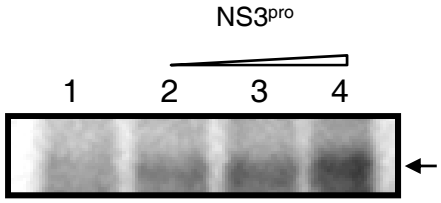
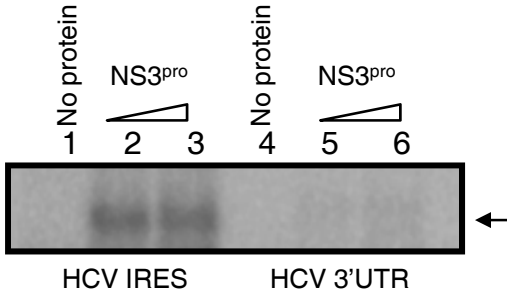
a



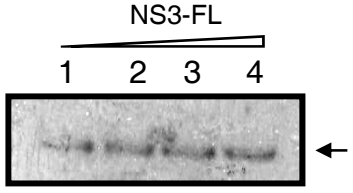
UV crosslinking assay: α[^{32}P]UTP labeled HCV IRES RNA was UV-crosslinked to increasing concentrations (0.1, 0.2 and 0.4μM) NS3^{pro} in RNA binding buffer containing 140mM KCl. The protein-nucleotide complex was resolved in 15% SDS-PAGE followed by phosphor imaging analysis. The position of the NS3^{pro} protein is indicated by an arrow.

b



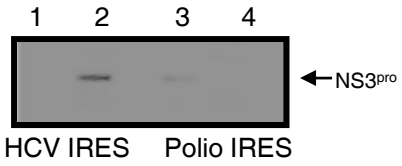
UV crosslinking assay: α[^{32}P]UTP labeled HCV IRES RNA (lanes 1-3) and HCV 3'UTR RNA (lanes 4-6) were UV-crosslinked to increasing concentration (0.1 and 0.2μM) of NS3^{pro}. The protein-nucleotide complex was resolved in 15% SDS-PAGE followed by phosphor imaging analysis. The position of the NS3^{pro} protein is indicated by an arrow.

c



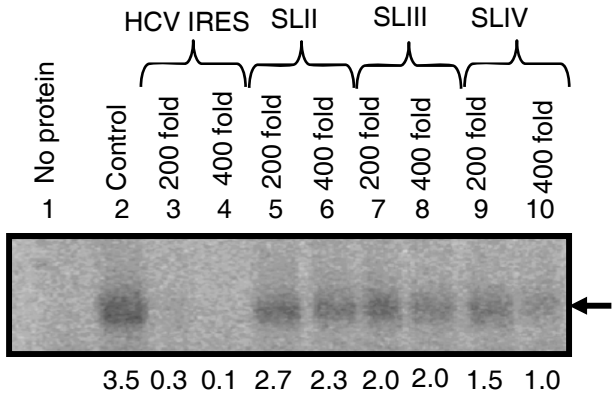
UV crosslinking assay: α[^{32}P]UTP labeled HCV IRES was UV crosslinked to increasing concentrations (0.1, 0.2, 0.4 and 0.8μM) of recombinant full length NS3 protein (lane1-4). The position of the full length NS3 protein is indicated by an arrow.

a

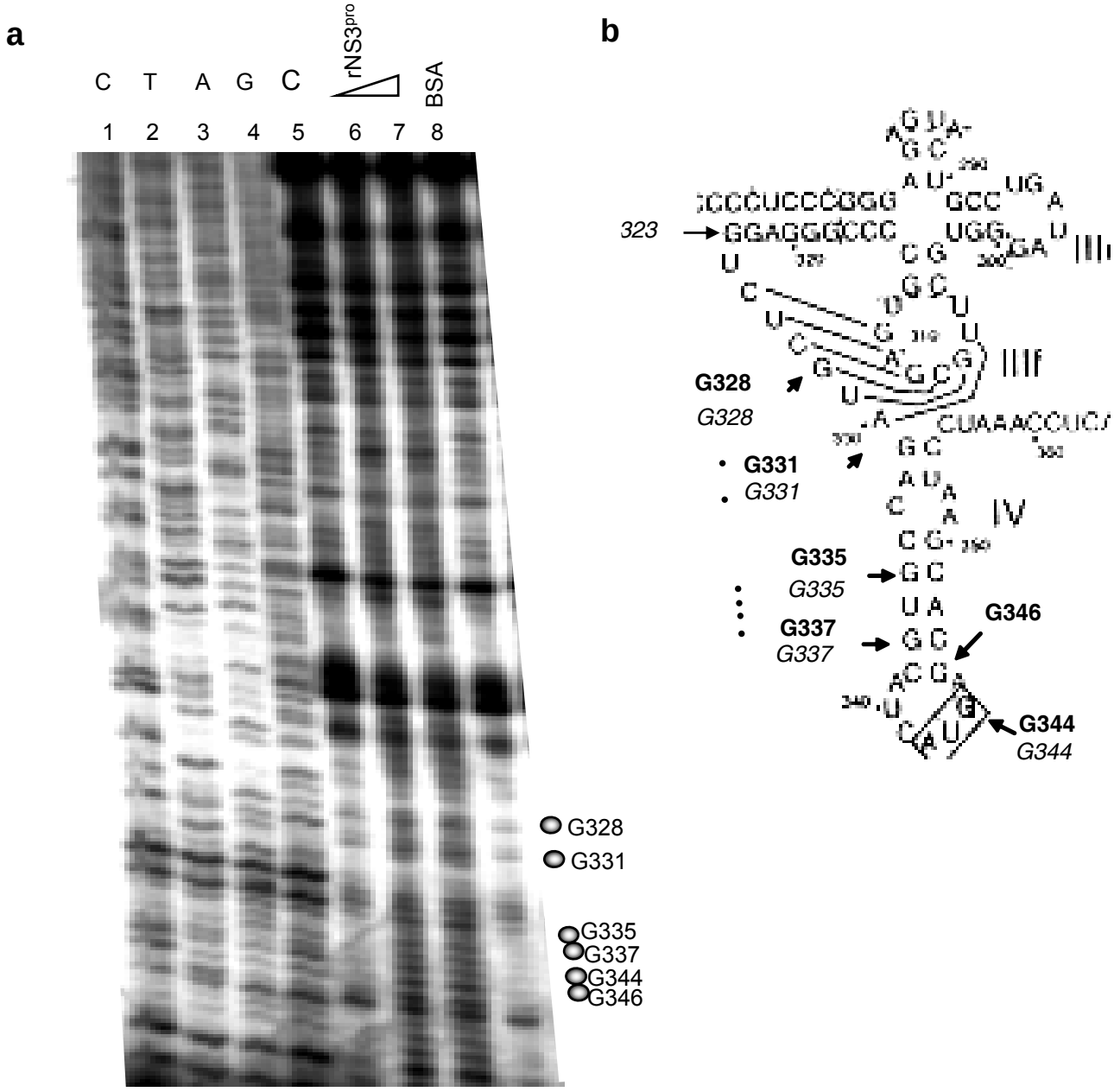


UV crosslinking assay: α[^{32}P]UTP labeled HCV IRES (lanes 1-2) and Polio IRES RNA (lanes 3-4) were UV-crosslinked to NS3^{pro} protein. The protein-nucleotide complex was resolved in 15% SDS-PAGE followed by phosphor imaging analysis. The position of NS3^{pro} protein is indicated by an arrow.

b



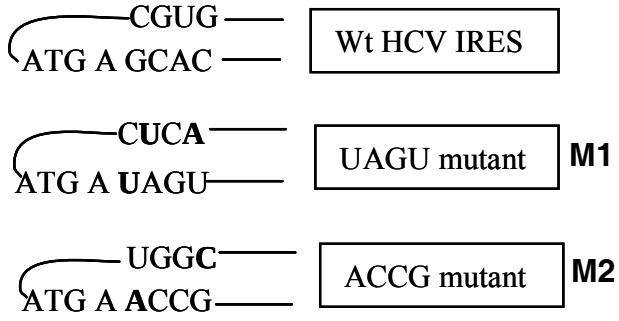
Competition UV crosslinking assay: α[^{32}P]UTP labeled HCV IRES was UV-crosslinked to NS3^{pro}. Unlabelled cold HCV IRES (lanes 3-4), SLII (lanes 5-6), SLIII (lanes 7-8) and SLIV RNA (lanes 9-10) were used for competition. Lane 2 is a control without cold RNA competition. The protein-nucleotide complex was resolved in 15% SDS-PAGE followed by phosphor imaging analysis. The position of NS3^{pro} protein is indicated by an arrow.



Panel a: Toe printing assay. Unlabeled HCV5′-UTR RNA was incubated in absence (lane 5) or presence of increasing concentration of purified recombinant NS3^{pro} (lanes 6-7) or BSA as non specific protein (lane 8) and then annealed to an end labeled primer. The RNA-protein complex was reverse-transcribed. The cDNA was resolved on a sequencing gel. In parallel to the cDNAs, sequencing reaction was run (lanes 1-4) to indicate the position of RT pauses. Major toe prints are indicated on the right of the panel.

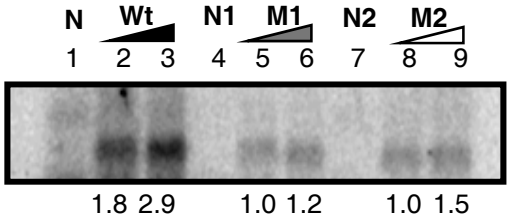
Panel b: Schematic representation of SLIV + pseudoknot region (adopted from Brown *et al*, 1992) showing the positions of Toe prints (bold) and foot prints.

a



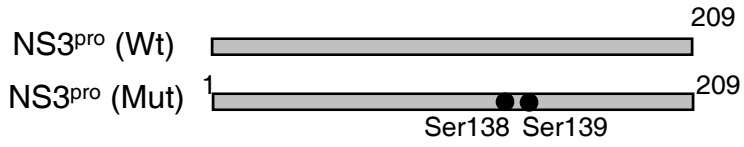
Schematic representation of wild type and the two mutant SLIV (UAGU and ACCG mutants) constructs showing the positions of the mutations.

b



UV crosslinking assay: α[^{32}P] UTP labeled HCV wild type (lanes 1-3), or M1 (lanes 4-6), and M2 IRES (lanes 7-9) RNAs were UV-crosslinked to increasing concentrations of NS3^{pro}. The protein-nucleotide complex was resolved in 15% SDS-PAGE followed by phosphor imaging analysis. The position of NS3^{pro} protein is indicated by an arrow. N, N1 and N2 represents no protein control.

a



Alignment of nucleotide sequence of wild type NS3 and mutant NS3 using NCBI BLAST

```
Score =  815 bits (441),  Expect = 0.0
Identities = 445/447 (99%), Gaps = 0/447 (0%)
Strand=Plus/Minus

Query  346
TGCACCTGCGGCAGCTCGGACCTTTACCTGGTCACGAGACATGCTGATGTCATCCCGGTG  405

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  207
TGCACCTGCGGCAGCTCGGACCTTTACCTGGTCACGAGACATGCTGATGTCATCCCGGTG  148

Query  406
CGCCGGCGGGGCGACACTAGGGGGAGCTTGCTCTCCCCTAGACCCATCTCCTACTTGAAG  465

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  147
CGCCGGCGGGGCGACACTAGGGGGAGCTTGCTCTCCCCTAGACCCATCTCCTACTTGAAG  88

Query  466
GGCTCTTCGGGTGGTCCATTGCTCTGCCCCTCGGGGCACGTTGTGGGCATCTTCCGGGCT  525
          |||  ||
||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87
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Query  526  GCCGTGTGCACCCGGGGGGTCGCGAAG  552
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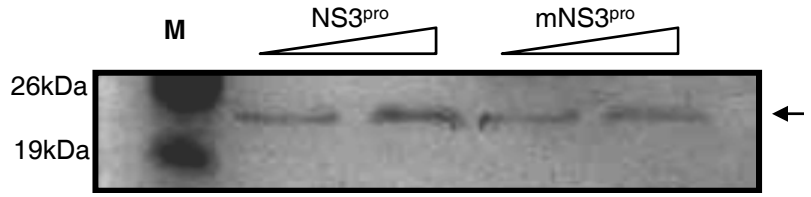
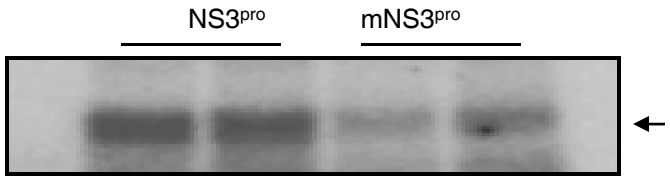
b



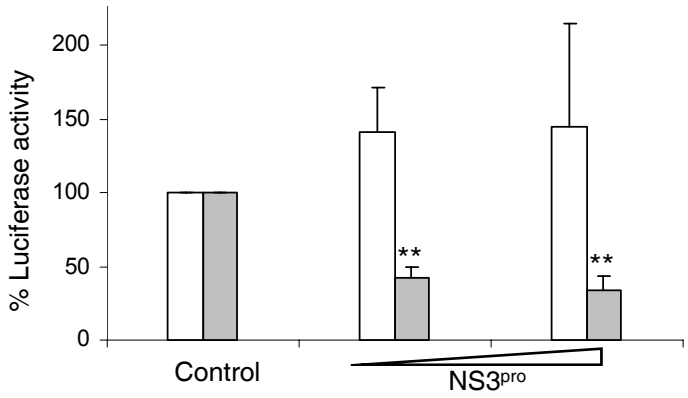
Silver stained gel: Purified wild type and mutant NS3^{pro} were resolved in 15% SDS-PAGE followed by silver staining. Lane M represents the protein marker.

c



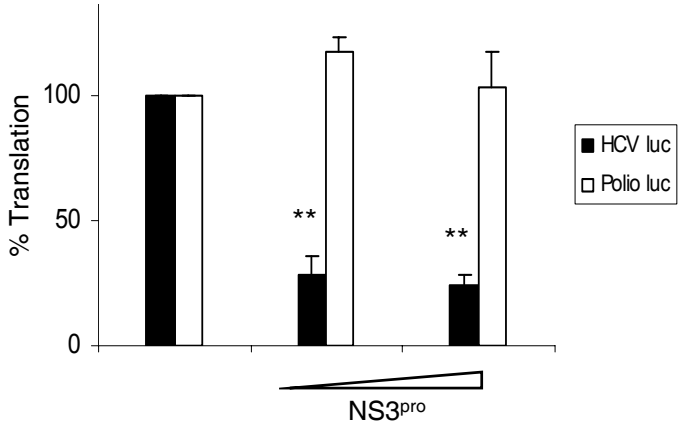
UV crosslinking assay: α[^{32}P]UTP labeled HCV IRES RNA was UV-crosslinked to NS3^{pro} or mNS3^{pro} (as indicated). The protein-nucleotide complex was resolved in 15% SDS-PAGE followed by phosphor imaging analysis. The position of the respective proteins are indicated by arrows.

a

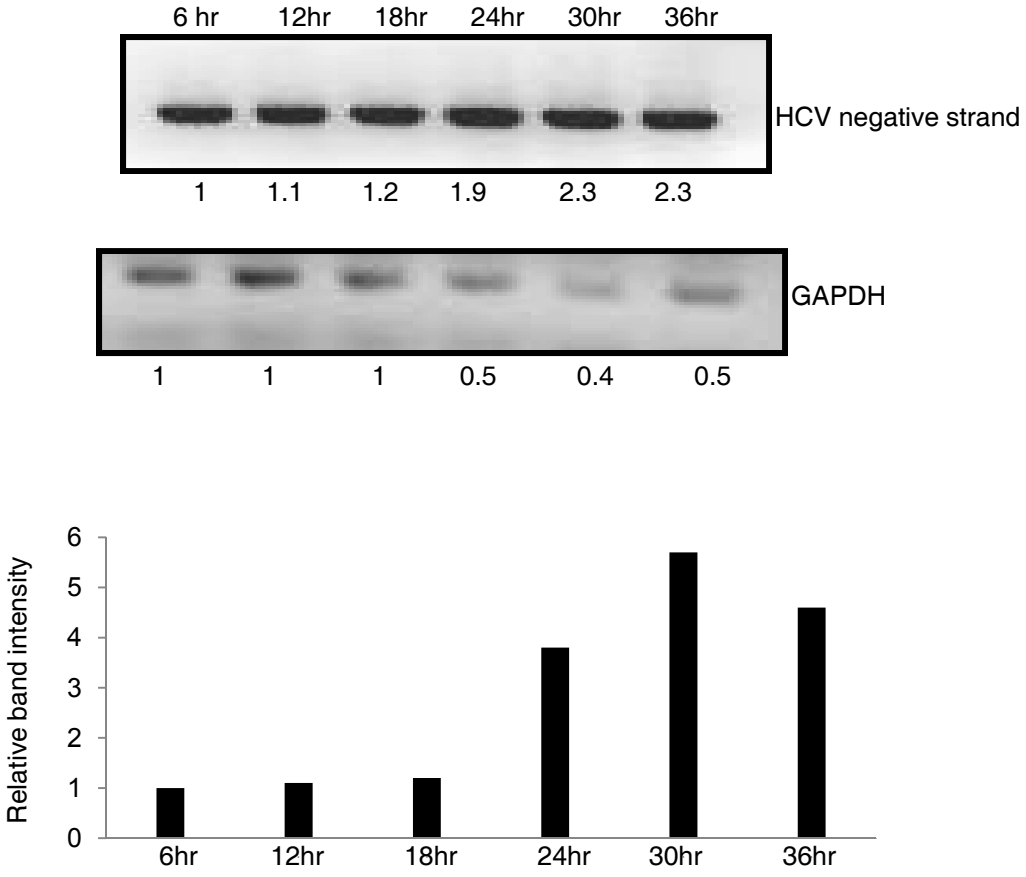


HCV bicistronic construct was transiently cotransfected with increasing concentrations of $NS3^{pro}$ mammalian expression construct. DNA concentrations were normalized using the empty vector DNA. 24 hours post transfection, cells were lysed and luciferase assay was performed. Percent luciferase activities corresponding to Rluc (white bar) and Fluc (grey bar) values were plotted against the protein concentrations. Values which significantly differ from control (P value<0.001) are indicated as asterisks.

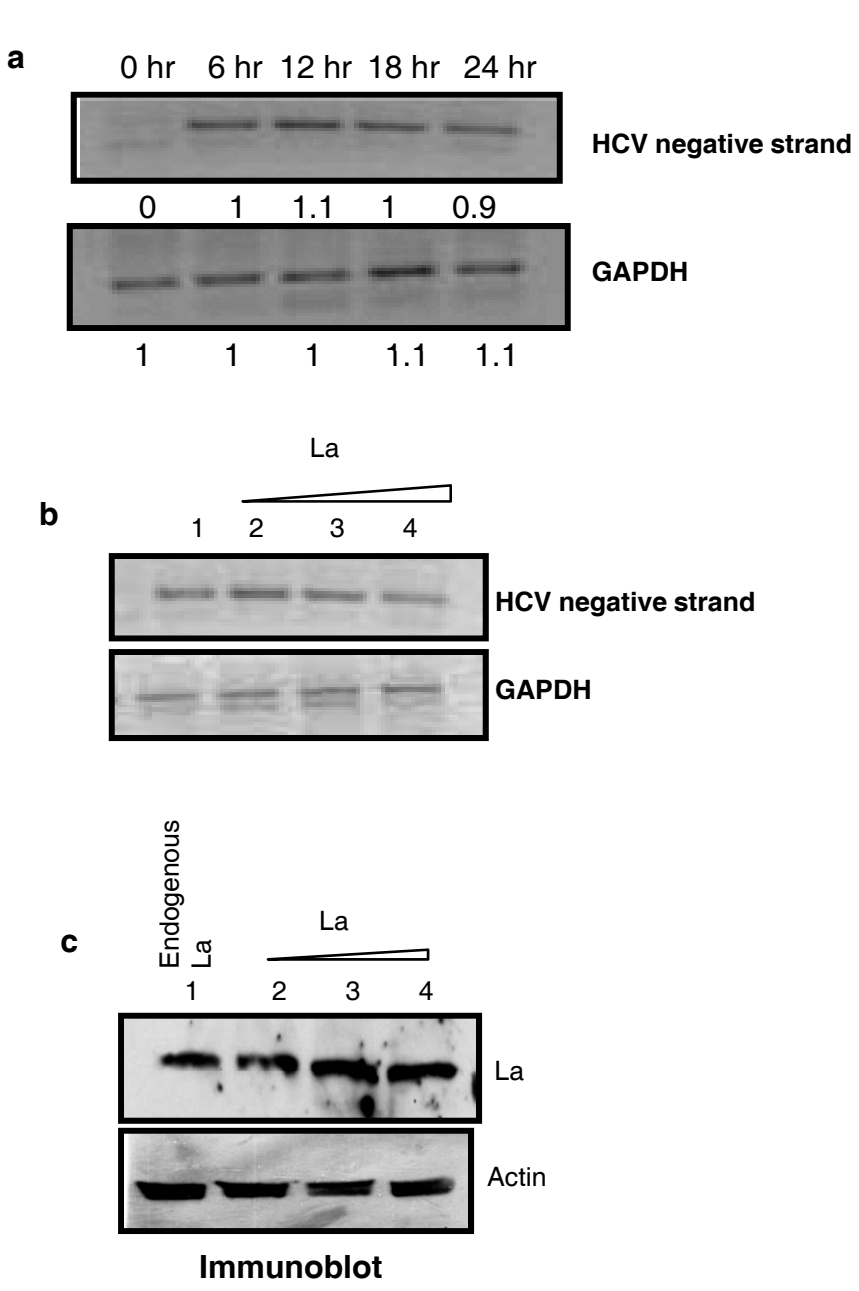
b



In vitro translation : HCV-IRES luc RNA and Polio-IRES luc RNA were translated *in vitro* in rabbit reticulocyte lysate in presence of increasing concentrations (0.2 and 0.4μM) of $NS3^{pro}$ protein. Values which significantly differ from control (P value<0.001) are indicated as asterisks.



Semi quantitative RT-PCR: Huh7 cells were transiently cotransfected with 5µg of pSGR JFH1/Luc RNA and 500ng of vector using Lipofectamine 2000 as transfection reagent. At 6th, 12th, 18th, 24th, 30th and 36th hour of transfection, RNA was isolated and HCV negative strand RNA was detected using semi quantitative RT PCR followed by densitometry of the band intensities. GAPDH was detected as internal control. Band intensities of the HCV negative strand amplification was normalized with that of GAPDH and plotted with time points on X axis and relative band intensity on Y axis

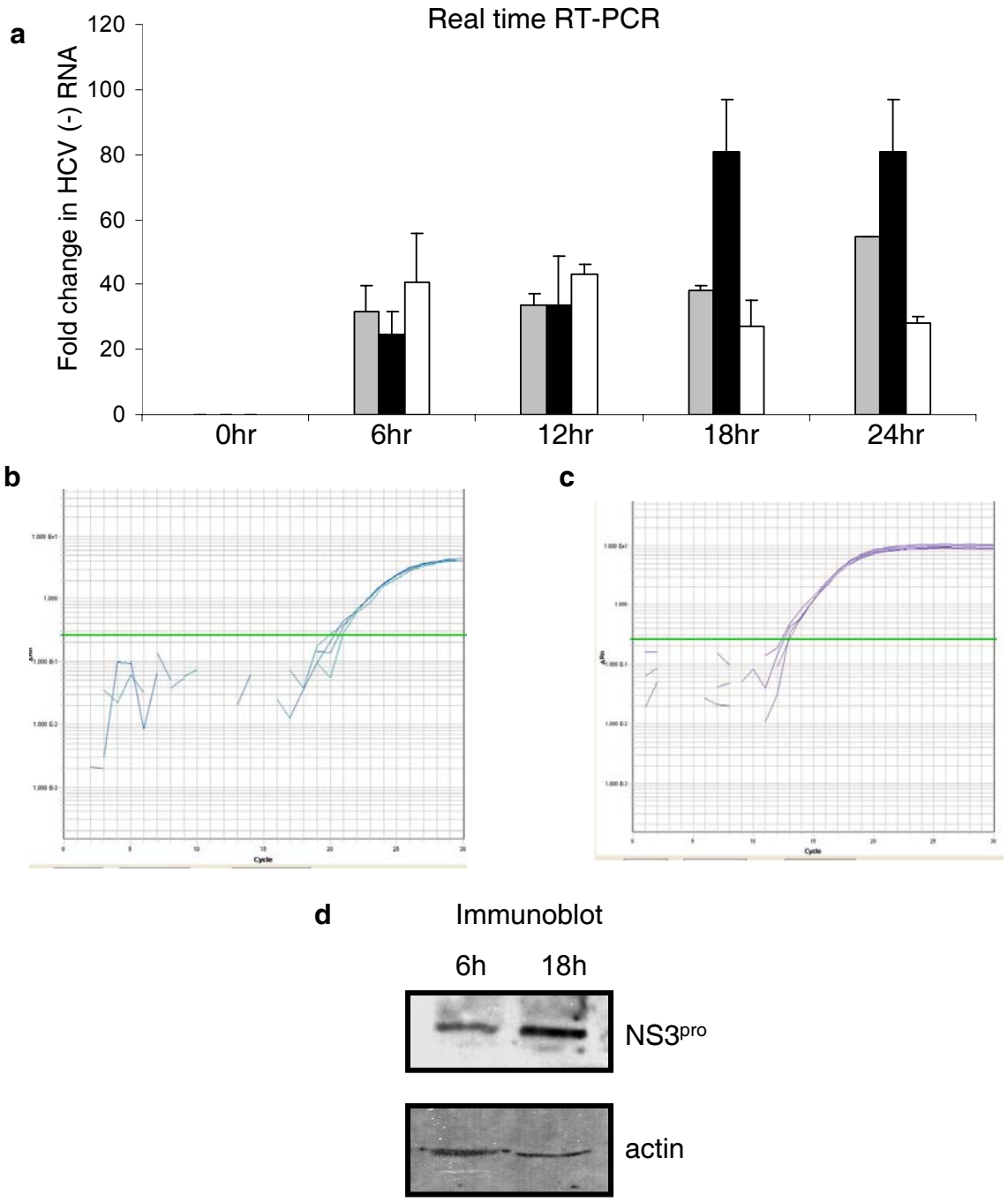


Huh 7 cells were transiently co-transfected with HCV dicistronic replicon (Kato et al 2005) along with La encoding plasmid. RNA was isolated at different time points (as indicated) and reverse transcribed with HCV 5' primer and GAPDH 3' primer using MMLV RT (Promega) for amplification of negative strand of HCV RNA. Resulting cDNA was used for PCR amplification corresponding to HCV IRES. The PCR products were run on 2% agarose gel. GAPDH was used as an internal control (bottom of each panel).

Panel a: effect of La over expression on HCV negative strand synthesis at different time intervals.

Panel b: effect of increasing concentrations of La overexpressions at 18 hours post transfection.

Panel c: Western blot analysis of the above reactions (described in panel b) to show over expression of La protein, actin was used as internal control

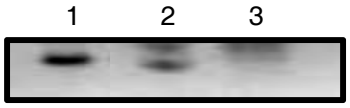


Real time RT-PCR: Huh7 cells were transiently cotransfected with 5µg of pSGR JFH1/Luc RNA and construct encoding either NS3 protease (black bar) or core (white bar) or vector alone (grey bar) using Lipofectamine 2000 as transfection reagent. At 0, 6th, 12th, 18th and 24th hour post transfection, RNA was isolated and HCV negative strand RNA was detected using real time RT PCR. GAPDH was used as an internal control (panel a). The data was analyzed by ABI-Prism's Real time PCR machine. Fold change in RNA level was calculated taking 0th hour time point as control.

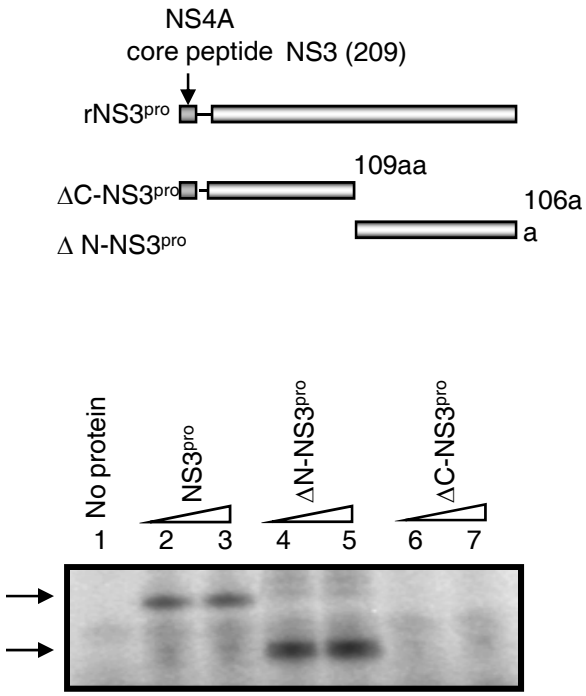
Panel b and c: Representative amplification plots for HCV IRES and GAPDH respectively as obtained in the real time RT-PCR mentioned above.

Panel d: Western blot analysis to show over expression of NS3^{pro} in the transfected cells.

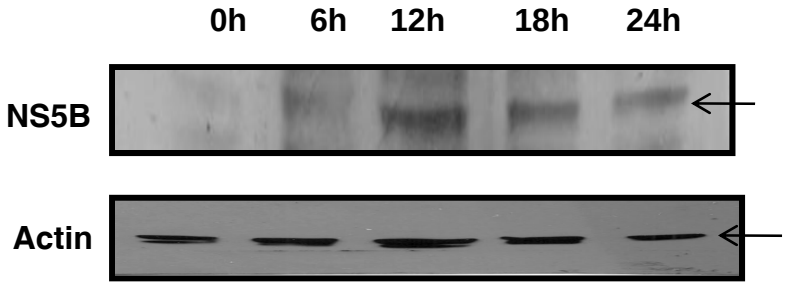
PCR: Possibility of DNA contamination was checked by PCR of the in vitro transcribed pSGRlucJFH1 RNA for HCV IRES (Lane 1). Lane 2 is the positive control for the PCR where pSGRlucJFH1 plasmid DNA was used



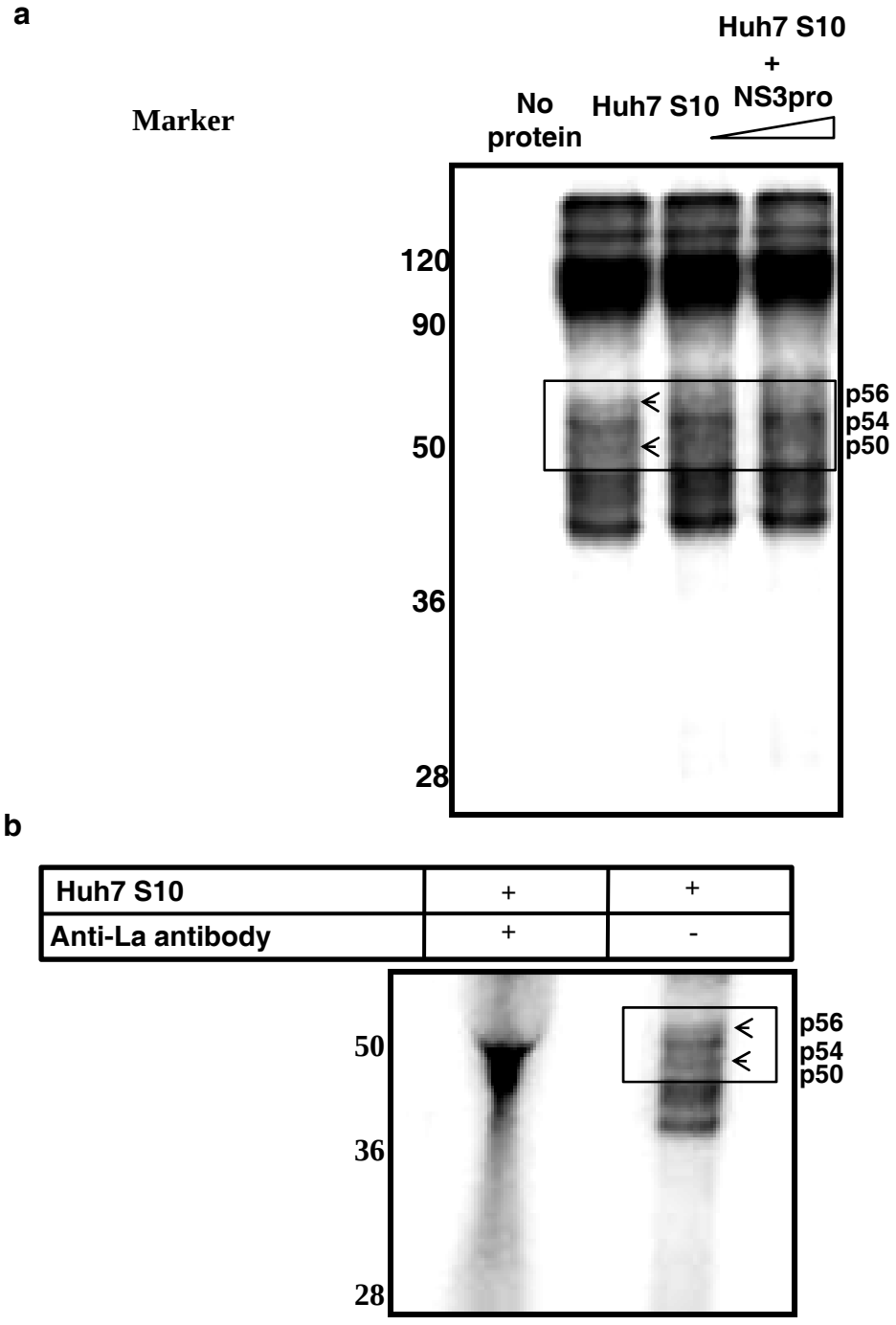
PCR: Strand specificity of reverse (Lane 2) and forward (Lane 3) HCV IRES primers was checked by RT-PCR of the in vitro transcribed pSGRlucJFH1 RNA for HCV IRES. Lane 1 is the positive control for the PCR where pSGRlucJFH1 plasmid DNA was used



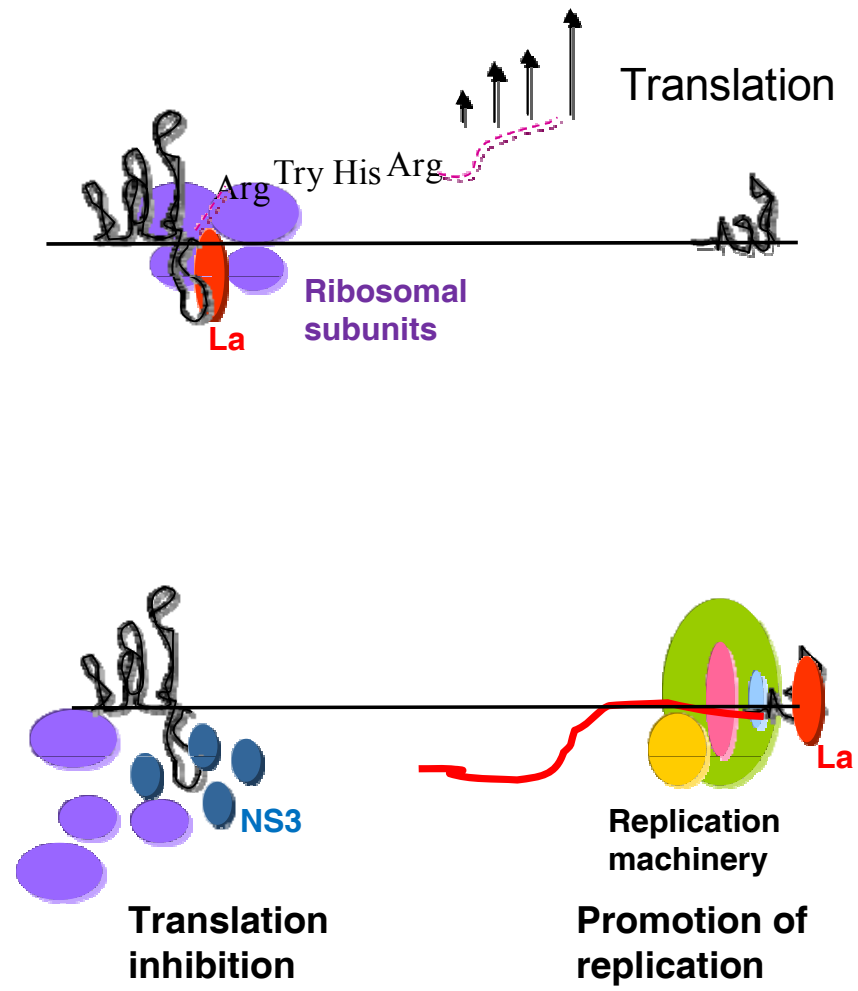
UV crosslinking assay: [α^{32}P] UTP labeled HCV IRES RNA was UV cross-linked with increasing concentrations of HCV NS3^{pro} (lanes 2-3), ΔN-NS3^{pro} (lanes 4-5) or ΔC-NS3^{pro} (lanes 6-7) as indicated on the top of the lanes.



Immunoblot: Huh 7 cells were transiently co-transfected with HCV dicistronic replicon (Kato et al 2005) along with $NS3^{pro}$ encoding plasmid. Western blot analysis of the above reactions was carried out using anti-NS5B antibody to show levels of NS5B protein. Actin was used as internal control



UV crosslinking assay: **Panel a:** α[^{32}P] UTP labeled HCV SLIV RNA was UV-crosslinked to Huh7 S10 protein in absence and presence of increasing concentration of NS3^{pro}. The protein-nucleotide complex was resolved in 12% SDS-PAGE followed by phosphor imaging analysis. The position of different cellular proteins interacting with the SLIV RNA is indicated. **Panel b.** Similarly the RNA protein complex was immuno precipitated using anti La antibody and resolved in SDS 15%PAGE. The direct binding of HCV SLIV with Huh7S10 was run alongside. The numbers at the left of each panel represent the molecular masses of marker run alongside



La protein stimulates HCV IRES mediated translation. NS3 binding competes out La from SLIV region. Dislodging of La leads to ribosome unloading and translation inhibition. La binds to 3'UTR, replication complex assembles and HCV RNA starts replicating.