was also found to be present in the host nucleus and thus believed to play a role in the termination of host transcription (Kurilla et al., 1982). For RPV, in the in vitro transcription system employing purified RNP derived from infected cells or from purified virus, addition of host cell extract stimulated the transcription of viral mRNAs, which indicated a role for cellular factors (Ghosh et al., 1996). In order to understand the role of *le*-RNA in the regulation of RNA synthesis and to identify the host proteins involved in viral RNA synthesis as well as their specific interaction with the *le*-RNA of RPV, we examined the specific interaction of cellular proteins with the 3'-leader RNA of Rinderpest virus. We demonstrate that cellular La protein forms a specific complex with RPV *le*-RNA (complementary to 3' genomic sequence) in vitro as well as in vivo. La protein was found to be co-localized with the viral RNA in RPV-infected cells. Further, La protein has been shown to increase viral replication/transcription in a minireplicon assay.

2. Methods

2.1. Viruses, cells, plasmids, and antibodies

A tissue culture-adapted vaccine strain of RPV (RBOK, the original attenuated Kabete 'O' strain of RPV) was obtained from the Institute of Animal Health and Veterinary Biologicals, Bangalore, India. A549 cell line (human lung carcinoma cells) was obtained from ATCC (USA). HeLa as well as BSC-1 (monkey kidney epithelial cells) cell lines were procured from National Centre for Cell Science, Pune, India. The RPV-L cDNA clone (pPol10) and RPV-N clone (pKSN1) (Baron and Barrett, 1995) were obtained from Dr. M. Baron, Institute for Animal Health, Pirbright laboratory, UK. The full-length P gene was sub-cloned from the parent plasmid p335 containing the P gene with one edited amino acid extra (508 aa) into pRSET-B vector for expression

(Kaushik and Shaila, 2004). Two oligonucleotides corresponding to 56 nucleotides of the 3' end of the viral genome, each containing *Eco*RI and *Not*I restriction enzyme sites, were synthesized. The length of each oligonucleotide was 80 nt. Equimolar concentration of the two sequences was annealed in 0.3 M NaCl by heating at 96 °C for 5 min and cooling slowly at room temperature for a period of 30 min. The double stranded oligonucleotide was digested with *Eco*RI and *Not*I and ligated to the pBS KS (+) vector digested with same enzymes and transformed into *Escherichia coli* DH5 α . Positive recombinant clones were identified by colony hybridization using end-labeled oligonucleotide. One strongly positive clone, pBS-LN-1, was used to synthesize (+) sense *le*-RNA (Niraj Dhar and M.S. Shaila, unpublished data). The human La cDNA present in pET-La (generous gift from Prof. Jack Keene, Duke University) was sub-cloned into *Bam*HI and *Eco*RI sites of pRSET-A vector (Invitrogen) after PCR amplification with the primers 5'-GACCGGATCCATGGCTGAAAATGG-3' and 5'-CGTAGAATTCCTACTGGTCTCCAG-3'. Similarly, the full-length La gene was released from pET-La by digestion with *Bgl*II and *Eco*RI, and the fragment was inserted between *Bam*HI and *Eco*RI sites in pcDNA3 vector (Invitrogen) to generate pcDNA3-La. Rabbit polyclonal antibodies against purified bacterially expressed RPV-P (Kaushik and Shaila, 2004), RPV-L (Chattopadhyay and Shaila, 2004), RPV-N protein (Kaushik et al., 2001), and La protein were used in this study.

2.2. Expression and purification of La protein

La protein was expressed as His-tag fusion at the N-terminus in *E. coli* strain BL21 (DE3). Transformed cells were grown in LB medium containing 100 μ g/ml ampicillin at 37 °C and induced with 0.4 mM isopropylgaloctopyranoside (IPTG) at OD of 0.6 and grown for further 4 h. The cells were harvested and lysed by sonication in lysis buffer (150 mM NaCl in 50 mM Tris-HCl, pH 8, supplemented with 2 mM PMSF). The supernatant after centrifugation was subjected to purification through Ni-NTA agarose (Qiagen, USA). The purified protein was dialyzed for 4 h against dialysis buffer containing 50 mM Tris (pH 7.4), 25 mM KCl, 1 mM DTT, 0.2 mM PMSF, and 20% (v/v) glycerol. The purity was verified by silver-stained gel analysis and immunoblotting using anti-La polyclonal antibodies raised in rabbit. The recombinant La migrates higher (around 55 kDa) than endogenous La protein due to the presence of His-tag upstream of La gene.

2.3. In vitro transcription

The first 56-nt region from the 3' end of genomic RNA of RPV was cloned in Bluescript KS (+) vector employing a synthetic oligonucleotide pair complementary to each other under T7 promoter (pBS-LN-1 clone). The anti-sense sequence is under the control of T3 promoter. The expected

size of the T7 transcript corresponding to (+) sense *le*-RNA transcript is 72 nt whereas that of the anti-sense RNA is 70 nt. The pBS-LN-1 DNA, linearized with *Hind*III, was used as the template for sense *le*-RNA synthesis by T7 RNA polymerase, whereas *Sac*I-digested DNA was used as the template for the synthesis of anti-sense *le*-RNA by T3 RNA polymerase. The reactions were carried out in transcription buffer containing 40 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 10 units of Rnasin, and 500 μM each of ATP, GTP, CTP, UTP, 1 μg linearized template, and 20 units of RNA polymerase at 37 °C for 1 h. For the synthesis of labeled RNA, instead of 500 μM UTP, 100 μM UTP was used along with 10 μCi α-³²P-UTP (3000 μCi/mmol). The transcripts were purified by phenol-chloroform extraction followed by ethanol precipitation in the presence of 2 μg yeast tRNA.

2.4. Gel mobility shift assay

[³²P]-labeled RPV *le*-RNA (50,000 cpm) was incubated with either total cell extract (2 μg) or 1.0 μg of purified recombinant La protein (unless otherwise mentioned) in RNA-binding buffer containing 10 mM Tris-HCl, pH 8.0, 15 mM KCl, and 5 mM MgCl₂ at 25 °C for 15 min in 10–20 μl reaction volume. The samples were subjected to electrophoresis on 6% non-denaturing acrylamide gel containing 0.5× TBE at constant voltage (120 V) for 2 h at 4 °C. For competition assay, purified La protein was incubated with unlabeled competitor RNAs in binding buffer for 15 min prior to incubation with the labeled RNA.

2.5. UV cross-linking of *le*-RNA–protein complex

[³²P]-labeled *le*-RNA was incubated with purified recombinant La protein (1 μg) at 30 °C for 15 min in RNA binding buffer (used for EMSA) and then irradiated with a UV lamp for 10 min. The mixture was treated with 20 μg RNase A (Sigma) at 37 °C for 30 min to digest the unbound RNA. The protein–nucleotidyl complexes were electrophoresed on 10% SDS-polyacrylamide gel (PAGE) followed by phosphorimaging.

2.6. Filter-binding assay

Filter-binding assay was carried out to measure the binding affinity of recombinant La protein with the positive and negative sense *le*-RNA. The incubation conditions are the same as that described for gel mobility shift assay, keeping the total RNA amount constant (50,000 cpm) while increasing the concentration of purified protein. The reaction mixtures were passed through nitrocellulose filters (0.45 μm, size), equilibrated with binding buffer, allowing protein–RNA complexes to be retained on the filter and unbound RNA to pass through. The filters were washed with 4 ml of binding buffer under low vacuum. The nitrocellulose filters were dried, and the radioactivity retained was mea-

sured in a liquid scintillation spectrometer. The percentage of counts retained was plotted against the concentration of protein used. The dissociation constant for binding (K_d) was determined as the concentration of the protein where 50% of the RNA remained bound to the protein as a complex.

2.7. Transfection

Confluent monolayer of A549 cells (1 × 10⁶ cells/35-mm dish) grown in F12 HAM (GIBCO-BRL) supplemented with 5% newborn calf serum was washed with PBS and then transfected with 5 μg of appropriate plasmid DNAs with lipofectamine according to the manufacturer's protocol (GIBCO-BRL) in OPTI-MEM medium.

2.8. Immunoprecipitation of La protein bound to leader RNA

A549 cells (9-cm dish) were infected with RBOK strain of RPV with or without transfection with 5 μg of pcDNA3-La DNA (12 h before virus infection). The cell lysates, prepared 36 h after transfection (100 μl) (in 10 mM Tris-HCl, pH 8.0, 0.01%, Triton X-100, 150 mM NaCl and 1 mM PMSF), were pre-cleared by treatment with rabbit pre-immune serum and protein A-sepharose beads. Anti-La antibody (1:3000 titer) was incubated with 10% suspension of protein A-sepharose beads for 1 h. The beads were washed twice with wash buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Noidet P-40) and added to the pre-cleared supernatant. After 4 h of incubation at 4 °C, the beads were washed thrice with wash buffer and suspended in 100 μl of 100 mM Tris-HCl, pH 8.0. The RNA present in the immunoprecipitates was extracted with phenol-chloroform and ethanol precipitated. The isolated RNA was subjected to Northern blot hybridization using [³²P]-radiolabeled antisense *le*-RNA probe (1 × 10⁷ cpm). A portion of the immunoprecipitated sample was also subjected to SDS-10% polyacrylamide gel electrophoresis and immunoblotted. The protein was detected after immunostaining using enhanced chemiluminescence detection kit (Amersham, USA) following the manufacturer's protocol.

2.9. Isolation of intracellular nucleocapsids

Nucleocapsids were purified from virus-infected cells as described earlier (Ghosh et al., 1996) with few modifications. Infected cells showing about 70% CPE were washed and scraped into PBS followed by centrifugation at 2000 × g. The cell pellet was resuspended and incubated in hypotonic buffer (10 mM sodium phosphate, pH 7.4 and 1 mM PMSF) for 10 min and lysed by the addition of NP-40 to a final concentration of 1% (w/v). The nuclei were removed by centrifugation at 1000 × g for 2 min. The supernatant was centrifuged at 170,000 × g in a Ti-50 rotor. The pellet was resuspended in PBS and loaded on a discontinuous CsCl step gradient (40, 30, and 20% on a 5% sucrose cushion). The

sample was subjected to centrifugation using SW50 rotor at $210,000 \times g$ for 4 h. The visible band of nucleocapsid was collected from the 30% region. The nucleocapsids were resuspended in PBS and recentrifuged at $170,000 \times g$ in a Ti-50 rotor for 30 min.

2.10. *In vivo* replication–transcription assay

The viral minigenome pMDB8A, carrying the 3' regulatory sequence (leader region), transcription/replication start regions, and 5' trailer sequences flanking a reporter gene (chloramphenicol acetyltransferase, CAT) ORF driven by T7 promoter and terminator and delta ribozyme, was used for the *in vivo* transcription replication assay (Baron and Barrett, 1997). The transcript from this minigenome is anti-genomic sense and it is replicated to genomic sense RNA by the virus proteins L, N, and P expressed by co-transfected plasmids in A549 cells infected with recombinant vaccinia virus expressing T7 RNA polymerase. The newly synthesized genomic RNA is then transcribed and translated to give CAT protein.

A549 cells (1×10^6 cells/35-mm dish) were infected with recombinant vaccinia virus, v TF7.3, at a multiplicity of infection of 10 at 37°C . At 1 h post-infection, the cells were washed with PBS, transfected with $1 \mu\text{g}$ each of pMDB8A and different ratio of pKS-N1 (the N cDNA in pBluescript KS (+) and pRP6 (wt P cDNA) to Pol10 (the L gene in pGEM5Zf+) in 1 ml OPTI-MEM. At 24 h post-transfection, the cells were harvested and the amount

of CAT protein made was estimated by enzyme-linked immunosorbent assay (Roche Molecular Biochemicals). The sub-optimal ratio of N and P to L at which the reporter gene product CAT could be measured was determined and used for subsequent experiments where variable concentrations of pcDNA3-La DNA was also co-transfected along with N, P, and L gene constructs.

2.11. Northern blot analysis

Isolated *in vitro* synthesized RNA was analyzed on 1.5% MOPS–formaldehyde agarose gel and transferred to a Hybond N membrane by capillary transfer. The transferred RNA was UV cross-linked. [^{32}P]-labeled sense or anti-sense *le*-RNA (1×10^7 cpm) was used for hybridization in 0.1% SDS, 50% formamide, $5 \times \text{SSC}$, 50 mM NaPO_4 , pH 6.8, 0.1% sodium pyrophosphate, $1 \times$ Denhardt's solution, 50 $\mu\text{g/ml}$ sheared Herring sperm DNA. The blot after washing was autoradiographed.

2.12. Confocal microscopy

Sub-confluent monolayer of A549 cells grown on coverslips was infected with RPV at a multiplicity of infection of 10. Cells after 36 h were washed with PBS and fixed with 70% acetone–30% methanol solution at -20°C . The cells were air-dried, followed by incubation with anti-La polyclonal antibody (1:1000 dilution). Uninfected cells were

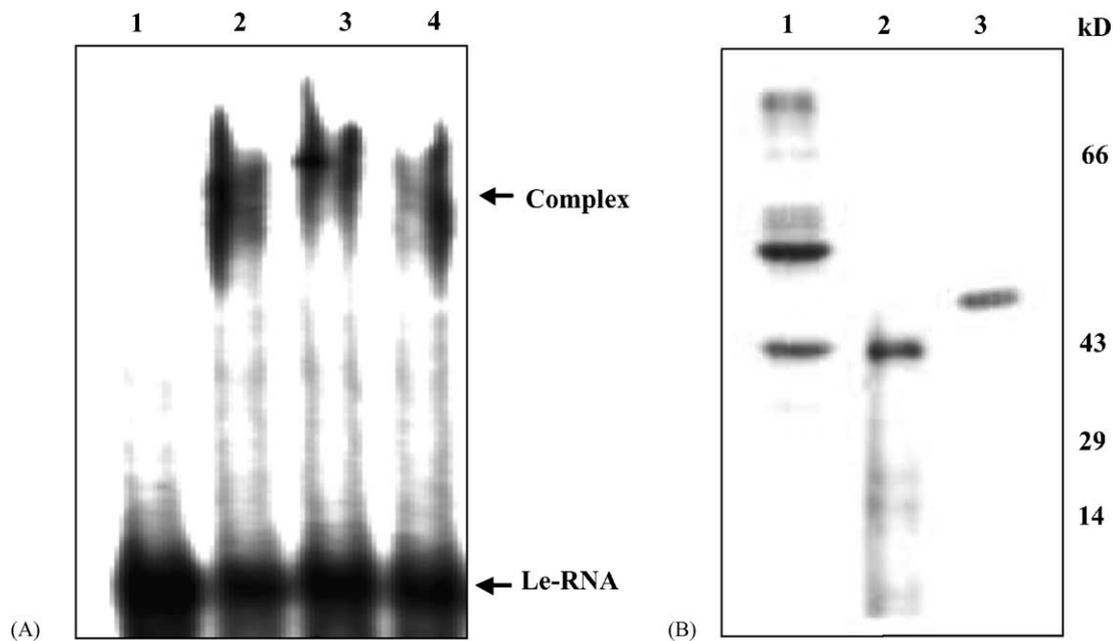


Fig. 1. (A) Formation of complexes between host proteins and the RPV leader RNA *in vitro*. [^{32}P]-labeled *le*-RNA of RPV was incubated with $2 \mu\text{g}$ HeLa cell extract (lane 2) or $2 \mu\text{g}$ A549 cell extract (lane 3) or $1 \mu\text{g}$ purified recombinant His-tagged La protein (lane 4). Protein–RNA complexes were analyzed on a non-denaturing 6% polyacrylamide gel. Lane 1 is minus protein control. (B) La protein interacts with *le*-RNA. A549 cell extract ($2 \mu\text{g}$) was incubated with [^{32}P]-labeled sense *le*-RNA of RPV (50,000 cpm). The RNA–protein complex was cross-linked by UV light and immunoprecipitated with anti-La polyclonal antibody. The immunoprecipitated samples were electrophoresed on 10% SDS-PAGE followed by autoradiography. Lane 1: direct binding with proteins from A549 cell extract. Lane 2: immunoprecipitated protein from A549 cell extract. Lane 3: Complex formed between purified recombinant La protein and *le*-RNA was similarly cross-linked immunoprecipitated and subjected to electrophoresis.

used as control. The cells after washing with PBS were further incubated with anti-rabbit IgG conjugated with Cy3. The cells were again washed with PBS followed by nuclear staining with DAPI and examined under a Leica confocal laser scanning microscope.

3. Results

3.1. Human La protein interacts with Rinderpest virus leader RNA in vitro

Cytoplasmic extracts were incubated with [32 P]-labeled RPV *le*-RNA and analyzed on 4% non-denaturing gel. Complexes of *le*-RNA with cellular proteins were seen (one major and a few minor) both with A549 as well as HeLa extracts (Fig. 1A, lanes 2 and 3). A similar shift was observed when *le*-RNA was incubated with purified recombinant La protein (lane 4). Based on this result, we speculated that La could be one of the cellular proteins, which binds to the *le*-RNA of RPV. When the [32 P]-labeled *le*-RNA was incubated with A549 cell extracts and UV cross-linked, digested with RNaseA, and analyzed by SDS-PAGE (Fig. 1B), several cellular proteins were seen to form complexes with *le*-RNA (lane 1). When the cross-linked complexes were subjected to immunoprecipitation using anti-La antibody, one of those cellular proteins associated with *le*-RNA migrated to a position corresponding to La protein, with a molecular weight of 48 kDa (lane 2). Purified recombinant La protein (His-tagged) also formed a similar complex with *le*-RNA suggesting that one of the cellular proteins could be La (lane 3). To further investigate the specificity of this interaction, different unlabeled RNAs were used as competitors during binding with *le*-RNA and the efficiency of the competition was monitored by UV cross-linking assay (Fig. 2A). Unlabeled sense *le*-RNA was found to inhibit the binding as expected, whereas bovine liver tRNA (non-specific RNA) or RPV mRNA (P) could not compete with the labeled *le*-RNA efficiently (Fig. 2A). The binding of labeled *le*-RNA was inhibited at 50-fold molar excess of unlabeled sense *le*-RNA, and the inhibition was almost 80% at 100-fold concentration (lanes 2–4). The competition with bovine tRNA (lanes 5–7) was ineffective even when 250-fold molar excess of the RNAs was added to the reaction mixture. To further prove the specificity of binding, the competition experiment was done with viral mRNA (P-mRNA, lanes 8–10). In this case also, even in presence of 250-fold higher concentrations of the viral mRNAs, there was no significant competition, indicating high specificity of *le*-RNA binding ability of La protein.

3.2. La protein binds both positive and negative sense *le*-RNA with high affinity

We observed that negative sense *le*-RNA (complementary to 3' end antigenome) also bound to endogenous La

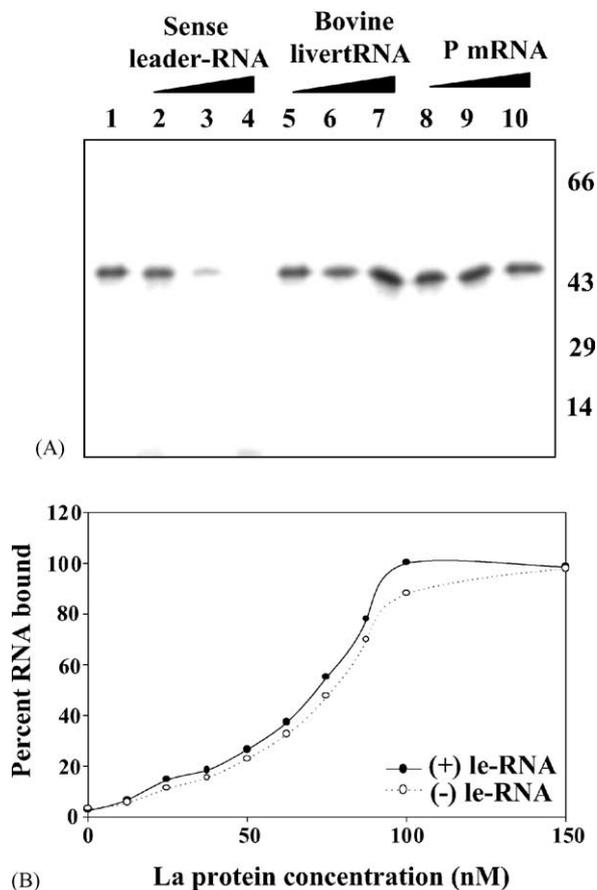


Fig. 2. (A) Specificity of La binding to *le*-RNA. Competition UV cross-linking assays were performed with purified recombinant His-tagged La protein and [32 P]-labeled *le*-RNA in the absence (lane 1) or presence of 50-, 100-, and 250-fold molar excess (as indicated on top of the lanes) of unlabeled *le*-RNA (lanes 2–4) or non-specific RNAs, bovine liver tRNA (lanes 5–7), and RPV P mRNA (lanes 8–10). The migration of marker proteins is indicated at the right of the panel. (B) Concentration dependence of P protein binding to *le*-RNA. Filter binding assays were performed with increasing concentrations of purified His-tagged La protein and 0.1 ng of [32 P]-labeled positive or negative sense *le*-RNA (50,000 cpm). The amount of bound RNA was determined by binding to nitrocellulose filters.

protein present in cell extracts or purified La protein, thus demonstrating the specificity of binding to *cis*-acting regulatory RNAs (data not shown). To determine the affinity of La protein for binding to both positive and negative sense *le*-RNA, a fixed amount of [32 P]-labeled RNA was incubated with increasing concentrations of purified La protein and the binding was monitored by nitrocellulose filter binding assay (Fig. 2B). The apparent K_d for binding of positive sense *le*-RNA was calculated to be 72.5 nM and that of negative sense *le*-RNA to be 77.5 nM, which indicates strong interaction between the La protein and *le*-RNA.

3.3. La protein interacts with leader RNA in virus-infected cells

The in vitro observations were further confirmed by demonstration of interaction of *le*-RNA synthesized in

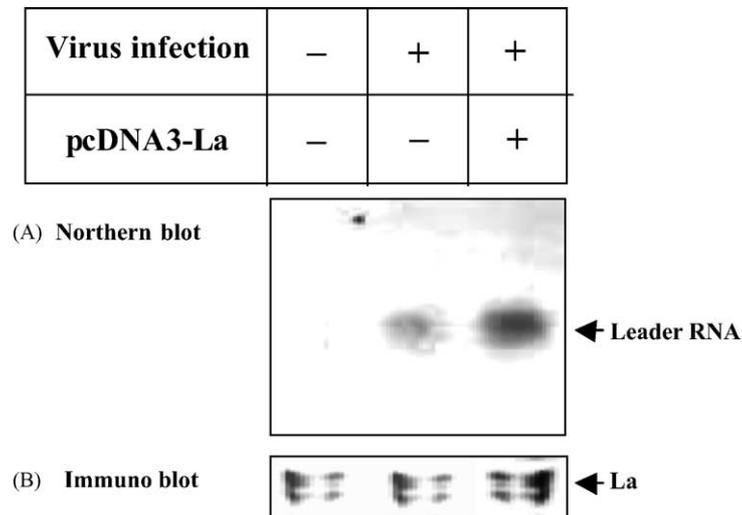


Fig. 3. (A) La protein interacts with sense *le*-RNA in vivo. A549 cells or pcDNA3-La transfected A549 cells were infected with RBOK strain of RPV. The *le*-RNA was co-immunoprecipitated from the cell lysate with polyclonal anti-La antibody. RNA was extracted from the immunoprecipitate and electrophoresed on a formaldehyde agarose gel and analyzed by Northern blotting. The blot was hybridized with [³²P] anti-sense *le*-RNA and visualized by autoradiography. Uninfected cell lysate was used as a control. (B) Lower panel shows the presence of La protein in the viral RNP as detected by Western blot analysis using enhanced chemiluminescence.

virus-infected cells with cellular La protein. The *le*-RNA made in the cells was co-immunoprecipitated with anti-La antibody, and *le*-RNA was extracted from the immunoprecipitate and subjected to Northern blot hybridization using [³²P]-labeled anti-sense *le*-RNA as the probe. The expressed protein was verified by Western blotting using anti-La antibody (Fig. 3). *le*-RNA was immunoprecipitated by anti-La antibody only from infected cell extract but not from uninfected cell extract, though both express similar amounts of La protein. The amount of immunoprecipitated *le*-RNA is more due to increased La expression when cells were transfected with La cDNA (pcDNA3) prior to infection (lane 3). These results conclusively demonstrate that RPV *le*-RNA made in infected cells interacts specifically with cellular La protein.

3.4. La protein is associated with viral nucleocapsid

After confirming the interaction of La protein with positive sense *le*-RNA of RPV, it was of interest to understand the significance of this interaction in virus life cycle. As a first step, the association of La protein with viral RNP was tested. Viral nucleocapsids were isolated from infected cells, and the proteins were electrophoresed and immunoblotted with anti-La antibodies. Parallel blots were immunostained with hyperimmune serum against purified virus. The results are shown in Fig. 4. Coomassie blue staining of RNP proteins reveals N protein as a major constituent of the nucleocapsid followed by P protein, with a faint band corresponding to the high molecular weight protein L. Immunoblotting of these proteins with hyperimmune serum against pure virus reveals their specificity (lane B). A protein band of molecular weight 48 kDa (lane A) is identified as La protein

as revealed by its reactivity with anti-La antibody (lane C). Thus, it is clear that La protein is associated with viral RNP.

3.5. La protein enhances viral replication–transcription in vivo

Baron and Barrett (1995) have successfully demonstrated viral replication and transcription in vivo by co-transfecting RPV minigenome with cDNA for N, P, and L genes of RPV, which lead to expression of the reporter gene CAT. This system was used to study the effect of La on viral replication and transcription. In order to see the effect of La on viral RNA synthesis using this assay, it was essential to use sub-optimal concentrations of N, P, and L plasmid DNAs. Under these conditions, measurable CAT activity should be seen in the absence of exogenous La protein. For this purpose, cells were transfected with viral minigenome with varying concentrations of the N/P and L plasmids and CAT synthesis was measured (Fig. 5A). At 10:1 ratio of N/P to L cDNA, CAT was expressed to a moderate level. In this condition, varying amounts of pcDNA3-La DNA was co-transfected and subsequent CAT expression was measured. We observe that with the increase in exogenous La protein synthesis, CAT expression also increased significantly (Fig. 5B). This indicates that the La protein has an effect on the RNA synthesis machinery of the virus by way of increasing the replication and transcription of viral RNA in vivo.

3.6. Detectable amount of La protein is redistributed to cytoplasm in RPV-infected cells

La protein is predominantly localized in the nucleus under normal conditions. However, it was earlier shown that

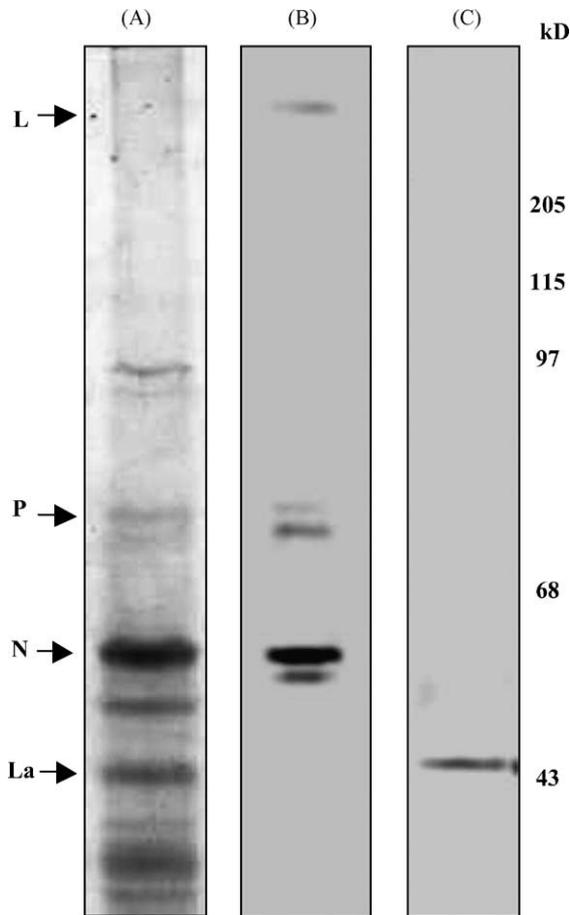


Fig. 4. In vivo association of La protein with RPV nucleocapsids in infected cells. Intracellular viral RNP from infected A549 cells was purified and electrophoresed on a 8% SDS-PAGE. (A) Coomassie blue staining. (B) ECL-Western immunoblot of the separated RNP proteins, probed with hyperimmune serum against RPV. (C) RPV hyperimmune immunoblot of the same gel probed with rabbit anti-La antibody.

in case of certain viral infections, La protein redistributes into the cytoplasm of the cell. Since the *le*-RNA in infected cells is synthesized in the cytoplasm, in order for La protein to interact, it should be relocalized to the cytoplasm. Hence, to examine the distribution of La protein in RPV-infected cells, we employed confocal microscopy. Sub-confluent monolayer of A549 cells grown on coverslips was infected with RPV (moi of 10 TCID₅₀ units/cell). Thirty-six hours post-infection, the cells were washed and fixed, followed by incubation with anti-La polyclonal antibody. Uninfected cells were used as control (Fig. 6, panels A through D). The cells after washing with PBS were further incubated with anti-rabbit IgG conjugated with Cy3. The cells were again washed with PBS followed by nuclear staining with DAPI and examined under a Leica confocal laser scanning microscope. Our results indicate that although La is mostly present in the nucleus of the uninfected cells, it comes out of the nucleus into cytoplasm upon infection with RPV (Fig. 6, panels B and F). Much less La protein came out of the nucleus when cells were infected with lower moi

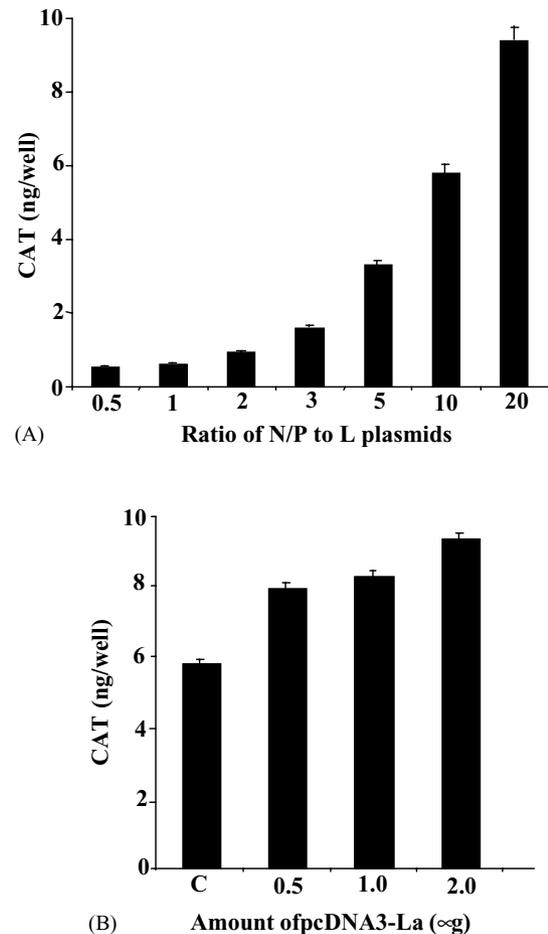


Fig. 5. (A) Viral replication/transcription assay in vivo. Recombinant vaccinia virus (vTF7.3)-infected A549 cells were transfected with RPV minigenome along with the cDNA plasmids of N, P, and L plasmids at different N/P:L ratio. Cells were harvested 48 h after transfection, and amounts of CAT synthesized were assessed by CAT-ELISA. (B) Effect of La protein on viral replication/transcription in vivo vTF7.3-infected A549 cells were transfected with RPV minigenome along with the cDNA plasmids of N, P, and L plasmids keeping the N/P:L ratio at 10:1 with different amounts of pcDNA3-La construct (0, 0.5, 1.0, and 2.0 μg). Cells were harvested 48 h after transfection, and amounts of CAT synthesized were assessed by CAT-ELISA and plotted against increasing concentration of the La encoding plasmid, pcDNA3-La.

(2 TCID₅₀ units/cell) (data not shown). This was done to eliminate the possibility of leakage of the La from the nucleus due to extensive cytopathic effects of the virus at high moi. Thus, these results strongly suggest that La protein becomes available for the interaction with viral RNA in the cytoplasm.

4. Discussion

DNA viruses use the host RNA polymerase for the synthesis of mRNAs with the help of other viral proteins. RNA viruses, on the other hand, are unique as they encode the RdRP, which is absent in the host. In spite of having the

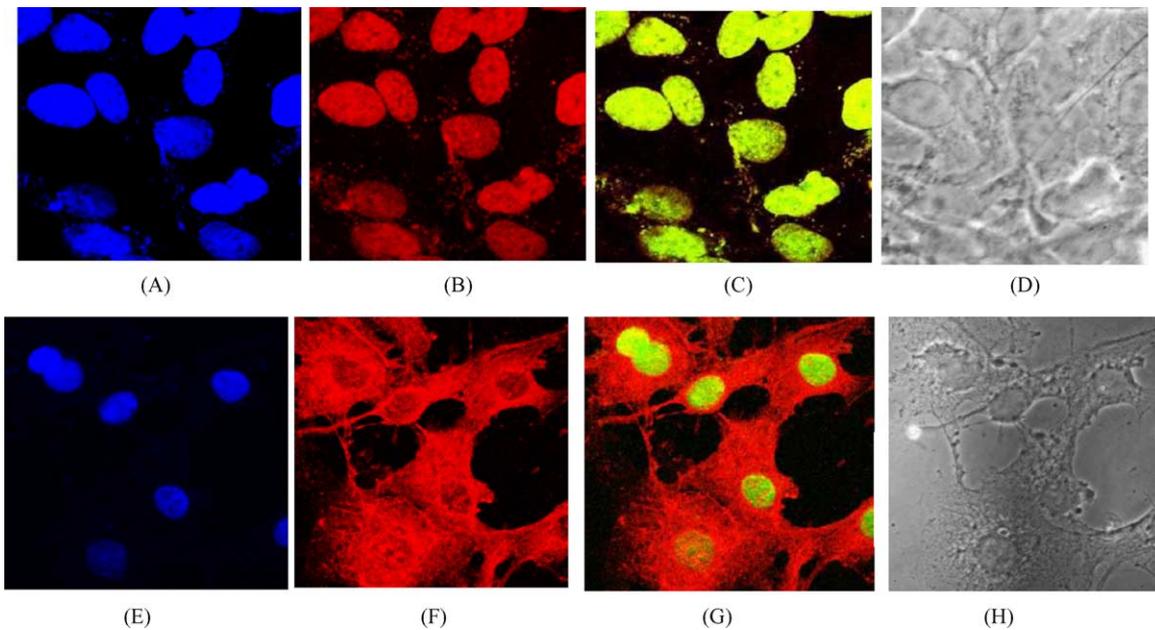


Fig. 6. Localization of the La protein in infected and uninfected cells. Panels A through D represent the uninfected control cells. At 36h (E through H) post-infection, cells were fixed and permeabilized. Coverslips were treated with anti-La antibody followed by Cy3-tagged secondary antibody. Cells in panels A and E were stained with DAPI showing nuclear staining. B and F show anti-La staining and panels C and G represent merged images showing localization of La in the nuclei and cytoplasm as a consequence of RPV infection. Panels D and H represent bright-field pictures of uninfected (D) and infected (H) cells.

virus-encoded RdRP, the paramyxoviruses need host factors to support different events throughout their life cycle. Cellular cytoplasmic proteins have been shown to activate mRNA synthesis by RNP (Moyer et al., 1986). In SeV, the cellular factor was identified as tubulin (Mizumoto et al., 1995). Requirement of actin was established in case of HPIV 3 (De et al., 1993), whereas in VSV, association of eukaryotic elongation factor, the α sub-unit of EF1, was found to be absolutely necessary for the activation of L protein (Mathur et al., 1996). Therefore, considerable interest has been focused on the elucidation of the interactions of cellular proteins with *cis*-acting regulatory elements of negative sense RNA viruses.

In the present study, we have identified and characterized one cellular protein, La, which is a nuclear protein that interacts with RPV *le*-RNA, in vitro as well as in vivo. Using gel mobility shift assay, we demonstrated that certain cellular proteins interact with RPV *le*-RNA in vitro. Immunoprecipitation of the *le*-RNA–protein complex with anti-La antibody showed that one of the *le*-RNA binding proteins is human La protein. In the infected cells, the *le*-RNA made by the virus was found to be immunoprecipitated with anti-La antibody, indicating that this interaction takes place in vivo. The association of La protein with RNP isolated from infected cells further showed that La protein may be involved in viral RNA synthesis. Using RPV minigenome to analyze in vivo replication–transcription, we could show that La protein can activate the viral RdRP in a dose-dependent manner. Additionally, by using reconstituted viral replication–transcription system, we have observed a

dose-dependent increase in viral replication upon addition of increasing concentrations of purified La protein (data not shown). However, we do not rule out the possibility that over-expression of La protein might also increase the transcription of other host factors involved in regulation of virus replication. Previously in VSV, the *le*-RNA was found to be immunoprecipitated with anti-La antibody and 80% of the La:*le*-RNA complex was detected in infected cell cytoplasm whereas 2% of La was found to be present in infected cell nucleus (Kurilla and Keene, 1983). La protein, an RNA polymerase III transcription factor having a molecular weight of 48 kDa, though present in the nucleus, was also detected in the cytoplasm of virus-infected cells. In HPIV 3, La protein was found to be associated with the 3' genomic non-coding sequence along with glyceraldehyde 3-phosphate dehydrogenase (De et al., 1996). In case of VSV, La protein interacts with *le*-RNA along with the nuclear RNP U (Gupta et al., 1998). In RPV, we have identified La protein as one of the *le*-RNA-binding protein. Results of confocal microscopy indicated that La protein present in the nucleus of uninfected cells could be detected in the cytoplasm after virus infection. The La protein has been shown to shuttle between the nucleus and the cytoplasm in uninfected cells and in herpes simplex virus type 1-infected cells (Bachmann et al., 1989a,b), and on poliovirus infection of CV-1 cells, La protein is redistributed to the cytoplasm (Meerovitch et al., 1993). This redistribution of La protein has been demonstrated to be specific for its role in enhancing poliovirus RNA translation, and another nuclear protein splicing factor, SC35, does not get redistributed on poliovirus infection

of the cells, thereby ruling out any stress-related effects. Detection of the *le*-RNA in the RNP or nucleocapsids from virus-infected cells suggests that *le*-RNA is present in the microenvironment of the RNA synthesis machinery to bring about its effect. Further studies are necessary to understand the mechanism by which La protein stimulates RNA synthesis and how it is recruited into the RNP complex.

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