Materials and methods
3.1 Cells lines, culture conditions and Virus

Human embryonic kidney (HEK) 293A and porcine stable kidney (PS) cells obtained from the National Centre for Cell Sciences, Pune, India were used to perform the experiments described in this thesis. HEK 293A cells were grown in Eagle’s Minimal Essential Medium (Sigma) while PS cells were grown in the Minimal Essential Medium (Sigma) supplemented with 10% fetal calf serum and antibiotics (Gibco BRL). The GP78 strain of JEV was used in these studies (Vrati et al., 1999) which was grown in PS cells.

3.2 Preparation of E. coli competent cells and transformation

Luria Bertani (LB) agar (1% bacto-tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0, 1.5% agar) plate was streaked with the desired strain of E. coli and a single colony from this plate was inoculated in 3 ml of LB medium and grown at 37 °C with shaking at 200 rpm. After 8 hrs, a small inoculum from this culture was used to start a 100 ml culture in the same medium. Once the culture reached log phase (OD600 ~0.5), it was chilled on ice for 30 min and centrifuged at 2000 g for 10 min at 4 °C. The pellet was gently suspended in 50 ml of chilled 100 mM CaCl2 and incubated on ice for 30 min. Following this, the cell suspension was spun at 2000 g for 10 min at 4 °C and the pellet was gently suspended in 5 ml of chilled 100 mM CaCl2 containing 15% glycerol. Cell suspension was immediately stored at -70°C in small aliquots of 0.2 ml. The competent cells were then transformed by the heat shock method. The competent cells usually had a transformation efficiency of ~10^6 CFU/μg of pUC18 DNA.

3.3 Plasmid miniprep

The plasmid DNA from transformed E. coli cells was isolated by the alkaline lysis method using the QIAprep spin miniprep kit (Qiagen, USA). Briefly, 3 ml LB was inoculated with a single bacterial colony and incubated at 37 °C overnight with vigorous shaking. The cells were pelleted by centrifugation at 8000 g for 5 min. The pellet was suspended in 250 μl of cell suspension buffer P1, transferred to a micro centrifuge tube and to this 250 μl cell lysis buffer P2 was added. The tube was inverted gently 4-6 times to mix the solution completely. Next, 350 μl of neutralization buffer N3 was added and the tube was immediately inverted 4-6 times. This was centrifuged for 10 min at 12000 g and
the supernatant was applied to a QIAprep column by decanting. The column was washed by adding 0.75 ml of buffer PE followed by centrifugation for 1 min. The flow-through was discarded and the column centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep column was then placed in a clean 1.5 ml micro centrifuge tube; 100 µl water was added to the center of the column, which was allowed to stand for 1 min followed by centrifugation for 1 min to elute the DNA. The DNA concentration was measured on a UV spectrophotometer (1 OD_{260} = 50 µg/ml for double stranded DNA) and stored at -20°C in small aliquots.

3.4 DNA sequencing

Sequencing of plasmid DNA was done by Sanger’s dideoxy chain termination method using the T7 Sequenase Quick-Denature plasmid sequencing kit (USB Corp. USA). Briefly, plasmid DNA (3-4 µg) was denatured using 1 M NaOH, annealed to primer (5 pmol) by incubation for 10 min at 37 °C and then chilling on ice. The mixture was then neutralized with 1 M HCl. The annealing was continued at 37 °C for 10 min. For the labeling reaction 1 µl of 0.1 M DTT, 2 µl of labeling mix (1.5 µM 7-deaza-dGTP, 1.5 µM dCTP, 1.5 µM dTTP, 0.5 µl of [35S] α-dATP (1250 Ci/mmol, NEN) and 2 µl T7 sequenase plasmid sequencing formulation (preblended T7 sequenase version 2.0 DNA polymerase and inorganic pyrophosphatase in 20 mM Tris-Cl, pH 7.5, 2 mM DTT, 0.1 mM EDTA, 50% glycerol) were added to the ice-cold annealed DNA. After incubating at room temperature for 2-5 min, 4.5 µl of the labeling reaction mix was added to each of the 4 different tubes containing 2.5 µl each of dideoxynucleotides ddATP, ddTTP, ddCTP, and ddGTP termination mixes. The termination mixes contained 80 µM each of 7-deaza-dGTP, dGTP, dCTP and dTTP in 40 mM Tris-Cl, pH 7.6 and 50 mM NaCl. Besides, ddG termination mix had 8 µM ddTTP and ddATP. Random incorporation of the dideoxynucleotides in the elongation chain would result in the termination of polymerization at specific site. The reaction was terminated by adding 4 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The samples were heated at 75 °C for 2 min and loaded onto a 6% polyacrylamide (acrylamide: bisacrylamide ratio 29:1) sequencing gel made in TBE buffer (50 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3) containing 7 M urea. The gel was pre-
Materials and Methods

electrophoresed for about 30 min at 2.0 KV before loading the samples. After the run, the gel was dried on Whatmann 3MM paper at 80 °C for an hr and exposed to X-ray film.

3.5 Preparation of S100 cytoplasmic extract

Brain tissue from one week-old Balb/c mice was harvested and washed twice with ice-cold phosphate-buffered saline (PBS) (0.137 M NaCl, 2.68 mM KCl, 4.3 mM NaH2PO4, 1.47 mM KH2PO4), resuspended and vortexed in cytolysis buffer (10 mM HEPES, pH 7.9, 5 mM DTT, 20% glycerol, 10 mM NaCl, 0.1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 1% Triton X-100) and stored on ice for 20 min. The nuclei were removed by centrifugation at 5000 g for 10 min at 4 °C. The supernatant was clarified by ultra centrifugation at 100,000 g for 1 hr at 4 °C. The resulting S100 supernatant was then concentrated by buffer exchange in storage buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 5 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin and 50% glycerol) and stored in small aliquotes at −70 °C. The concentration of protein in the cytoplasmic extract was determined using the BCA™ Protein Assay kit (Pierce), which is a detergent-compatible formulation based on bicinechonic acid (BCA) for the colorimetric detection and quantitation of total protein.

3.6 Cloning of noncoding regions of JEV for in vitro RNA transcription

The RNA transcripts used to study RNA-protein interactions were generated by in vitro transcription of plasmid constructs containing cDNAs encoding JEV NCR sequences cloned under the bacteriophage T7 promoter. pJE3SL, containing cDNA of JEV 3'-SL was available in the lab. A cDNA representing the 5'-NCR of JEV was cloned in pGEM-T easy vector (Promega) under the control of the T7 promoter. The recombinant plasmid pJE5NCR was able to synthesize RNA representing JEV 5'-NCR. Synthesis of cDNA to JEV 5'-NCR RNA was carried out by reverse transcription-polymerase chain reaction (RT-PCR) of viral RNA using oligonucleotides SV431 and SV 217 (Table 3.1), avian myeloblastosis virus (AMV) reverse transcriptase and Taq DNA polymerase. The upstream oligonucleotide SV217 contained an Apa I site followed by 18-nucleotide JEV 5'-NCR sequence (nucleotide 1-18). The downstream oligonucleotide SV431 contained a BamH I site and 18-nucleotide sequence complementary to bases 78-95 located within JEV 5'-NCR. The RT-PCR product which contained 3'-A overhangs due to the use of Taq
DNA polymerase, was cloned into pGEM-T easy vector (Promega) using the TA cloning strategy. The desired clones with correct cDNA orientation were confirmed by sequencing the constructs. The cDNA insert contained an Apa I site at the 5'-end of the 5'-NCR sequence. The pGEM-T easy vector had another Apa I site just upstream of the insert and downstream to the T7 promoter sequence. To minimize the vector sequence that would be fused to the 5'-end of the 5'-NCR RNA that could be transcribed from the plasmid, Apa I digestion of the recombinant plasmid was carried out. The digestion removed the extra stretch of ~ 40 bases between the insert and T7 promoter. The digested product was separated on agarose gel; the large DNA fragment was purified, self-ligated and transferred to DH5α *E. coli* cells. The recombinant plasmid pJE5NCR containing JEV 5'-NCR cDNA close to the T7 promoter was confirmed by nucleotide sequencing.

### 3.7 Preparation of RNA transcripts

Plasmid DNAs containing cDNAs encoding JEV NCR sequences were linearized with the appropriate restriction enzymes and gel purified using Gel purification kit (Amersham Biosciences). Plasmid pJE3SL was linearized with *Xba* I, while pJE5NCR was linearized with *Bam* HI. For producing the radiolabelled transcript, *in vitro* transcription was performed at 37 °C for 1 hr in a 20 µl reaction containing 40 mM Tris-Cl (pH 7.5), 6 mM MgCl₂, 10 mM NaCl, 2 mM spermidine, 10 mM DTT, 0.5 mM each of ribonucleotides (A, C and G), 12 µM UTP (Promega), 50 µCi [³²P]-UTP (3000 Ci/mmol NEN), 1 µg of linearized DNA template, 20 U of RNasin (Promega) and 20 U of T7 RNA polymerase (Promega). The reaction product was then treated with 10 units of RNase-free DNase (Promega) for 20 min at 37 °C. The transcript was then treated with phenol-chloroform, precipitated with ethanol, and resuspended in 20 µl RNase-free water. RNA yield was determined by the trichloroacetic acid (TCA) precipitation protocol as described below.

Unlabeled (cold) RNAs were synthesized in 50 µl transcription reactions from linearized plasmids. The reaction conditions were as described above except that no [³²P]-rUTP was added, and the concentration of all four rNTPs was 0.5 mM. After transcription, the reaction products were DNase digested. Following the phenol-chloroform treatment,
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cold RNAs were ethanol precipitated, pelleted, and resuspended in 100 µl RNase-free H₂O. The RNA amounts were measured spectrophotometrically.

3.8 Determination of RNA yield by TCA precipitation

The RNA transcription reaction (20 µl) was diluted 1:10 in distilled water. One µl of the diluted reaction was spotted onto glass fiber filter (Sartorius) in duplicate. The filters were air dried before scintillation counting to determine the total radioactivity measured in cpm. In duplicate tubes, 1 µl of the 1:10 dilution of the reaction was added to 100 µg yeast tRNA (Sigma) in a total volume of 100 µl, to which 0.5 ml ice-cold 5% TCA was added and mixed. This was chilled on ice for at least 5 min. The samples were applied to the glass fiber filter which had been pre-wetted with 5% TCA. The filters were rinsed with 2 ml acetone and allowed to air dry. Scintillation fluid was added to the dried filters in scintillation vials and radioactivity counts were taken.

The percent incorporation of the radioactivity and total cpm incorporated, the total amount of RNA synthesized and its specific activity were calculated as follows:

% Incorporation = [Incorporated cpm/ total cpm] X 100

Total cpm incorporated = Incorporated cpm X dilution factor X [reaction volume/volume counted]

nmol of labeled rNTP = µCi rNTP in reaction/isotope concentration in µCi/nmol

nmol of limiting cold rNTP = µl of limiting cold rNTP X 100 µM rNTP X [10³ nmol / 1 µmol] X [1 L/10⁶ µl]

Total nmol of limiting rNTP = nmol of labeled rNTP + nmol of limiting cold rNTP

Max. theoretical RNA yield = total nmol of limiting rNTP X 4 rNTPs X [330 ng/nmol rNTP]

Total µg of RNA synthesized = % incorporation X maximum theoretical RNA yield

Specific activity = total incorporated cpm/total µg of RNA synthesized.

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3.9 **Synthesis of recombinant human La protein (rhLa) and its mutants in *E. coli***


To express recombinant proteins, *E. coli* BL 21 (DE3) cells were transformed with pRSETA-La or other plasmids expressing truncated versions of La protein. A single colony was inoculated in LB and grown overnight at 37 °C. The overnight grown culture was diluted 1:50 LB and grown till A$_{600}$ reached ~0.8. The cells were then induced with 0.8 mM isopropyl-1-thio-beta-D-galactopyranoside (IPTG). After 4 hrs of further growth, cells were centrifuged and resuspended in 10 ml of bacterial lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole) and lysed by sonication. The lysates were centrifuged at 12,000 g for 30 min and the resultant supernatant was loaded onto a Ni-NTA agarose column (Lysate of one liter culture / ml resin). After washing the column with bacterial wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 40 mM imidazole), protein was eluted with the elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 500 mM imidazole). Eluted protein was dialyzed against recombinant protein storage buffer (50 mM Tris, pH 7.4, 100 mM KCl, 7 mM β-mercaptoethanol, 20% glycerol), aliquoted, snap frozen and stored at -70 °C. Protein concentration was determined by the BCA method and used for various assays as freshly thawed.

3.10 **Electro-mobility shift assay (EMSA)**

Ten microgram recombinant protein or S100 cytoplasmic extract was incubated with yeast tRNA and poly ICU in binding buffer (14 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT and 60 mM KCl) containing 1 unit RNasin (Promega) in a final volume of 30 μl and incubated at 37 °C for 10 min. 500 pg labeled RNA was heated at 90 °C, chilled on
ice and added to the reaction mixture. Following the incubation, 6 \mu l of a dye mixture (50% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol) was added, and the reaction mixture was immediately loaded on a 5% native polyacrylamide gel (50:1 acrylamide - bisacrylamide), electrophoresed at 50V at 4 °C in 0.5 X TBE gel running buffer. After electrophoresis, the gel was dried under vacuum at 80 °C for an hr. The RNA-protein complex was visualized by autoradiography at -70 °C. For competition studies, appropriate amount of unlabeled specific or nonspecific competitor RNA was added to the reaction mixture, incubated for 15 min and then labeled RNA was added.

3.11 Ultra-violet (UV) light-induced crosslinking of RNA –Protein complexes

Following the RNA-protein binding reaction, the products in the micro centrifuge tube were irradiated for 30 min with 254 nm UV lamp held at 3 cm distance from the reaction mixture. After irradiation, 5 \mu g RNase A (Sigma) was added and the mixture was incubated for 30 min at 37 °C to digest unprotected RNA. The Laemmli sample buffer (0.125 M Tris buffer, pH 6.8, 2% SDS, 5% \beta-mercaptoethanol or 20 mM DTT, 10% glycerol and 0.001% bromophenol blue) was then added, boiled for 10 min and the reaction products separated by discontinuous 12% SDS-polyacrylamide gel (PAGE) (acrylamide: bisacrylamide ratio 29:1) in Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). The gel was fixed in 7% acetic acid, dried at 80 °C for 1 hr and autoradiographed.

3.12 Immunoprecipitation of UV-induced cross-linked proteins

The cross-linked RNA-protein complexes after RNase digestion (as mentioned above) were incubated with Sepharose A CL-4B beads (Pharmacia) and the antibody for 2 hrs at 4 °C. The antibody-Sepharose complexes were washed six times with RIPA buffer (1% Sodium deoxycholate, 0.1% SDS, 10mM Tris-HCl, pH 8.0, 140 mM NaCl, 0.5% Triton X100, 5 mM Iodoacetamide, 0.002% Sodium Azide). Beads were collected by centrifugation for 30 seconds at 14,000 rpm. The Immunoprecipitates were analyzed by 12% SDS-PAGE as mentioned above and visualized by autoradiography.
3.13 Northwestern analysis of RNA binding protein

Protein samples were resolved on a 12% SDS-PAGE and transferred to nitrocellulose membranes at 30 V for 16 hrs in transfer buffer (39 mM glycine, 48 mM Tris and 20% methanol, pH 8.3) at 4 °C. The transferred proteins were renatured overnight at 4 °C in binding buffer containing 1% bovine serum albumin (BSA) and 16 µg/ml salmon sperm DNA. Membranes were then incubated for 30 min with 10 µg/ml yeast tRNA at 37 °C. ³²P-labeled RNA probe was added next and the binding continued for 8-12 hrs at 37 °C. Nonspecifically bound radioactivity was removed by washing the membranes three times for 5 min each with binding buffer at room temperature. Protein-RNA binding was visualized by autoradiography.

3.14 Filter-binding Assay

The various amounts of protein (1-10 nM) were incubated in 20 µl reaction with the 100 femtomoles ³²P-labeled RNA at 30 °C in binding buffer (14 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT and 60 mM KCl) containing yeast tRNA (500 ng) for 15 min. The reaction products were then loaded onto nitrocellulose filters (Millipore) pre-equilibrated with 2 ml binding buffer and filtered under vacuum. The filters were then washed twice with 2 ml binding buffer and dried, and the counts retained were measured in a liquid scintillation counter. The graph was plotted with protein concentration (nM) on X axis and the percentage of bound RNA as the percentage of counts retained, on the Y axis. The relative binding affinity constant (Kd) was calculated by fitting the data into binding curve (Langmuir) equation using Graph Pad Prism software.

3.15 End labeling of oligonucleotide with ³²P-gamma ATP

10 picomoles of oligonucleotide was incubated with 30 µCi ³²P-gamma ATP in the presence of T4 polynucleotide kinase buffer (50 mM Tris buffer pH 7.5, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine) and 10 units T4 polynucleotide kinase in a reaction of 10 µl for 30 min at 37 °C. The enzyme was then heat inactivated at 92 °C for 2 min and the
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oligonucleotides separated from free ATP using microspin G-25 column (Amersham). The volume was then raised to 100 μl, aliquoted and stored at -20 °C.

3.16 Primer Extension Inhibition (toe printing) Analysis

In vitro transcribed, gel purified RNA (60 femtomoles) was incubated with 0.5 pmole 32P-end-labeled oligonucleotide primer complementary to the 3'-end of the RNA (Table 3.1) in the primer extension buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 10 mM dNTP, 0.5 mM spermidine) and binding was allowed to take place in a final volume of 10 μl at 58 °C for 20 min, followed by cooling at room temperature for 10 min. Increasing amounts of protein (0-100 ng) were then added to the reaction and again incubated for 30 min at 30 °C. The primer was then extended using 1 unit of AMV-reverse transcriptase at 42 °C for 90 min. The DNA thus synthesized was precipitated using sodium acetate and ethanol, resuspended in 20 μl loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) and electrophoresed on a 6% polyacrylamide, 7 M urea denaturing gel along with the nucleotide sequence ladder of the corresponding cDNA generated using the same end-labeled primer.

3.17 Co-Immunoprecipitation and RT-PCR

For Co-Immunoprecipitation of RNA bound to endogenous La protein, HEK 293A cells (5 X 10⁶) infected with JEV at a multiplicity of infection of 10 were harvested, washed twice with ice cold PBS and collected in 500 μl cell lysis buffer (10 mM HEPES, pH 7.0, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin and 0.5% Nonidet P-40). 150 units of RNasin (Promega) and protease inhibitor mixture (Roche) were added freshly. Cells were incubated for 10 min on ice, pipetted up and down to burst open the cells and again incubated for 10 min on ice. The lysate was then centrifuged at 16,000 g for 15 min at 4 °C and the supernatant was collected in a separate tube which was centrifuged again. The supernatant was stored on ice and 100 μl was used for immunoprecipitation.

Protein A sepharose beads (10 μl) (Amersham) were washed with PBS, blocked with 2% BSA and incubated for 1 hr with antibody diluted to 1 ml. Mouse anti-La antibody 4B6 was kindly provided by M. Bachmann (Johannes Gutenberg University,
Mainz, Germany) and anti-JEV Envelope (E) protein antibody 3B9 was prepared in our laboratory. Antibodies were then washed 6 times with buffer B (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.05% Nonidet P-40). The beads were then resuspended in 850 μl buffer B, 40 μl 0.5 M EDTA, 150 units Rnasin (Promega) and 100 μl of lysate was then added. The reaction tubes were incubated on an end-to-end rotor overnight at 4 °C. Beads were then collected and washed 6 times with ice cold buffer B and resuspended in 100 μl buffer B supplemented with 0.5% SDS and incubated at 37 °C for 30 min. RNA was then extracted using the TriPure isolation reagent (Roche) and precipitated in presence of 20 μg glycogen. The RNA pellet was resuspended in 20 μl diethyl pyrocarbonate (DEPC)-treated water and used as template for reverse transcription followed by polymerase chain reaction (RT-PCR) using JEV specific oligonucleotide primers.

3.18 Yeast Transformation

JEV 3'-SL cDNA sequence was fused with the 5'-end of the MS2 RNA sequence between Age I and Sma I sites to generate RNA-hybrid vector pRH5 (Invitrogen). The cDNA encoding La protein was fused at the 3'-end of the B42 domain-encoding sequence in plasmid pYESTrp2 (Invitrogen). Yeast host strain, L40uraMS2 was co-transformed with the RNA-hybrid and the protein-hybrid plasmids using lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Geitz and Woods, 2002). The yeast culture was started from the single colony streaked on YPD (1% yeast extract, 2% peptone, and 2% dextrose) agar plate. An overnight culture was grown in YPD medium at 30 °C. The culture was diluted into 50 ml YPD to get an A₆₀₀ of 0.4 and continued growing for additional 4-5 hrs. The cells were pelleted at 3000 g for 2 min, washed twice with sterile water and resuspended in 1 ml sterile water. One microgram of each of the plasmid DNA was then added to a tube containing 100 μl of the above prepared yeast cells and 346 μl of transformation mix (40% polyethylene glycol 3350, 100 mM lithium acetate, 100 μg salmon sperm DNA) and vortexed vigorously to mix the components. The tubes were incubated at 40 °C with intermittent mixing. The cells were washed with 1 ml sterile water, resuspended in 100 μl sterile water and plated onto respective selection media plates and incubated at 30 °C.
3.19 Liquid β-galactosidase Assay

The transformed yeast colonies were grown in 3 ml selection medium overnight at 30 °C. This culture was diluted to 10 ml and grown for additional 4-5 hrs. Cells equivalent to 2 A600 were pelleted and washed with 1 ml Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, pH 7.0) and resuspended in 100 µl Z buffer. Cells were disrupted by 3 cycles of freeze-thawing by alternatively transferring cells from liquid nitrogen to 37 °C and back. 100 µl of Z buffer was set up as blank. To the lysed cells or blank, 700 µl Z buffer containing 1.9 µl of β-mercaptoethanol was added and mixed thoroughly. To this, 160 µl of 4 mg/ml O-nitrophenyl β-D-galactopyranoside (ONPG) solution in Z buffer was added, mixed thoroughly and incubated at 30 °C for color development. The reaction was stopped by adding 400 µl of 1 M Na2CO3 and mixing properly. The reaction tubes were centrifuged at 12,000 rpm for 20 min and supernatant was transferred to fresh micro centrifuge tubes and was optical density measured at 420 nm.

3.20 Viral RNA Isolation

Culture supernatant of JEV GP78-infected cells was used to isolate the viral RNA using the RNeasy kit (Qiagen) as per the manufacturer’s protocol. All the reagents were provided in the kit. However, their composition was not mentioned. Briefly, 500 µl of buffer RLT (containing 10 µl β-mercaptoethanol per ml of buffer RLT) was added to 500 µl culture supernatant following which 500 µl of 70% ethanol was mixed by pipetting. The mixture was transferred to an RNeasy mini spin column sitting in a 2 ml collection tube and centrifuged for 15 sec at 10,000 rpm. Next, 700 µl buffer RW1 was pipetted onto the RNeasy column and spun for 15 sec at 10,000 rpm for washing. The flow through was discarded. The RNeasy column was then transferred to a new collection tube and 500 µl of buffer RPE was pipetted onto the column and centrifuged for 15 sec at 10,000 rpm. Next, 500 µl RPE was pipetted onto RNeasy column and centrifuged for 2 min at 13,000 rpm to dry the RNeasy membrane. The column was then transferred to a new micro centrifuge tube and 50 µl of RNase-free water was pipetted directly onto the RNeasy
column and centrifuged for 1 min at 10,000 rpm to elute the RNA. The RNA was stored at -70 °C.

3.21 Site Directed Mutagenesis

QuickChange site-directed mutagenesis kit (Stratagene) was used to create mutants of pJE3SL and pJE5NCR. Briefly, 50 ng template DNA, 125 ng of complementary oligonucleotides containing desired mutation and dNTPs were mixed in presence of reaction buffer and pfuTurbo DNA polymerase provided in the kit. The mix was subjected to 18 cycles of temperature cycling with denaturation at 95 °C, annealing at relevant temperature determined by the oligonucleotide sequence, and extension at 68 °C. The mixture was then incubated at 4 °C for 20 min. Dpn I restriction enzyme was then added to the reaction and incubated at 37 °C for 1 hr. The reaction mixture was then transformed in E. coli XL1-blue competent cells and screening was done for the desired plasmid clones using restriction analysis and sequencing.

pJE3SL was mutated to pJE3SL~1 using oligonucleotides SV624 and SV625, and to pJE3SL~2 with oligonucleotides SV626 and SV627. Similarly pJE5NCR was mutated to pJE5NCR(S) using oligonucleotides SV472 and SV473, to pJE5NCR~5 using oligonucleotides SV474 and SV475, and to pJE5NCR~8 using oligonucleotides SV476 and SV477. Sequence of the oligonucleotides is given in table 3.1.
Table 3.1  List of Oligonucleotides used in this study.

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