

# Y Box-binding Protein-1 Binds to the Dengue Virus 3'-Untranslated Region and Mediates Antiviral Effects\*

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Dengue virus, a member of the family Flaviviridae, poses a serious public health threat worldwide. Dengue virus is a positive-sense RNA virus that harbors a genome of ~10.7 kb. Replication of dengue virus is mediated coordinately by cis-acting genomic sequences, viral proteins, and host cell factors. We have isolated and identified several host cell factors from baby hamster kidney cell extracts that bind with high specificity and high affinity to sequences within the untranslated regions of the dengue virus genome. Among the factors identified, Y box-binding protein-1 (YB-1) and the heterogeneous nuclear ribonucleoproteins (hnRNPs), hnRNP A1, hnRNP A2/B1, and hnRNP Q, bind to the dengue virus 3'-untranslated region. Further analysis indicated that YB-1 binds to the dengue virus 3' stem loop, a conserved structural feature located at the 3' terminus of the 3'-untranslated region of many flaviviruses. Analysis of the impact of YB-1 on replication of dengue virus in YB-1<sup>+/+</sup> and YB-1<sup>-/-</sup> mouse embryo fibroblasts indicated that host YB-1 mediates an antiviral effect. Further studies demonstrated that this antiviral impact is due, at least in part, to a repressive role of YB-1 on dengue virus translation via a mechanism that requires viral genomic sequences. These results suggest a novel role for YB-1 as an antiviral host cell factor.

Dengue virus (DENV)<sup>2</sup> is the etiologic agent of dengue fever, currently the most prevalent arthropod-borne viral disease of humans. DENV is related to other medically important flaviviruses, including West Nile, yellow fever, and Japanese encephalitis viruses, and is transmitted to humans by the mosquitoes *Aedes aegypti* and *Aedes albopictus*. DENV contains a positive-sense RNA genome of 10.7 kb that is translated in the cytoplasm of infected cells as a single polyprotein and subsequently cleaved to yield three structural proteins (capsid, envelope, and pre-membrane) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (1). Following a requisite

first round of viral translation in the cytoplasm, the subsequent steps of genome replication, second round translation, and assembly are coordinated to generate infectious DENV virions. Four serotypes of DENV (DENV1–4) co-circulate in most endemic countries, increasing the incidence of the more severe forms of the disease, dengue hemorrhagic fever and dengue shock syndrome. To date, only palliative treatments and supportive therapies are available for tens of millions of patients who acquire the disease annually. The identification of host cell factors that play a role in DENV infection remains an essential and largely unexplored area in the characterization of the mechanism of viral replication and the development of effective antiviral therapies.

The DENV genome contains a 7mG cap and is flanked by 5'- and 3'-untranslated regions (UTRs) of ~96 and 451 nucleotides, respectively. The 3'-UTR of DENV differs from nearly all host cell mRNA 3'-UTRs in that it lacks a poly(A) tail. The DENV 5'- and 3'-UTRs contain sequences and secondary structures that are highly conserved among flaviviruses and are important for the regulation of translation and replication (1–12). Viral end-to-end interactions have been demonstrated directly (3) and shown to be involved in DENV RNA synthesis (5). 5'-to-3'-UTR interactions are presumed to be involved in the regulation of viral translation as well (13). The ~100 nucleotides at the 3' terminus of the 3'-UTR of all flaviviruses form a thermodynamically stable and conserved structural element, the 3' stem loop (3'SL), that has been demonstrated to play an important role in viral replication. Deletion of the 3'SL significantly reduces translation of viral reporter RNAs (6), whereas mutagenesis of the 3'SL has been demonstrated to impair DENV translation and replication (7, 12). The loop region of the 3'SL, referred to as the pentanucleotide loop, contains sequences that are necessary for replication of flaviviruses, including West Nile and dengue viruses (10). Phosphorodiamidate oligomers targeted to this region interfered with both translation and replication of DENV reporter replicons (7, 8). Despite the delineation of many important cis-elements in the DENV genome, our understanding of the molecular basis of their action remains unclear.

In particular, very little is currently understood about the role of host factors in maintenance and modulation of DENV genomic structure and function. Although host-derived proteins, including La autoantigen (La) (14–17), eukaryotic elongation factor 1A (eEF1A) (14, 18), and the polypyrimidine tract-binding protein (14), have been shown to interact with the 3'-UTRs of DENV and West Nile virus genomes, their precise roles in viral replication are not clear. For example, investigators have demonstrated that La can bind to both the 5'- and

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<sup>2</sup> The abbreviations used are: DENV, dengue virus; hnRNP, heterogeneous nuclear ribonucleoprotein; UTR, untranslated region; HIV-1, human immunodeficiency virus, type 1; GMP-PNP, guanosine-5'-[(β,γ)-imido]triphosphate; RT, reverse transcription; MEF, mouse embryo fibroblast; EMSA, electrophoretic mobility shift assay; m.o.i., multiplicity of infection; FCS, fetal calf serum; BHK, baby hamster kidney; MALDI, matrix-assisted laser desorption ionization; MS/MS, tandem mass spectrometry; 3'SL, 3' stem loop.

## YB-1 Binds Dengue Virus 3'-UTR and Has Antiviral Effects

3'-UTRs of DENV (15). Although subsequent experiments indicated that La undergoes changes in cellular localization during DENV infection and that La can repress viral RNA synthesis *in vitro* (16), using a dominant negative La protein, we were unable to demonstrate any impact of La on DENV propagation in cultured cells.<sup>3</sup> Clearly, the identification and characterization of host cell factors that are involved in the orchestration of different stages of the DENV life cycle will extend our understanding of the molecular mechanisms of DENV replication.

One of the challenges to identification of host cell proteins that interact with a viral genome is to ensure that isolated proteins bind with sufficient specificity and affinity to suggest biological significance. To increase the likelihood of biochemical isolation of physiologically relevant host cell binding factors, we have utilized an *in vitro* biochemical system that mimics properties of DENV infection observed *in vivo*. Specifically, we employed RNA affinity chromatography to isolate DENV UTR-interacting host cell factors from a baby hamster kidney cell extract that recapitulates DENV translation dynamics observed *in vivo*. In further experiments, we investigated the role of one of the host cell factors identified, Y box-binding protein 1 (YB-1), in DENV replication. YB-1 is a cold shock domain protein that has been shown previously to be involved in diverse cellular processes, including the regulation of cap-dependent translation, transcription, and signal transduction (reviewed in Ref. 19). YB-1 has previously been implicated in oncogenesis and has been shown to associate with several DNA viruses. Our results indicate that YB-1 elicits an antiviral effect on DENV replication that is mediated, in part, through repression of DENV viral translation.

### EXPERIMENTAL PROCEDURES

**Cell Lines, Extracts, Virus Infection, and Flow Cytometry**—Baby hamster kidney (BHK)-21 cells (clone 15) were maintained in minimum essential media  $\alpha$  (Invitrogen) containing 5% fetal calf serum (FCS, HyClone, Logan, UT), 2 mM Glutamax (Invitrogen), and 100 units of penicillin/100  $\mu$ g of streptomycin (P/S) (Invitrogen) at 37 °C in 5% CO<sub>2</sub>. Immortalized mouse embryo fibroblasts (MEFs) generated from YB-1<sup>+/+</sup> and YB-1<sup>-/-</sup> mice (20) were obtained from Zhi Hong Lu and Timothy Ley (Washington University, St Louis). Cells were maintained in Dulbecco's modified Eagle's media (Invitrogen) containing 10% FCS, 0.1 mM nonessential amino acids (Invitrogen), 2 mM Glutamax (Invitrogen), and P/S. C6/36 *A. albopictus* mosquito cells were maintained in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FCS, P/S, and 100 mM HEPES, pH 7.2.

For extract preparation, BHK cells were adapted for growth in suspension by maintenance in a rich growth medium. Specifically, cells were grown in RPMI 1640 medium (Invitrogen) containing 10% FCS, 10 mM HEPES, pH 7.2, and P/S. Cells were seeded in a spinner bottle and supplemented with growth medium every 2–3 days to maintain a density of 10<sup>6</sup> cells/ml. Twelve to 24 h prior to harvesting, growth medium was added to ensure that harvested cells were in growth phase. Translation

extracts were prepared as described previously (21). Briefly, BHK cells were harvested, washed with PBS, resuspended in hypotonic lysis buffer (21), and lysed via passage through a 21-gauge needle. Lysates were subjected to centrifugation (10,000  $\times$  g) for 5 min. Supernatant was harvested and supplemented with glycerol to a final concentration of 10% prior to flash-freezing in liquid nitrogen. Translation extracts were thawed and supplemented with a freshly prepared mixture of ATP, GTP, creatine phosphokinase, phosphocreatine, and protease inhibitors prior to use.

Infection of MEFs by DENV2 strain 16681 was performed as described previously (22). Briefly, cells were incubated with virus at an indicated m.o.i. for 2 h in a volume of medium sufficient to cover the cells. At 2 h post-infection, cells were washed four times and then incubated in growth medium. At indicated times post-infection, cell supernatants were collected and supplemented with FCS to 20%. Detached cells and cellular debris were removed by centrifugation at 1200 rpm for 3 min. Viral supernatants were stored at -80 °C until plaque assays were conducted. Plaque assays were performed on BHK cells as described previously (22).

Flow cytometric analysis was performed to determine intracellular levels of DENV2 NS3 protein in infected MEF cells. Flow cytometry was conducted as described previously (22, 23). DENV NS3 protein was detected using a mouse monoclonal antibody to DENV NS3.<sup>4</sup> Following overnight incubation with the primary antibody at 4 °C, samples were washed with PBS and stained with a secondary Alexa 488-conjugated goat anti-mouse antibody (Invitrogen) for 1–2 h at 25 °C. NS3 expression was measured using an EPICs XL flow cytometer (Beckman-Coulter, Fullerton, CA).

**RNA Constructs, *In Vitro* Transcription, and Transfection**—A region consisting of five nucleotides of NS5 and the entire 3'-UTR of DENV2 (451 nucleotides) was amplified from infectious clone pD2/IC (a gift of Richard Kinney, Centers for Disease Control and Prevention, Fort Collins, CO) using a forward primer containing an EcoRI site and a T7 promoter (5'-CCG-GAATTCTAATACGACTCACTATAGGTAGAAAGCAA-AAC-3') and a reverse primer containing an XbaI restriction site (5'-GACTTCTAGCCTTGTTCATGTTAG-3'). PCR products were cloned into pBSKS II (Stratagene, La Jolla, CA) to create a vector from which sense and antisense 3'-UTR could be transcribed in separate reactions following digestion with different restriction enzymes and gel purification. DENV2 3'SL constructs were generated from the DENV2 3'-UTR constructs using a forward primer containing an EcoRI site, a T7 consensus site, and homology to the 5' end of the 3'SL (5'-CGGAAT-TCTAATACGACTCACTATAGGGAAAGACCAGAGATC-CTGCTGTCTCC-3') and a reverse primer with homology to the 3' end of the 3'SL (5'-GGTCGACTCTAGAGAACCTGT-TGATTCAAAC-3'). PCR fragments were subcloned into pTOPO (Invitrogen), excised, and cloned into pBSKS II (Stratagene). DENV2 3'-UTR RNA and firefly luciferase reporter RNAs, 5DLuc3D and 5DLuc3 $\Delta$ SL (6), and DENV2 *Renilla* luciferase replicons, (p)DRrep and (p)DRrep-RdRpmut,<sup>5</sup> were

<sup>3</sup> J. Walker, S. Paranjape, and E. Harris, unpublished results.

<sup>4</sup> P. R. Beatty and E. Harris, unpublished results.

<sup>5</sup> K. Clyde, J. Barrera, and E. Harris, submitted for publication.

transcribed using a Ribomax T7 RNA polymerase kit (Promega, Madison, WI). A 7-methyl-GpppA nucleotide was incorporated at the initial adenosine residue during transcription of luciferase reporter constructs (New England Biolabs, Ipswich, MA). Unincorporated nucleotides were removed using NucAway columns (Ambion, Austin, TX). RNA was concentrated, when necessary, by ethanol/ammonium acetate precipitation. DENV2 luciferase reporter constructs were transfected into YB-1<sup>-/-</sup> and YB-1<sup>+/+</sup> MEF and BHK cells using Lipofectamine 2000 (Invitrogen) as described previously (7). MEF and BHK cells transfected with *Renilla* luciferase replicons were harvested at various times post-transfection as indicated. MEF cells transfected with 5DLuc3D and 5DLuc3ΔSL firefly luciferase reporter constructs were harvested at 4–7 h post-transfection. Translation was monitored by luciferase production as described previously (6, 7). Briefly, cells were washed with PBS and lysed with Cell Culture Lysis Reagent (Promega) for assessment of firefly luciferase levels or with *Renilla* Luciferase Assay Lysis Buffer (Promega) for measurement of *Renilla* luciferase. Luciferase production was monitored using Luciferase Assay Reagent Substrate or *Renilla* Luciferase Assay Substrate (Promega) and a TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA).

Assessment of transfection efficiency was determined by quantitative RT-PCR analysis as described elsewhere (7).<sup>5</sup> Specifically, RNA was harvested from cells at 2 h post-transfection using the RNeasy system (Qiagen, Valencia, CA) or mini RNA Isolation II kits (Zymoresearch, Orange County, CA) and then quantitated using the Lux system (Invitrogen) for RNA reporter constructs or the Taqman system (Applied Biosystems, Foster City, CA) for DENV replicon constructs. Relative transfection efficiencies of YB-1<sup>-/-</sup> and YB-1<sup>+/+</sup> cells were determined and used to normalize luciferase activity.

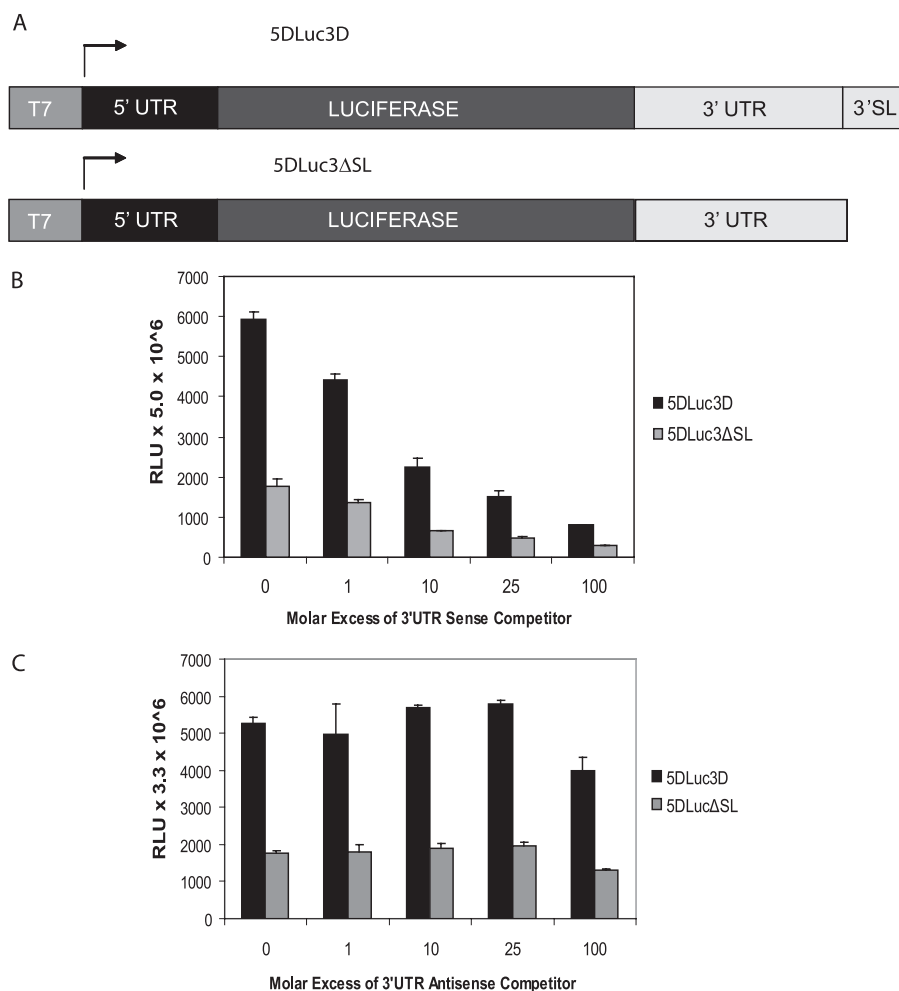
**Affinity Chromatography**—To prepare affinity resin, 5 μM of DENV reporter RNA was oxidized by incubation in 0.1 M sodium periodate, 0.1 M NaOAc, pH 5.0, for 1 h at 25 °C. Following oxidation, RNA was precipitated by addition of 1 ml of ice-cold ethanol and isolated by centrifugation at 10,000 × g for 15 min. Precipitated RNA was washed with 70% ethanol, dried briefly in a speedvac, and resuspended in 0.1 M NaOAc, pH 5.0. Coupling of RNA to the resin was accomplished by incubating oxidized RNA with 100 μl of hydrazide-agarose resin (Sigma) (equilibrated in 0.1 M NaOAc, pH 5.0) on an end-over-end rotator at 4 °C for 12–18 h. Uncoupled RNA was removed by washing three times each with 2.0 M NaCl, 0.1 M NaOAc, and translation buffer (21). Equilibrated resin was incubated with BHK translation extract at 30 °C for 30–60 min. Reactions were conducted in the presence of translation inhibitors and/or RNA competitors as indicated. Translation competence of the extract was monitored by luciferase production using Luciferase Assay Reagent (Promega) as above. Following binding, protein-RNA complexes were washed extensively in buffer containing 100 mM KCl and then eluted with a step gradient of KCl (0.1–1 M). Eluted protein fractions were concentrated with 20% trichloroacetic acid containing deoxycholate salt and resolved by SDS-PAGE. Proteins were stained with Coomassie Colloidal Blue (Invitrogen). Protein bands of interest were excised and submitted for MS/MS mass spectrometry as described below.

**Mass Spectrometry**—Gel bands of interest were excised and digested with trypsin (Promega) (25). The resulting digests were purified with C18 μZipTips (Millipore) and subjected to mass spectrometric analysis on an Applied Biosystems 4700 Proteomics Analyzer, which is a MALDI-time-of-flight tandem mass spectrometer (26). The purified sample was mixed 1:1 with α-cyano-4-hydroxycinnamic acid matrix (10 g/liter), and 1.2 μl of the mixture was spotted onto the MALDI target plate. A reflector mode mass spectrum of the digest was first obtained, after which several individual peptide peaks were manually selected for MS/MS analysis. The peptide sequence for each was manually deduced from its MS/MS spectrum.

**Viral RNA Immunoprecipitation**—BHK cells were infected with DENV2 strain 16681 at an m.o.i. of 1. Specifically, BHK cells at 80% confluency were incubated with DENV2 16681 in RPMI medium with 2% FCS and P/S. Following incubation for 2 h, cells were washed four times to remove unincorporated virus and then incubated in RPMI growth media. At the indicated times post-infection, cells were harvested and cross-linked with 1% formaldehyde. Cells were disrupted by bead-beating in lysis buffer (50 mM HEPES/KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM aprotinin, and 20 units/ml Supersasin (Ambion)), and resultant cell lysates were immunoprecipitated with anti-YB-1 polyclonal rabbit antibody (gift of Valentina Evdokimova, University of British Columbia, Vancouver, Canada). Co-immunoprecipitated RNA was isolated using an RNeasy mini kit (Qiagen). One-step RT-PCR (Qiagen) was conducted using forward (5'-TTCCACACAATGTGGCAGTCAC-3') and reverse (5'-GGAGATCCTGACGTTCCRRGG-3') primers homologous to DENV NS3. RT-PCR products were resolved via electrophoresis on 1% agarose and visualized with ethidium bromide.

**Footprinting and Electrophoretic Mobility Shift Analysis**—RNA encoding the 3'SL of DENV was transcribed *in vitro* with T7 RNA polymerase. Following purification using NucAway columns (Ambion) to remove unincorporated nucleotides, RNA probes were labeled with [ $\gamma$ -<sup>32</sup>P]ATP. Probes were resolved on a 5% denaturing acrylamide gel, excised, and eluted. Gel-purified probe was extracted with phenol/chloroform/isoamyl alcohol, ethanol-precipitated, and resuspended in double distilled H<sub>2</sub>O. Probe concentration was determined by measurement of absorbance at 260 nm, and specific activity was determined by scintillation counting. Recombinant His-YB-1 was expressed in *Escherichia coli* using pHISYB-1 (gift of Valentina Evdokimova) following the procedure of Evdokimova *et al.* (27). For footprinting reactions, 10 pM of probe was incubated with purified His-YB-1 protein (at molar ratios indicated) for 15 min at room temperature. Subsequently, RNase T2 (Sigma), RNase T1 (Ambion), or RNase VI (Ambion) was added at the concentrations indicated, and reactions were incubated for an additional 10 min at room temperature before being stopped by the addition of phenol/chloroform/isoamyl alcohol. Following phenol/chloroform extraction, reactions were ethanol-precipitated in the presence of 3 μg of glycogen (Ambion). Samples were resuspended in formaldehyde loading buffer, heated to 95 °C for 3 min, and immediately cooled on ice. One-third of each sample was resolved on a 10% denaturing polyac-

## YB-1 Binds Dengue Virus 3'-UTR and Has Antiviral Effects



**FIGURE 1. Characterization of *in vitro* translation extract using DENV reporter constructs.** *A*, schematic representation of DENV wild type reporter construct, 5DLuc3D, and 3'SL deletion variant, 5DLuc3ΔSL. T7, T7 promoter. *B*, addition of increasing molar excess of sense DENV 3'-UTR significantly reduces translation *in vitro* of WT DENV reporters (5DLuc3D) and reporter constructs harboring a deletion of the 3'SL (5DLuc3ΔSL). *C*, addition of DENV 3'-UTR antisense RNA does not repress translation of 5DLuc3D or 5DLuc3ΔSL *in vitro*. In all experiments, RNA reporter constructs were incubated in BHK S-10 translation extract with competitors as indicated. Data shown are representative of four independent experiments. Error bars indicate S.D. ( $n = 3$ ).

rylamide gel. For electrophoretic mobility shift assays, 3'SL RNA probes were synthesized via *in vitro* transcription with T7 RNA polymerase, and [ $\alpha$ -<sup>32</sup>P]CTP was incorporated during transcription. 3'SL probe (10,000 cpm per reaction) was incubated with buffer or 5- or 10-fold molar excess of YB-1 at 25 °C for 15 min. Samples were resolved by electrophoresis on a 5%, 0.5 × TBE nondenaturing acrylamide minigel. Gels were dried and visualized using a PhosphorImager (GE Healthcare).

## RESULTS

**Isolation and Identification of Host Cell Proteins That Bind to the DENV 3'-UTR**—To isolate and identify mammalian host factors that are involved in various stages of DENV replication, we employed an RNA affinity chromatography strategy that involved the use of a mammalian cell extract that was competent for translation. BHK cells support high levels of DENV replication and, consequently, are widely employed to study DENV infection. This prompted us to develop a methodology for large scale production of BHK extracts. The BHK extract faithfully

recapitulated DENV 3'-UTR-dependent translation observed in cells (6). For example, translation of DENV reporter RNAs (described in Fig. 1*A*) harboring the full-length 3'-UTR (5DLuc3D) was significantly more efficient than translation of constructs containing a deletion of the 3'SL (5DLuc3ΔSL) or lacking the DENV 3'-UTR (Fig. 1*B* and data not shown), similar to results obtained in cultured cells (6). Moreover, translation in BHK cell extracts was effectively competed with excess sense (Fig. 1*B*) but not antisense DENV 3'-UTR RNA (Fig. 1*C*). At high concentrations (100-fold molar excess) of antisense RNA, nonspecific titration of translation factors is likely responsible for the slight decrease in translation that is observed (Fig. 1*C*). BHK extracts were distinct from rabbit reticulocyte lysates; translation of 5DLuc3D reporter constructs could not be efficiently competed by excess 3'-UTR sense competitor when reactions were performed in rabbit reticulocyte lysate extracts (data not shown). We reasoned that RNA affinity chromatography employing this functional BHK extract should therefore enable us to identify factors present in BHK cells that are potentially important for mediating DENV translation by the 3'-UTR.

To isolate proteins that associate with the UTRs of the DENV genome, 5DLuc3D RNA reporter constructs (Fig. 1*A*) were transcribed *in vitro* with T7 RNA polymerase, oxidized with sodium periodate, and coupled to hydrazide-agarose resin. Neither oxidation of RNA nor immobilization of reporter constructs prevented translational competency of reporter RNAs (data not shown). After extensive washing to remove uncoupled RNA, immobilized reporter constructs were incubated at 30 °C in a BHK S-10 translation extract. Importantly, the BHK S-10 lysate was not nuclease-treated, thereby ensuring the presence of endogenous competitor RNAs and more closely mimicking physiologic conditions. In these experiments, BHK extracts were treated with the nonhydrolyzable GTP analogue, GMP-PNP, which prevents translation by inhibiting the formation of the 80 S complex, thus enabling capture of proteins poised on the DENV message prior to translation elongation. Following incubation, immobilized protein-RNA complexes were washed extensively, and proteins bound to the reporter RNAs were eluted with a step gradient of KCl (0.1–1.0 M). Eluted fractions were

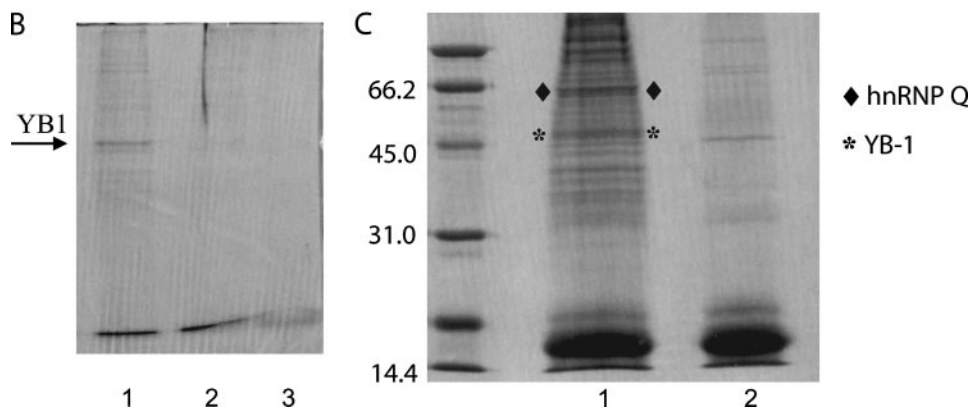
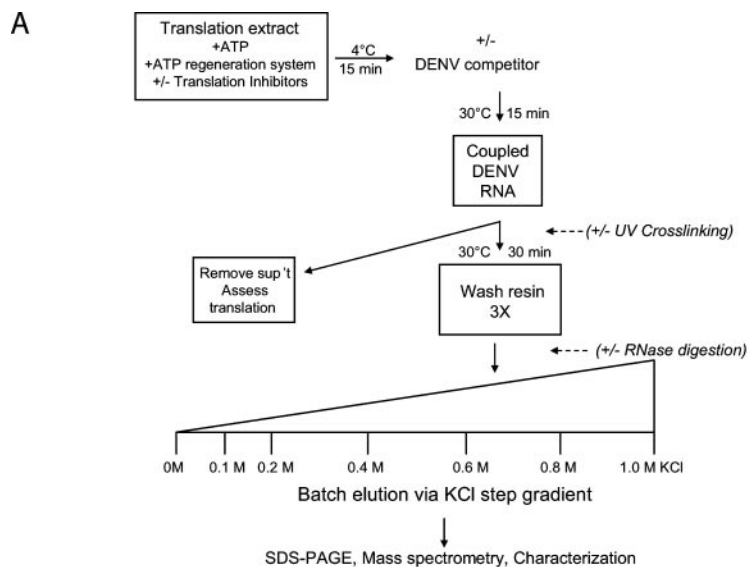
precipitated with trichloroacetic acid, resolved on 10% SDS-polyacrylamide gels, and stained with colloidal Coomassie Blue. Proteins of sufficient abundance were excised from the gel, digested with trypsin, and identified by MS/MS. Control purifications conducted in parallel using resin alone enabled

ascertainment of nonspecifically associating proteins. Using this approach, three proteins were determined to bind the DENV reporter constructs with high affinity as follows: eEF1A, hnRNP U, and eEF1B $\gamma$  (Table 1). eEF1A has been demonstrated previously to associate with the 3'SL of West Nile virus, DENV, and other flaviviruses (18), confirming the validity of the RNA affinity chromatography method that we employed.

**TABLE 1****Host factors that interact with the DENV2 UTRs**

DENV2 RNA sequence	Identified host protein	Molarity of elution buffer (KCl)
3'-UTR	Y box-binding protein-1 (YB-1)	0.75–1.0 M
3'-UTR	hnRNP A1	0.25–0.5 M
3'-UTR	hnRNP A2/B1	0.25–0.5 M
3'-UTR	hnRNP Q	0.25–0.5 M
3'-UTR	hnRNP H	NA <sup>a</sup>
5D3D reporter	Elongation factor 1B $\gamma$ (eEF1B $\gamma$ )	0.2–0.4 M
5D3D reporter	hnRNP U	0.2–0.4 M
5D3D reporter	eEF1A	0–0.1 M
No RNA	Actin	
No RNA	Annexin A2	

<sup>a</sup> NA indicates not applicable (eluted with sense 3'-UTR).



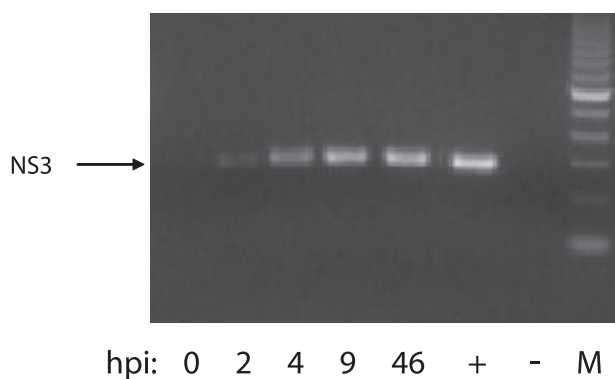
**FIGURE 2. DENV RNA affinity chromatography.** *A*, overview of RNA affinity chromatography strategy. *B*, DENV 3'-UTR RNA affinity chromatography with competition. Proteins eluted from a DENV 3'-UTR affinity column at 0.75–1.0 M KCl were resolved by 10% SDS-PAGE and stained with colloidal Coomassie Blue prior to excision of bands for mass spectrometry analysis. *Lane 1*, proteins eluted after binding reaction conducted in the absence of any competitor. Mobility of YB-1 is indicated by the arrow. *Lane 2*, binding reaction performed in the presence of DENV 3'-UTR competitor. *Lane 3*, negative control; no RNA coupled to affinity resin. *C*, cross-linking RNA affinity chromatography. Proteins were cross-linked to DENV 3'-UTR RNA affinity column with formaldehyde. Following extensive washing, proteins were released by treatment with RNase A and resolved by 10% SDS-PAGE. *Lane 1*, cross-linking reaction performed in the presence of BHK extract and immobilized DENV 3'-UTR RNA. *Lane 2*, negative control; no RNA coupled to resin.

To identify the factors that associate with the DENV 3'-UTR, DENV 3'-UTR RNA was coupled to hydrazide-agarose resin. RNA affinity resin was subsequently incubated with a BHK S-10 extract pretreated with 3'-UTR sense RNA or mock-treated to enable evaluation of binding specificity (Fig. 2*A*). Following incubation at 30 °C for 45 min, resin was washed extensively and then eluted with a KCl step gradient in translation buffer containing ATP and GTP. ATP and GTP were added during all steps of purification to ensure that factors that required nucleotides for binding would not be excluded. Fractions were

resolved by SDS-PAGE, and proteins of interest were excised and submitted for identification by MS/MS. Several proteins were identified that associated with the DENV 3'-UTR only in the absence of 3'-UTR sense competitor (Table 1). Among the proteins identified, hnRNP A1, hnRNP A2/B1, and hnRNP Q associated with the DENV 3'-UTR with moderate affinity (0.25–0.5 M KCl). Another RNA-binding protein, YB-1, associated with high affinity (1 M KCl) to DENV 3'-UTR sequences (Table 1 and Fig. 2*B*). Competition with excess sense-DENV 3'-UTR RNA blocked association of these proteins with the affinity resin, indicating that the binding of these factors was specific. Other proteins, including actin and annexin A2, bound nonspecifically to the DENV 3'-UTR, as deduced from the association of these proteins with mock-coupled resin lacking RNA.

To confirm association of these proteins with the DENV 3'-UTR, additional RNA affinity chromatography strategies were pursued. DENV2 3'-UTR RNA was coupled to hydrazide-agarose resin and incubated with BHK translation extract. In one set of experiments, the resin was subsequently washed, and bound proteins were eluted by the addition of 3'-UTR sense RNA. Eluted proteins were resolved by SDS-PAGE, excised, and identified by MS/MS. Results again indicated

## YB-1 Binds Dengue Virus 3'-UTR and Has Antiviral Effects



**FIGURE 3. Immunoprecipitation of DENV RNA from infected cells using anti-YB-1 antibody.** Infected BHK cells were cross-linked with formaldehyde, and cell lysate was precipitated with an antibody to YB-1. Following immunoprecipitation, cross-links were reversed, and complexes were incubated with proteinase K. RNA was purified and analyzed by RT-PCR using primers directed to the DENV NS3 coding region. Arrow indicates expected PCR product size of 278 bp. RNA amplified from cells harvested at 0, 2, 4, 9, and 46 h post-infection (*hpi*) is indicated. +, purified DENV2 RNA; -, double distilled H<sub>2</sub>O; M, molecular weight marker.

that YB-1 specifically associates with the DENV2 3'-UTR (data not shown). In another experiment, protein-RNA complexes assembled on immobilized DENV2 3'-UTR sense RNA were cross-linked by UV irradiation. Unassociated proteins were removed by extensive washing with buffer containing 0.1 M KCl, and resultant cross-linked RNA-protein complexes were digested with RNase, enabling resolution of bound proteins by SDS-PAGE and identification by MS/MS. Under these conditions, we identified several proteins, including YB-1, hnRNP Q, and hnRNP H, that associated with the DENV 3'-UTR (Table 1, Fig. 2C, and data not shown).

**YB-1 Associates with DENV RNA during Infection**—Given the observation that YB-1 associated with high affinity and specificity to DENV 3'-UTR *in vitro*, we sought to determine whether YB-1 associates with DENV RNA during viral infection. To accomplish this, viral RNA immunoprecipitation analysis was conducted. BHK cells were infected with DENV2 strain 16681 at an m.o.i. of 1 for 0, 2, 4, 6, 9, and 24 h. Following infection, cells were washed, harvested, and treated with formaldehyde to cross-link protein-RNA complexes. Cell lysates were prepared, and protein-RNA complexes were immunoprecipitated with an antibody to YB-1. Following reversal of cross-links, RNA was isolated, and RT-PCR was conducted with primers targeted to DENV NS3. As shown in Fig. 3, YB-1 associates with DENV RNA during viral infection at times as early as 2 h post-infection. These results confirm the physiologic association of YB-1 with the DENV genome during the course of viral infection.

**YB-1 Associates with the 3' SL of DENV**—Recent reports indicate that YB-1 levels are tightly controlled within the cell via a mechanism of translational autoregulation, wherein YB-1 binds to consensus sites within the 3'-UTR of its cognate mRNA (28). Inspection of the DENV UTRs revealed a sequence in the 3'-UTR with high homology to regulatory consensus sequences in the YB-1 3'-UTR (Fig. 4A). The region of homology overlapped sequences within the top loop of DENV 3' SL

(Fig. 4B, *dashed line*), referred to as the pentanucleotide loop, that is highly conserved among flaviviruses and is important for replication and translation of DENV (6, 7, 12) and West Nile virus RNA (18, 29).

To determine whether YB-1 associates with this sequence in the DENV 3'-UTR, EMSA and enzymatic footprinting analyses were conducted. Sequences encoding the 3' SL were cloned immediately downstream of a T7 RNA polymerase promoter to enable *in vitro* transcription of probe RNAs. The EMSA probe was radiolabeled by incorporation of [ $\alpha$ -<sup>32</sup>P]CTP during transcription. Purified recombinant His-YB-1 was incubated with the 3' SL probe at molar ratios of 0, 5, and 10 molecules/molecule of RNA probe. Protein-RNA complexes were then resolved on a non-denaturing polyacrylamide gel, and the mobility of the complexes was determined by visualization using a PhosphorImager. As demonstrated in Fig. 4C, YB-1 binds to the 3' SL probe, producing shifts that correspond to a single molecule of YB-1 per RNA molecule (*lane 2*, 5-fold molar excess, complex I) and a higher order protein-RNA complex (*lane 3*, 10-fold molar excess, complex II) in which multiple YB-1 molecules are presumed to be binding to RNA. From this we conclude that YB-1 binds to the DENV 3' SL and, in the presence of high amounts of YB-1, that higher order RNA-protein complexes are formed.

To determine the precise binding site of YB-1 within the 3' SL, enzymatic RNA footprinting reactions were performed. DENV2 3' SL RNA was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP, and purified probes were incubated with His-YB-1 at molar ratios of 0, 5, and 10 molecules/molecule of RNA. The protein-RNA complexes were then incubated with ribonuclease VI, which preferentially digests double-stranded RNA, and RNase T2, which digests single-stranded RNA with a preference for adenosine nucleotides (30). As shown in Fig. 4, D and E, YB-1 does not significantly protect nucleotides within the stem but does protect sequences within the first several nucleotides of the loop (Fig. 4B, *loop A*), including the first two nucleotides that comprise the pentanucleotide loop sequence. The apparent lack of protection by YB-1 of sequences within the stem may be due to an inability to bind DENV in this region, or alternatively, the stem sequences adjacent to the loop may "breathe," adopting single-stranded structures that cannot be cleaved by ribonuclease VI because of its preference for double-stranded RNA. In addition to marked protection of loop A, YB-1 also appeared to protect the side loop (Fig. 4B, *loop B*). Although we cannot specify if this binding is due to the interaction of a single YB-1 molecule with both loop sequences or the binding of several YB-1 molecules to a single RNA molecule, these results demonstrate that YB-1 binds specifically to functionally important regions within the DENV 3' SL that include sequences of the YB-1 consensus binding site.

**YB-1 Represses DENV Replication *in Vivo***—Given the association of YB-1 with the DENV 3'-UTR, we next investigated the function of YB-1 during DENV replication. YB-1 knock-out mice have been generated and shown previously to have an embryonic lethal phenotype after day 13.5 (20). Immortalized YB-1<sup>+/+</sup> and YB-1<sup>-/-</sup> MEF lines generated

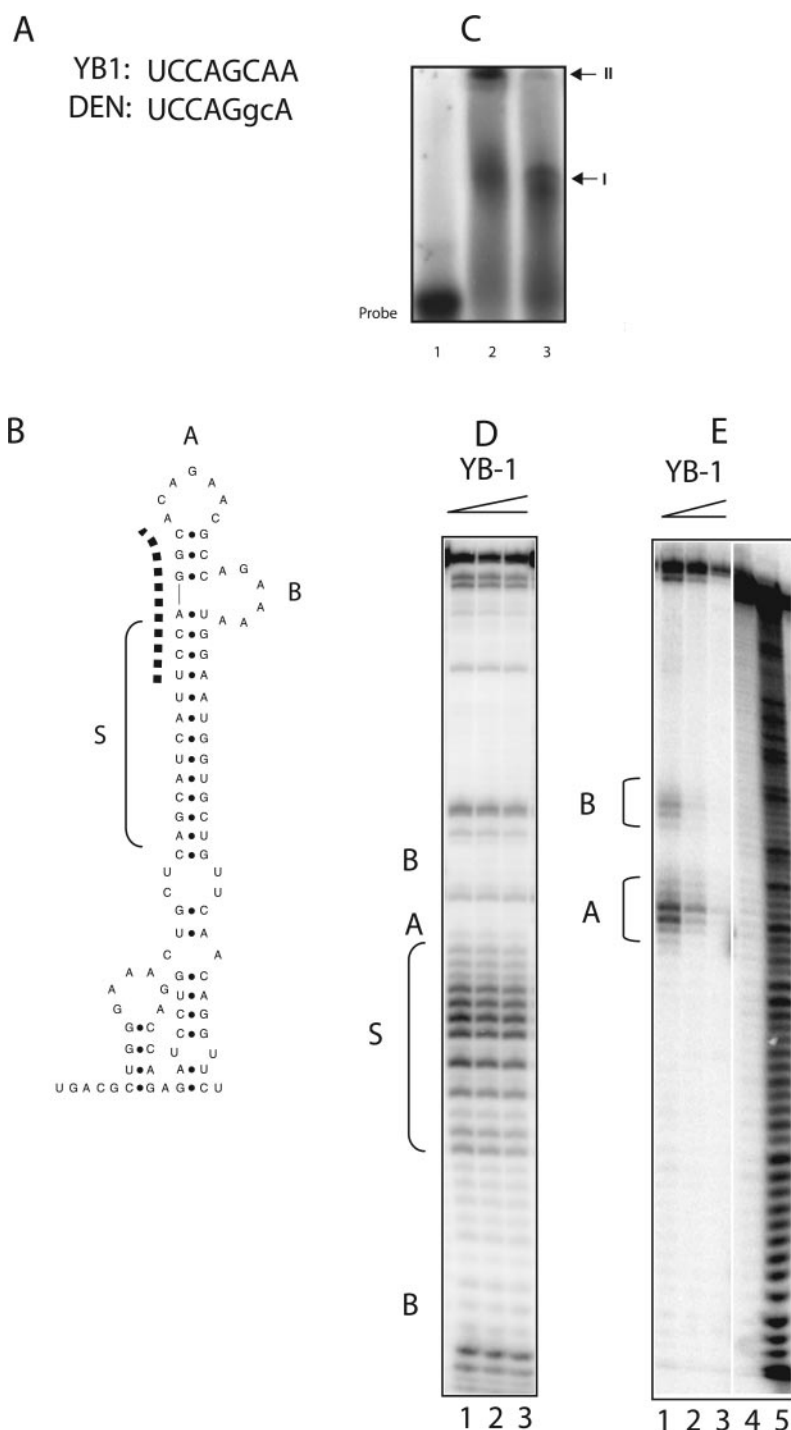
from day 13.5 embryos were infected with DENV2 strain 16681. At 24 and 48 h post-infection, cell supernatants were harvested, and viral production was quantified by plaque assay. As shown in Fig. 5A, YB-1 knock-out cells produce 10–20-fold more virus during the first round of viral replication (24 h post-infection) than do YB-1<sup>+/+</sup> MEFs when infected at an m.o.i. of 2. By 48 h post-infection, this difference is ~5-fold. When cells are infected at an m.o.i. of 5, we also observe higher viral replication in YB-1<sup>-/-</sup> cells compared with YB-1<sup>+/+</sup> cells (data not shown). Similar results were obtained from experiments conducted in additional congenic lines of YB-1<sup>+/+</sup> and YB-1<sup>-/-</sup> MEFs (Fig. 5B). These data demonstrate that host cell YB-1 mediates antiviral effects that lead to the repression of DENV replication.

**YB-1 Represses Expression of DENV Proteins in Infected MEFs**—To confirm the enhancement of viral replication in the absence of YB-1, we determined the extent of viral infection within the cell population. To achieve this, flow cytometric analysis was conducted to determine the percentage of YB-1<sup>+/+</sup> and YB-1<sup>-/-</sup> cells expressing DENV NS3 at various times after infection. Cells were infected with DENV2 at an m.o.i. of 2, and at 20, 24 and 50 h post-infection, the cells were harvested and processed for flow cytometry. Cells were labeled with a monoclonal antibody directed against DENV2 NS3 and subsequently with a fluorescently labeled secondary antibody and then analyzed to determine the percentage of infected cells. As shown in Fig. 5C, at 20 h post-infection, ~3 times more YB-1<sup>-/-</sup> MEFs than YB-1<sup>+/+</sup> MEFs were infected with DENV. At 24 and 50 h post-infection, this difference was ~2-fold. These data indicate that YB-1<sup>-/-</sup> MEFs are more efficiently infected with DENV than YB-1<sup>+/+</sup> MEFs.

**YB-1 Represses Translation of DENV**—YB-1 has been implicated in diverse cellular processes, including transcription, translation, interferon- $\gamma$  production, oncogenesis, and phosphatidylinositol 3-kinase signaling (19). As such, several mechanisms could potentially mediate the antiviral effect of YB-1 on DENV infection. To gain further insight into the mechanism by which YB-1 represses viral production, we conducted experiments to assess the impact of YB-1 on post-entry molecular events of translation and RNA synthesis of a DENV2 *Renilla* luciferase reporter replicon. DENV2 subgenomic replicon, (p)DRrep, derived from the DENV2 infectious clone pD2/IC, contains the DENV 5' and 3'-UTRs, a *Renilla* luciferase reporter gene, and DENV nonstructural genes but lacks the DENV structural genes.<sup>5</sup> YB-1<sup>-/-</sup> and YB-1<sup>+/+</sup> MEFs were transfected with RNA transcribed from (p)DRrep. Cells were harvested at indicated times post-infection and processed for quantitation of *Renilla* luciferase activity. Data were normalized for transfection efficiency by quantitation of input RNA at 2 h post-transfection by real time RT-PCR. As demonstrated by the representative data shown in Fig. 6A, we observed a single peak of luciferase activity between 2 and 18 h post-transfection, with the highest levels observed at 9–13 h post-transfection. As shown in Fig. 6A, translation of DENV2 subgenomic replicons was ~2-fold greater in YB-1<sup>-/-</sup> MEFs than in YB-1<sup>+/+</sup> MEFs. In different experiments ( $n = 4$ ), peak translation was 1.8- to 3.0-fold higher in YB-1<sup>-/-</sup> versus YB-1<sup>+/+</sup> MEFs ( $p$  value =

0.04, Wilcoxon signed rank test, two-sided). When YB-1<sup>-/-</sup> cells and YB-1<sup>+/+</sup> cells were transfected with a DENV2 *Renilla* replicon containing a mutation in the polymerase gene NS5 (GDD  $\rightarrow$  GVD), (p)DRrep-RdRpmut, that prevents replication of the replicon, luciferase activity was comparable with luciferase activity produced by (p)DRrep replicon in each of the MEFs. These data indicate that the observed luciferase production occurs during the first round of translation of the DENV replicons. In parallel experiments, (p)DRrep produced two distinct peaks of luciferase activity in BHK cells, at ~8 and 24 h post-transfection, respectively (data not shown), corresponding to the first (input) and second (post-replicative) round of translation. However, when (p)DRrep-RdRpmut was transfected into BHK cells, only a single peak was seen at ~8 h post-transfection (data not shown). The production of only a single peak of luciferase activity in MEFs transfected with the DENV replicons is likely due to the activation of interferon pathways in MEF cells (31, 32), which are absent in BHK cells. Nonetheless, these experiments enabled us to analyze the role of YB-1 during the presumptive primary round of DENV2 replicon translation. The 2-fold reduction in DENV translation observed here in the presence of wild type levels of YB-1 in YB-1<sup>+/+</sup> MEFs parallels the 2-fold reduction in viral protein synthesis observed in YB-1<sup>+/+</sup> MEFs in flow cytometric experiments and strongly suggests that significant repression of input virus translation can be mediated directly by YB-1.

**Dengue 5'- and 3'-UTR Sequences Are Not Sufficient for YB-1-mediated Translational Repression**—Given that YB-1 associates with regions of the 3'SL that have been shown to be involved in translational regulation of DENV, we next wished to determine whether binding of YB-1 to the 3'-UTR of the DENV genome is required for translational repression. Other investigators have demonstrated that auto-regulation of translation of YB-1 mRNA involves binding of YB-1 to sequences within the 3'-UTR and consequent displacement of poly(A)-binding protein (28), suggesting that an analogous mechanism may be involved in DENV translational repression. To determine whether binding of YB-1 to the 3'-UTR is sufficient for translational repression, experiments were performed to assess the impact of YB-1 on translation of the DENV reporter construct 5DLuc3D (Fig. 1A). 5DLuc3D reporter RNA was transcribed and transfected into YB-1<sup>-/-</sup> and YB-1<sup>+/+</sup> MEFs. Cells were harvested, and luciferase levels were quantitated by luminometry. Data were normalized for transfection efficiency. Contrary to results obtained with the DENV *Renilla* replicons, translation of the RNA reporter construct 5DLuc3D was not reduced by the presence of YB-1 (Fig. 6B, *0 hpi*). In data from four independent experiments, an average difference in luciferase production in YB-1<sup>-/-</sup> compared with YB-1<sup>+/+</sup> MEFs of 1.1-fold was observed. Statistical analysis indicated that the difference in each experiment was insignificant ( $p$  value  $>0.05$ ). Given that the reporter construct lacks genes encoding DENV nonstructural proteins, these data suggest that the repressive impact of YB-1 on translation of DENV2 is dependent upon the presence of certain elements of DENV



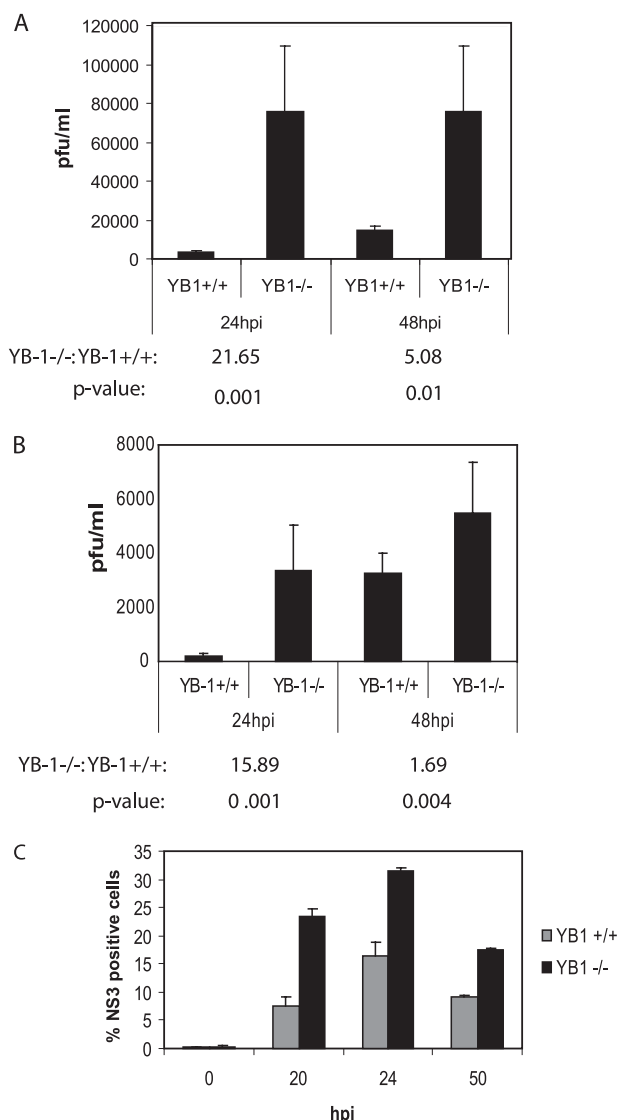
**FIGURE 4. Identification of YB-1-binding site within the DENV 3'SL.** *A*, alignment of YB-1 consensus binding site identified in the 3'-UTR of YB-1 mRNA with a potential binding region within the DENV 3'SL. *B*, schematic representation of the DENV 3'SL. *S*, stem; *A*, loop A; *B*, loop B. *Thick dotted line* indicates putative YB-1 consensus binding site. *C*, electrophoretic mobility shift assay. DENV 3'SL probes radiolabeled with [ $\alpha$ - $^{32}$ P]CTP were incubated with YB-1 at molar ratios of 0 (*lane 1*), 10 (*lane 2*), and 5 (*lane 3*) and resolved on a 5% nondenaturing polyacrylamide gel. Two complexes corresponding to a single molecule of YB-1 (*I*) and a higher order YB-1-RNA complex (*II*) were formed. *D*, footprint analysis of YB-1 binding to double-stranded regions of the DENV 3'SL. 3'SL probe end-labeled with [ $\gamma$ - $^{32}$ P]ATP was incubated with increasing amounts of YB-1. *Lane 1*, no YB-1; *lane 2*, 10 pmol of YB-1; *lane 3*, 100 pmol of YB-1. All samples were treated with RNase VI. *E*, footprint analysis of YB-1 binding to single-stranded loop regions of the 3'SL. 3'SL probe end-labeled with [ $\gamma$ - $^{32}$ P]ATP was incubated with increasing amounts of YB-1. *Lane 1*, no YB-1; *lane 2*, 10 pmol YB-1; *lane 3*, 100 pmol of YB-1; *lane 4*, probe alone; *lane 5*, RNA probe ladder generated by alkaline hydrolysis. *Lanes 1–3* were treated with RNase T2. Protection at loops *A* and *B* is indicated. Data are representative of three independent experiments.

genomic RNA and/or factors such as nonstructural proteins produced during infection.

To determine whether *trans*-acting DENV proteins could mediate this effect, experiments were conducted to assess translation of reporter constructs in cells infected with DENV2. YB-1<sup>+/+</sup> and YB-1<sup>-/-</sup> MEFs were infected with DENV2 strain 16681 at an m.o.i. of 2. At 12–14 h post-infection, when translation of NS3 is detectable by flow cytometric analysis (data not shown), cells were transfected with 5DLuc3D reporter RNA. Cells were harvested, and luciferase activity was assessed by luminometry. As indicated in the representative data shown in Fig. 6*B* (12 hpi), prior infection with DENV2 did not affect the translation of DENV reporter constructs. In separate independent experiments, the average ratio of luciferase production in YB-1<sup>-/-</sup> compared with YB-1<sup>+/+</sup> MEFs was 1.2 with *p* values >0.05, indicating no significant difference in translation. These results suggest that DENV viral proteins do not function in *trans* to mediate translational repression via YB-1; instead, DENV translational repression by YB-1 appears to require *cis*-acting genomic sequences and, potentially, *cis*-acting DENV nonstructural proteins.

## DISCUSSION

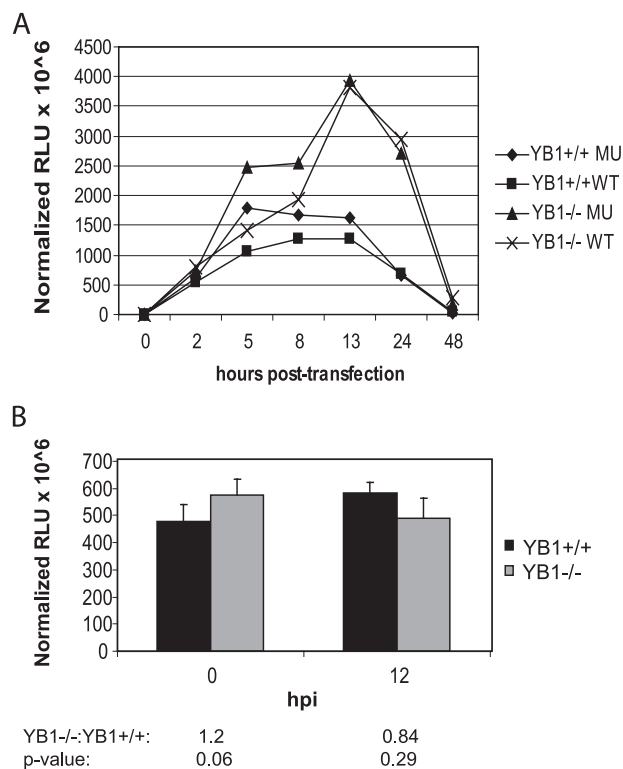
The identification of host cell factors that mediate DENV replication remains an important yet underexplored area that should enable not only greater insight into the molecular mechanisms of viral replication but also identification of potential therapeutic targets. To address this question, we employed a biochemical approach that takes advantage of a robust BHK extract that recapitulates DENV translational regulation observed *in vivo*. Using RNA affinity chromatography in conjunction with mass spectrometry, several host cell factors that associate with the DENV2 genome were identified. When translation reactions



**FIGURE 5. Higher levels of DENV replication in YB-1<sup>-/-</sup> versus YB-1<sup>+/+</sup> MEFs.** A and B, YB-1<sup>-/-</sup> and YB-1<sup>+/+</sup> MEFs were infected with DENV2 strain 16681 at an m.o.i. of 2 for 24 and 48 h. A and B represent data from two different congenic pairs of immortalized MEFs. Viral supernatants were harvested and assayed by plaque assay. Data shown are representative of five independent experiments. Fold differences in titers between YB1<sup>-/-</sup> versus YB-1<sup>+/+</sup> cells are indicated along with the *p* value as determined by Student's *t* test. Error bars indicate S.D. (*n* = 3). C, flow cytometric analysis of YB-1<sup>-/-</sup> and YB-1<sup>+/+</sup> MEFs. YB-1<sup>-/-</sup> and YB-1<sup>+/+</sup> MEFs were infected with DENV2, 16681 at an m.o.i. of 2, harvested at indicated times post infection (0, 20, 24, and 50 hpi), and processed for flow cytometric analysis. Data are representative of four independent experiments. *hpi*, hours post-infection. Error bars indicate S.D. (*n* = 3).

were conducted in the presence of the nonhydrolyzable GTP analogue GMP-PNP, eIF1A, eIF1 $\gamma$ , and hnRNP U associated with DENV reporter constructs. It is likely that these factors are poised on DENV RNA prior to translation elongation and may be important for configuration of a translationally active DENV ribonucleoprotein complex. Significantly, eIF1A has been shown previously to bind to the 3'SL of the 3'-UTR of West Nile virus, DENV, and other flaviviruses (18).

Several RNPs, hnRNP Q, hnRNP A1, hnRNP A2/B-, and hnRNP H, and YB-1 were found to bind specifically and with



**FIGURE 6. Cis-acting DENV genomic sequences required for increase in DENV translation in YB-1<sup>-/-</sup> cells.** A, translation of DENV *Renilla* luciferase replicon is increased in YB-1<sup>-/-</sup> versus YB-1<sup>+/+</sup> MEFs. Wild type (WT) (p)DRrep replicons and replication incompetent (p)DRrep-RdRpmut (MU) *Renilla* luciferase replicons were transfected into YB-1<sup>-/-</sup> and YB-1<sup>+/+</sup> MEFs. Cells were harvested at the indicated times post-transfection, and luciferase activity was monitored by luminometry. Luciferase activity was normalized for relative transfection efficiency of YB1<sup>+/+</sup> versus YB1<sup>-/-</sup> MEFs, using real time RT-PCR values for RNA amount at 2 h post-transfection. Data are representative of four independent experiments, each performed in duplicate. B, DENV genomic sequences are needed for YB-1-mediated translational inhibition. Firefly luciferase reporter RNA harboring DENV2 5'- and 3'-UTRs flanking the firefly luciferase gene (*5DLuc3D*) was transfected into YB-1<sup>-/-</sup> and YB-1<sup>+/+</sup> MEFs that had been infected with DENV2 strain 16681 or mock-infected for 12 h. Cells were harvested 7 h post-transfection, and luciferase activity was monitored by luminometry. As above, luciferase activity was normalized for relative transfection efficiency of YB1<sup>+/+</sup> versus YB1<sup>-/-</sup> MEFs, using real time RT-PCR values for RNA amount at 2 h post-transfection. *hpi*, hours post-infection. Data are representative of three independent experiments each performed in triplicate. *p* values were determined by Student's *t* test. Error bars indicate S.D. (*n* = 3).

high affinity to the DENV 3'-UTR, suggesting that these molecules may play a biologically significant role in the DENV life cycle. hnRNP A1 has been demonstrated to mediate molecular processes of numerous viruses, including splicing of HIV-1 (33) and human T-cell lymphotropic virus, type I (34), and RNA synthesis of coronaviruses (35) and hepatitis C (36). Several investigators have demonstrated that hnRNP A2/B1 and hnRNP U play an important role in the life cycle of HIV-1 by regulating RNA trafficking (37, 38) and transcription (39), respectively. Similarly, YB-1 has been demonstrated to regulate transcription of HIV-1 (40), JC virus (41), and adenovirus (42). RNA-binding proteins clearly play important roles in the regulation of many viruses; defining the roles of specific ribonucleoprotein complexes is likely to provide insight into the molecular dynamics of host-virus interactions.

## YB-1 Binds Dengue Virus 3'-UTR and Has Antiviral Effects

Of the RNA-binding proteins isolated, YB-1 displayed the most stringent association with the DENV 3'-UTR, binding only in the absence of 3'-UTR sense competitor and eluting from the affinity resin only when the KCl concentration was increased to 1.0 M. EMSA and footprinting experiments enabled us to define a specific YB-1-binding site within the 3'SL of the DENV 3'-UTR. This YB-1-binding site includes a portion of a consensus sequence with high homology to the YB-1-binding site within the 3'-UTR of the YB-1 mRNA. The area of protection by YB-1 in the DENV 3'SL includes several nucleotides within the pentanucleotide loop sequence, which have been demonstrated previously to play an important role in DENV translation and replication (7, 12). Of note, eEF1A has been shown to bind to the DENV 3'SL (18); it will be interesting to determine whether YB-1 and eEF1A bind cooperatively and function synergistically.

YB-1 is a cold-shock domain protein that is pivotally involved in diverse cellular functions, including the regulation of transcription, translation, and mRNA stability (19). Functional analysis of YB-1 in DENV replication indicated that YB-1 plays an antiviral role during infection in that YB-1<sup>-/-</sup> MEF cells produce 10–20-fold more infectious viral particles than YB-1<sup>+/+</sup> cells. YB-1 has been demonstrated previously to inhibit translation via an interaction with the 5' cap (59) as well as through association with the 3'-UTR of cellular messages (28). Examination of the effect of YB-1 on DENV translation was achieved by using DENV reporter replicons. Transfection of these replicons enabled circumvention of viral entry and direct examination of the impact of YB-1 levels on DENV translation. In the absence of YB-1, we determined that a 2-fold increase in DENV translation was reproducibly observed in infected cells as well as cells transfected with DENV *Renilla* luciferase replicons. This translational enhancement was not observed with DENV reporter constructs harboring the DENV 5'- and 3'-UTRs but lacking other DENV genomic sequences. Moreover, introduction of DENV viral proteins by prior infection did not restore a difference in translational capacity in YB1<sup>+/+</sup> versus YB1<sup>-/-</sup> MEFs. We therefore conclude that YB-1 mediates antiviral effects upon DENV and that this antiviral effect appears to require *cis*-acting DENV genomic sequences and, possibly, *cis*-acting DENV nonstructural proteins.

Translational repression via *cis*-elements in the DENV genome could be mediated by binding of YB-1 to consensus elements throughout the viral genome. Analysis of the DENV2 genome revealed the presence of several partial YB-1-binding sites within the 5'-UTR, NS2A, NS4A, and NS5 genes as well as a near-perfect YB-1 consensus within the NS5 coding region. One possibility is that translational down-regulation mediated by YB-1 requires cooperative binding of YB-1 along the viral RNA. In support of this, investigators have demonstrated that YB-1 forms higher order, chromatin-like structures on RNA molecules (43). These repressive complexes may become activated by competition of poly(A)-binding protein for YB-1-binding sites within the 3'-UTR, as is observed in regulation of YB-1

translation (28). Thus, it is possible that the natural role of YB-1 in DENV translation might be in enabling a switch between translation and replication of the viral genome.

The involvement of YB-1 in translational repression of DENV also suggests that different intracellular levels of YB-1 may play a role in determining the tropism of DENV during an infection. YB-1 levels in cells are very closely regulated by a negative feedback mechanism wherein YB-1 associates with the 3'-UTR of its cognate mRNA to repress translation (28). This regulation is essential for coordination of developmental and cell cycle regulation in mice (20, 44–46). Importantly, dysregulation of YB-1 expression has been correlated with oncogenesis and multidrug resistance (47–58). Given the tight regulation of YB-1 within different cell types and the antiviral role of YB-1 in DENV infection, it is possible that YB-1 levels may influence DENV cellular tropism.

Our results indicate that the antiviral impact of YB-1 on DENV replication is mediated, at least in part, by translational repression. It is of note, however, that the 2-fold impact on translation is significantly less than the 10–20-fold impact on virus production. It is plausible that subsequent rounds of viral RNA synthesis and translation lead to amplified viral production such that a 2-fold difference in first-round translation results in a 10–20-fold difference in viral output. However, it is also possible that YB-1 is involved in other antiviral mechanisms in infected cells. YB-1 may, for example, play a role in the mediation of early innate immune responses. In support of this hypothesis, recent reports have indicated that YB-1 is phosphorylated by activated AKT (59, 60), resulting in increased nuclear localization of YB-1 (59, 60). AKT has recently been shown to mediate early innate immune responses (61, 62) and, interestingly, has been shown to be activated early in DENV infection (63). It is possible that DENV infection may elicit a response similar to that observed during adenovirus infection, which has been shown to induce increased nuclear localization of YB-1. As a transcription factor, YB-1 could promote activation of early innate immune response genes such as *ISG54* and *ISG56*, which would, in turn, further down-regulate translation and replication of DENV. We postulate that DENV infection induces YB-1 activation and a subsequent antiviral cascade that could occur in a manner similar to the role of YB-1 in mediating interferon- $\gamma$  signaling via Jak1 (24).

The identification of YB-1 as a UTR-associating host cell factor with antiviral properties has important implications for our understanding of DENV infection as well as antiviral mechanisms in cells. Although our results have demonstrated that YB-1 is directly involved in repression of DENV translation, it remains likely that YB-1 is also involved in other antiviral activities in cells. We are currently pursuing experiments to determine whether YB-1 is directly involved in early innate immune responses during DENV infection. Furthermore, analysis of the role of other RNP molecules in the DENV life cycle will advance our understanding of the role of these important RNA regulatory molecules in the regulation of infection by RNA viruses and should facilitate identification of potential antiviral targets.

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## YB-1 Binds Dengue Virus 3'-UTR and Has Antiviral Effects

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