

Evidence That Hepatitis C Virus Resistance to Interferon Is Mediated through Repression of the PKR Protein Kinase by the Nonstructural 5A Protein

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Hepatitis C virus (HCV) is the major cause of non-A non-B hepatitis and a leading cause of liver dysfunction worldwide. While the current therapy for chronic HCV infection is parenteral administration of type 1 interferon (IFN), only a fraction of HCV-infected individuals completely respond to treatment. Previous studies have correlated the IFN sensitivity of strain HCV-1b with mutations within a discrete region of the viral nonstructural 5A protein (NS5A), termed the interferon sensitivity determining region (ISDR), suggesting that NS5A may contribute to the IFN-resistant phenotype of HCV. To determine the importance of HCV NS5A and the NS5A ISDR in mediating HCV IFN resistance, we tested whether the NS5A protein could regulate the IFN-induced protein kinase, PKR, a mediator of IFN-induced antiviral resistance and a target of viral and cellular inhibitors. Using multiple approaches, including biochemical, transfection, and yeast genetics analyses, we can now report that NS5A represses PKR through a direct interaction with the protein kinase catalytic domain and that both PKR repression and interaction requires the ISDR. Thus, inactivation of PKR may be one mechanism by which HCV avoids the antiviral effects of IFN. Finally, the inhibition of the PKR protein kinase by NS5A is the first described function for this HCV protein.

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INTRODUCTION

HCV, a member of the *Flaviviridae* (Kato *et al.*, 1990), is the major cause of non-A non-B hepatitis, increasingly becoming a source of morbidity worldwide (Cuthbert, 1994; Mansell and Locarnini, 1995). The HCV virion contains a 9.4-kilobase single-stranded positive-sense RNA genome which encodes a single polyprotein of approximately 3010 amino acids (aa) that is posttranslationally cleaved into individual structural and nonstructural proteins within the infected host cell (Selby *et al.*, 1993; Shukla *et al.*, 1995; Grakoui *et al.*, 1993). Chronic HCV infection leads to liver cirrhosis in a high frequency of cases and long-term virus persistence is epidemiologically associated with the development of hepatocellular carcinoma (Bisceglie, 1995). The public health impact of chronic HCV infection is currently being realized. In the United States alone approximately 3.5 million individuals are believed to be infected with HCV, reflected in the high frequency of adult liver transplants which are indicated due to complications from HCV infection (Alter, 1995). This is underscored by the high proportion of HCV-infected individuals (60–80%) who fail to respond to IFN therapy or who relapse after therapy cessation (Hoof-

nagle, 1994; Iino *et al.*, 1994). Importantly, type I IFN is currently the only approved therapy for chronic HCV infection (Hoofnagle, 1994; Iino *et al.*, 1994).

Response to IFN therapy differs among the six HCV genotypes, but is observed, at some level, in all HCV genotypes worldwide. Of the two predominant HCV genotypes, HCV-1a and HCV-1b, the former predominates in the Americas and Europe while the latter predominates throughout most Asian countries; both exhibit a high level of resistance to IFN therapy (Fried and Hoofnagle, 1995). Recently, others have sequenced clinical isolates of the HCV genome and correlated mutations within a discrete region of the nonstructural 5A protein (NS5A), termed the IFN sensitivity determining region (ISDR), of HCV-1b with the IFN-sensitive phenotype (shown in Fig. 1A). These studies demonstrated that strains closely matching the prototype HCV-1b NS5A sequence correlated with complete IFN resistance (Enomoto *et al.*, 1995, 1996).

Prior to the current report, possible mechanisms underlying this HCV resistance to IFN therapy were unknown. The IFN-induced cellular antiviral response is mediated in part by the actions of the Mx proteins, the 2'-5' oligoadenylate synthetase, RNase L, and PKR (review by Sen and Lengyel, 1992; Sen and Ransohoff, 1993). Induced by IFN, these antiviral effector proteins block viral gene expression at multiple levels. Perhaps

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the most widely studied of all the IFN-induced gene products is the PKR protein kinase (Meurs *et al.*, 1990). Activated by binding to double-stranded RNA (dsRNA), including viral RNAs and regions of secondary RNA structure, PKR phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF-2 α) resulting in a global cessation of protein synthesis and a concomitant block in viral replication within the infected cell (Hershey, 1991; Clemens, 1996; Merrick and Hershey, 1996). To counteract the deleterious effects of IFN induction and PKR activation, many eukaryotic viruses have evolved mechanisms to block the activity of PKR, including directing the physical interaction of PKR with specific kinase inhibitory molecules (summarized in Fig. 1B) (Katze, 1993, 1995). Examples include influenza virus which functionally recruits the cellular PKR inhibitor, P58^{IPK}, that binds to PKR to block kinase activity (Gale *et al.*, 1996; Lee *et al.*, 1990, 1994; Polyak *et al.*, 1996). The virally encoded vaccinia virus K3L protein, human immunodeficiency virus (HIV), Tat protein and adenovirus VAI RNA similarly bind to PKR to block kinase activity (Gale *et al.*, 1996; Katze *et al.*, 1987; McMillan *et al.*, 1995; Carroll *et al.*, 1993; Beattie *et al.*, 1991, 1995).

The high frequency of IFN-resistant HCV in the human population suggests that HCV, like these many other animal viruses, has evolved a mechanism(s) to block the IFN-mediated response to viral infection. Although the function of NS5A and its role in viral replication had been unknown, it has been suggested that NS5A, by an ISDR-directed mechanism, may mediate IFN resistance by interacting with and repressing one or more IFN-induced antiviral gene products. In the current report, we now provide direct proof that NS5A interacts with and inhibits the IFN induced protein kinase, PKR. Importantly, we found that the ISDR was required for NS5A interaction with PKR and repression of PKR activity. These data thus provide the first evidence for the molecular mechanisms underlying HCV resistance to IFN therapy.

MATERIALS AND METHODS

Plasmid construction

To generate wild-type HCV 1a NS5A constructs, the complete NS5A coding region from pSPns5 (HCV-1a; a generous gift from Dr. C. Seeger) was amplified by PCR using the oligonucleotide primers A, 5'-GGAATTCGAGCTCGCCCG and B, 5'-GCTCTAGAAGCACACGACATCTTC (*EcoRI* and *XbaI* sites underlined). The resulting product was directly cloned into pCR2.1 (Invitrogen) to yield plasmid pNS5A/CR2.1. The NS5A coding region was removed from pNS5A/CR2.1 as an *EcoRI* fragment for insertion into pBD (Stratagene) to yield pBD-NS5A, or as an *EcoRI*-*XbaI* fragment for insertion into pcDNA3.1/His and pYES2 (Invitrogen), to give pcDNA3.1/His-NS5A and pYES2-NS5A, respectively. To generate the ISDR deletion mutant of NS5A, we first generated individual N-terminal

and C-terminal coding fragments, each of which lacked the ISDR. The N-terminal region, encoding amino acids 1–236, was amplified by PCR using primer A and primer C, 5'-CCACTCGAGCGGACAGTTGGCTGG (*XhoI* site underlined). The C-terminal region, encoding amino acids 278–447, was amplified using primer B and primer D, 5'-CCGCTCGAGTGGTGATTCTGGTCTC (*XhoI* site underlined). The resulting PCR products were cloned directly into pCR2.1 to yield pN/CR2.1 and pC/CR2.1, respectively. The N-terminal coding region of NS5A was then removed from pN/CR2.1 for insertion into pcDNA3.1/His to generate pcDNA3.1/His-NS5A 1-236. An ISDR deletion construct was prepared by insertion of the C-terminal coding fragment into the *XhoI* and *XbaI* sites of pcDNA3.1/His-NS5A 1–236 to generate pcDNA3.1/His- Δ ISDR. This construct encodes NS5A amino acids 1–236 fused in-frame with amino acids 278–447, deleting the entire ISDR. The insert from pcDNA3.1/His- Δ ISDR was then subcloned into the 2 μ yeast expression vectors pBD and pYES2 to give pBD- Δ ISDR and pYES2- Δ ISDR, respectively. To obtain the NS5A coding region from HCV-1b, viral RNA was extracted from 100 μ l of serum (Chomczynski and Sacchi, 1987) obtained under informed consent from a genotype 1b patient who failed to respond to IFN therapy. Response to IFN was determined by RT-PCR and bDNA assay, as previously described (Gretch *et al.*, 1993). Verification of HCV genotype was determined by a combination of RFLP and genotype-specific PCR analyses of the viral 5' untranslated region and sequences encoding the core protein, respectively (Davidson *et al.*, 1995; Okamoto *et al.*, 1992). Viral cDNA was synthesized by reverse transcription using the priming oligonucleotide 5' GTGGTGACGCAGCAGAGAGT (corresponding to nt 7681–7700 of HCV-J (Bukh *et al.*, 1995)), followed with first-round PCR by the addition of the upstream primer 5' CAGCCTCACCATCACTCAGC (corresponding to HCV-J nt 6256–6275). For directional cloning of NS5A, first-round PCR products were further amplified using the NS5A-specific nested-set oligonucleotide primer pair 5' CCTTCCATGGGCTCCGGCTCGTGGCTAAAG and 5' ATCGGATCCTTAGGACATTGAGCAGCAGACGA (*NcoI* and *BamHI* sites underlined, respectively). After restriction enzyme digestion, the purified PCR products were cloned into the corresponding sites of pACT2 (Clontech) to give pAD-NS5A, which encodes an AD-NS5A fusion protein corresponding to HCV-1b. While the relationship between the ISDR aa sequence of HCV-1a and IFN sensitivity has not been precisely determined, this region of NS5A (aa 237–276) possessed significant aa identity to the prototypic IFN-resistant ISDR sequence defined previously (Enomoto *et al.*, 1995, 1996) and present in our HCV-1b NS5A clone (Fig. 1A). Construction of PKR plasmids pBD-PKR K296R, pAD-PKR aa constructs K296R, 1-242, 244-551, 244-366, 367-551, and pAD-P58^{IPK}wt were described previously (Gale *et al.*, 1996). pBD-PKR 98-551 was constructed by recovering

the 1.6-kb *NdeI/BamHI* fragment from pET11a-PKR M7 (Barber *et al.*, 1995) and cloning it into the corresponding sites of pGBT10 (Gale *et al.*, 1996). pEMBLyex4-K3L contains the entire vaccinia virus K3L gene inserted into pEMBLyex4 and is described in a separate paper (M. Kobayashi, E. Locke, J. Silverman, T. Ung, and T. Dever, manuscript submitted for publication). GST-NS5A was produced by introducing the *BamHI* fragment from the HCV-1a NS5 clone pSPns5 into the plasmid pGEX2T (Smith and Johnson, 1988) to give pGST-NS5A. This construct encodes NS5A aa 1–427 fused in frame with the glutathione *S*-transferase protein. The nt sequences of all constructs used in this study were confirmed by double-stranded DNA sequence analysis using an Applied Biosystems automated sequencer.

Analysis of protein interaction *in vitro*

Escherichia coli harboring pGEX2T or pGST-NS5A were grown in liquid culture and GST or GST-NS5A expression was induced by the addition of isopropyl thiogalactopyranoside into the culture medium. Bacteria were harvested and extracts prepared for binding analyses as described previously (Gale *et al.*, 1996). For binding analyses, we first confirmed expression of GST or GST-NS5A in our recombinant extracts by immunoblot analysis using either a GST-specific antiserum or an antiserum specific to a superoxidodismutase-NS5 fusion protein (anti-NS5; Chiron Corp.). Positive recombinant extracts were used for *in vitro* binding analysis. Wild-type human PKR and PKR deletion mutants were *in vitro* transcribed from the T7 promoter of pcDNA1neo and translated in the presence of [³⁵S]methionine as described previously (Katze *et al.*, 1991). For *in vitro* binding analyses, approximately 1×10^5 counts of trichloroacetic acid-precipitable material from each translation reaction were added to a crude *E. coli* extract containing either GST or GST-NS5A and processed as described (Gale *et al.*, 1996). Labeled proteins which bound to GST or GST-NS5A were separated by SDS-PAGE and visualized by autoradiography of the dried gel.

In vitro assay of PKR function

For PKR *in vitro* kinase assays GST and GST-NS5A were purified from *E. coli* extracts using glutathione-agarose affinity chromatography. Native PKR was affinity purified from IFN-treated human 293 cells (Galabru and Hovanessian, 1987). The concentration of all purified proteins was assessed by quantitative SDS-PAGE using BSA as a standard. *In vitro* kinase assays were carried out essentially as described (Tang *et al.*, 1996), except that PKR was preincubated with increasing concentrations of GST or GST-NS5A prior to the addition of histone substrates and [γ -³²P]ATP. Kinase reaction products were separated by SDS-PAGE and the dried gel was subjected to autoradiography for visualization of phos-

phorylated substrates. We have shown previously that there is a perfect correlation between phosphorylation of histones and purified eIF-2 α by PKR *in vitro* (Katze *et al.*, 1991).

Analysis of protein interaction *in vivo*

For analysis of protein interactions *in vivo* we employed the *Saccharomyces cerevisiae* strain Hf7c (*MATa ura3-52 his3-200 lys2-801 ade2-101 trp1-901 lea2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17-mers)₃-CYC1-lacZ*) in a 2-hybrid assay (Fields and Song, 1989). For the 2-hybrid assay and the yeast assays described below, cells were transfected by the lithium acetate method with the indicated expression constructs. For 2-hybrid analysis, we first verified expression of all AD- and BD-fusion proteins in the transfected yeast clones by immunoblot analysis, using anti-AD or anti-BD monoclonal antibodies (Clontech) as described by the manufacturer. In yeast strain Hf7c, synthesis of histidine is dependent upon interaction of the 2-hybrid proteins. Thus in the presence of a 2-hybrid protein interaction strains will exhibit growth on histidine-deficient medium. We thereby used the synthesis of the HIS reporter protein to assay for 2-hybrid protein interaction, as recently described (Gale *et al.*, 1996). Initial transfectants were selected on SD medium lacking the aa Trp and Leu (+His medium). Transfectants were subsequently streaked onto SD medium lacking the aa His, Trp, and Leu (–His medium) and were grown for 3–6 days to allow depletion of residual histidine stores. Colonies were restreaked onto –His medium and scored for growth after 3–7 days. We confirmed induction of reporter gene expression in our 2-hybrid yeast clones (data not shown) by measuring *LacZ* expression using a liquid assay and the fluorogenic substrate 4-methylumbelliferyl- β -D-galactoside (MUG) as described (Cao and Geballe, 1994).

Yeast growth repression assay for determination of NS5A and PKR function *in vivo*

For determination of NS5A and PKR function *in vivo* we used *S. cerevisiae* strain RY1-1 (*MATa ura3-52 leu2-3 leu2-112 gcn2 Δ trp1- Δ 63 LEU2::(GAL-CYC1-PKR)₂*) which carries two copies of human PKR integrated into the *LEU2* locus, under control of the galactose inducible *GAL1-CYC1* promoter (Romano *et al.*, 1995). When grown on inducing medium, this strain exhibits a slow growth phenotype due to the PKR-mediated phosphorylation of yeast eIF-2 α . Protein expression was verified by immunoblot analysis using either an anti-PKR mAb (Laurent *et al.*, 1985) or antisera generated against a recombinant NS5 protein (α -NS5) (a gift from Chiron). Both NS5A and Δ ISDR constructs were efficiently expressed from the pYes plasmid (data not shown). In addition, we found that coexpression of NS5A or Δ ISDR had no effect on PKR levels which were expressed efficiently to equal

levels in both transfected strains (data not shown). To assess growth of RY1-1 transfectants, yeast colonies were streaked onto uracil-deficient SD medium and SGAL medium as described by Romano *et al.* (1995). Growth was assessed at 3–7 days poststreaking of colonies.

Analysis of NS5A function in mammalian cells

To measure the effect of NS5A upon PKR function in mammalian cells, we used a secreted embryonic alkaline phosphatase (SEAP) reporter assay (Berger *et al.*, 1988). pcDNA1neo-SEAP and pcDNA3.1/His-NS5A were co-transfected into COS1 cells using the DEAE-dextran/chloroquin method (Cullen, 1987). Details of the SEAP assay (Berger *et al.*, 1988) have been described previously by our laboratory (Garfinkel *et al.*, 1993). Transfected cells were maintained in G418-selective medium and culture supernatants assayed at 24 hr posttransfection for SEAP activity. Cells were subsequently harvested and extracts prepared for SDS-PAGE and immunoblot analysis. For immunoblot detection of NS5A expressed from pcDNA3.1/His, we used α -NS5 sera.

eIF-2 α phosphorylation analysis

To determine the *in vivo* phosphorylation state of eIF-2 α within RY1-1 yeast harboring various expression plasmids, extracts were prepared from the respective strains and subjected to isoelectric focusing and immunoblot analyses as described (Dever *et al.*, 1993). Proteins (20 μ g) were first separated by isoelectric focusing and then transferred to a nitrocellulose membrane which was subsequently probed with a rabbit polyclonal antiserum specific to yeast eIF-2 α . The relative levels of basal- and PKR-phosphorylated eIF-2 α from each extract were quantitated by scanning laser densitometry and are presented as a percentage of the total eIF-2 α in a given sample.

RESULTS

NS5A and PKR interact *in vitro*

The mechanism(s) of HCV-mediated IFN resistance remain unknown, due in part to the inability to propagate the virus in cultured cells. However, molecular epidemiologic evidence suggests that NS5A may play a role in repressing the antiviral effects of IFN during HCV infection (Enomoto *et al.*, 1995, 1996). This led us to investigate the mechanism of HCV-mediated IFN resistance and the contribution of NS5A toward establishing the IFN-resistant phenotype of HCV. We tested the hypothesis that HCV NS5A may possess functions similar to those of other viral inhibitors of the IFN response (Katze, 1993, 1995), directly repressing the activity of PKR through a physical interaction with the kinase. We began

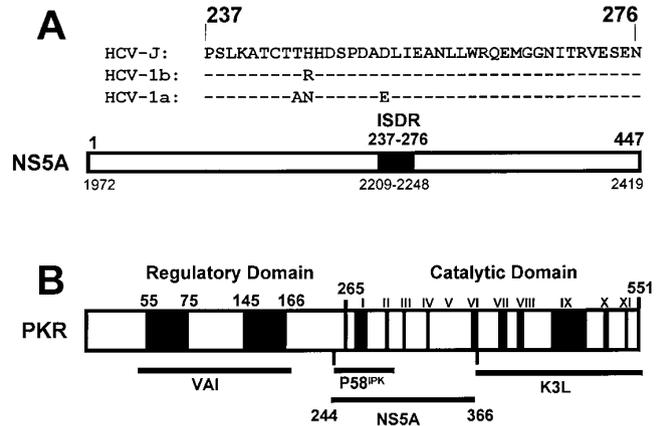


FIG. 1. (A) Top: Amino acid sequence comparison of the prototypic ISDR of NS5A from IFN-resistant HCV strain J and the corresponding region of NS5A from HCV strains 1a and 1b which were used in this study. Bottom: Structural representation of HCV NS5A representing the 447-aa protein from HCV-1a and -1b strains contained in our NS5A constructs. The ISDR is indicated in black and is deleted from the Δ ISDR construct derived from HCV-1a. Amino acid positions within NS5A are shown above (boldface type), and those corresponding to positions within the HCV polyprotein are shown below (normal type). (B) Structural representation of PKR. The positions of the two dsRNA binding motifs within the regulatory domain are shown in black. The catalytic domain spans aa 265–551 and includes the 11 protein kinase catalytic domain conservation regions (Hanks *et al.*, 1988) indicated by roman numerals. The regions of interaction with the PKR-inhibitory molecules adenovirus VAI RNA, vaccinia virus K3L and P58^{PK} are indicated (Katze *et al.*, 1987; Gale *et al.*, 1996). Also indicated is the NS5A-binding region (this study).

by employing NS5A from HCV 1-a (Fig. 1A) to test the ability of NS5A to directly interact with PKR. NS5A, expressed as a glutathione S-transferase (GST) fusion protein in *E. coli* (GST-NS5A), specifically interacted with *in vitro* translated full-length PKR using a GST pull-down assay. As seen in Fig. 2A, PKR aa 244–551 were both necessary and sufficient for complex formation with GST-NS5A. Thus, recombinant NS5A can specifically form a complex with PKR *in vitro* which maps to within the protein kinase catalytic domain-inclusive PKR C-terminus.

NS5A interacts with the PKR protein kinase catalytic domain *in vivo*

To confirm and extend these results *in vivo*, we carried out NS5A–PKR interaction studies using the yeast 2-hybrid system, scoring for growth on histidine-deficient medium as a positive 2-hybrid protein interaction. For comparison we included an assessment of the P58^{PK}–PKR interaction described previously (Gale *et al.*, 1996). We found that full-length NS5A from an IFN-resistant isolate of strain HCV-1b fused to the GAL4 activation domain (AD-NS5A) specifically interacted with the inactive full-length PKR mutant, PKR K296R, and the truncated mutant PKR 98-551, each of which were fused to the GAL4 DNA binding domain (BD) (Fig. 2B). BD-PKR 98-551 lacks the first dsRNA binding domain (Fig. 1B) and is deficient in

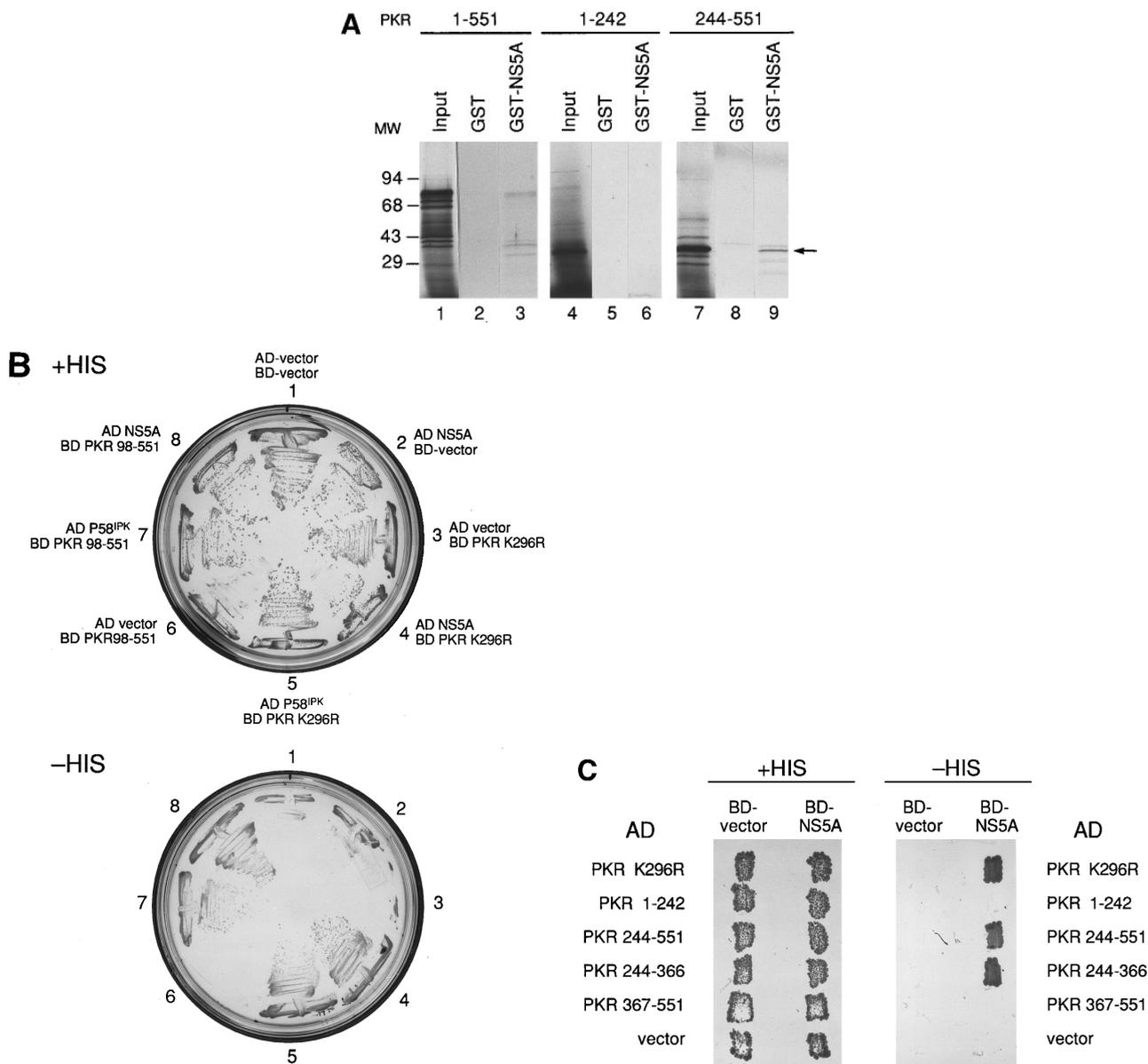


FIG. 2. NS5A interacts with PKR. (A) *In vitro* binding analysis. An autoradiogram of [³⁵S]methionine-labeled PKR *in vitro* translation products alone (lanes 1, 4, and 7) or selected by binding to GST or GST-NS5A within crude *E. coli* extracts, as indicated above each lane. Bound complexes containing PKR aa 1–551 (lanes 2 and 3), 1–242 (lanes 5 and 6), or 244–551 (lanes 8 and 9) were recovered by selection on glutathione–agarose. Bound translation products were eluted in SDS sample buffer and separated by 12.5% acrylamide SDS–PAGE. Arrow at right shows the position of PKR aa 244–551, which migrates slightly faster than the background band visible in lanes 8 and 9. Positions of molecular mass standards are shown in kDa (left). (B and C) Yeast 2-hybrid analysis. (B) NS5A from HCV-1b interacts with PKR *in vivo*. Full-length wt NS5A from HCV-1b was fused to the GAL4 transcription activation domain (AD) and coexpressed in yeast with the GAL4 DNA binding domain (BD) (position 2) or the BD-PKR fusion constructs BD-PKR K296R and BD-PKR 98-551 (positions 4 and 8, respectively). Coexpression of control GAL4 constructs for the 2-hybrid assay included AD-vector/BD-vector (position 1), AD-vector/BD-PKR K296R (position 3), AD-P58^{IPK}/BD-PKR K296R (position 5), AD-vector/BD PKR 98-551 (position 6), and ADP58^{IPK}/BD-PKR 98-551 (position 7). Transfected yeast were streaked onto +His (top) and –His medium (bottom). Growth on –His is indicative of 2-hybrid protein interaction. (C) NS5A from HCV-1a interacts with the PKR protein kinase catalytic domain. Yeasts expressing either the BD alone (BD-vector) or a BD-NS5A fusion protein from HCV-1a (BD-NS5A) were cotransfected with plasmids expressing the indicated AD-PKR fusion constructs or the control AD-vector (bottom row), replica plated onto +His (left) and –His medium (right) and assayed for growth.

binding dsRNA (Barber *et al.*, 1995). Thus, similar to the cellular PKR inhibitor, P58^{IPK}, HCV-1b NS5A can physically interact with PKR *in vivo*. Furthermore, these results indicate that the NS5A-PKR interaction is not a dsRNA-mediated event.

We next tested the ability of BD-NS5A, from an HCV-1a isolate, to interact with AD-PKR *in vivo* using the 2-hybrid assay. As seen in Fig. 2C, BD-NS5A from HCV 1a specifically interacted with AD-PKR K296R in yeast cotransfectants, as determined by growth in –His me-

dium. Thus, NS5A from the independent strains HCV-1a and HCV-1b can physically interact with PKR *in vivo*. To map the NS5A-interactive region of PKR, we employed a series of AD-PKR mutants to transfect yeast harboring the HCV 1a BD-NS5A construct. BD-NS5A specifically interacted with the PKR catalytic domain, mapping to within PKR aa 244–366, as AD- constructs PKR 244–551 and PKR 244–366 both mediated growth on –His medium (Fig. 2C). The NS5A-interactive region of PKR was exclusive of both the C-terminal 184 aa and the N-terminal 242 aa, as yeast cotransfectants harboring AD-PKR 367–551 and AD-PKR 1–242 failed to grow on –His medium. These results indicate that HCV NS5A interacts with structures within the PKR protein kinase catalytic domain *in vivo*. The NS5A-interactive region of PKR (aa 244–366) includes the region that interacts with the cellular PKR inhibitor, P58^{IPK} (Fig. 1B) (Gale *et al.*, 1996). This region of the protein kinase catalytic domain functions cooperatively in nucleotide binding and catalysis (Hanks *et al.*, 1988; Bossemeyer, 1995; Taylor *et al.*, 1993). Thus, similar to the proposed mechanisms of P58^{IPK}-mediated inhibition of PKR (Gale *et al.*, 1996), formation of an NS5A–PKR complex may interfere with kinase activation to render PKR nonfunctional.

NS5A represses PKR function

Inhibition of PKR function is critical for establishment of viral infection. A common theme shared among the divergent family of virally encoded PKR inhibitors and the cellular inhibitor, P58^{IPK}, is that inhibition of PKR function is mediated through a direct physical interaction with the kinase (reviewed by Katze, 1993, 1995). We sought to determine if the NS5A–PKR interaction could similarly result in an inhibition of PKR function. We first carried out an *in vitro* analysis of PKR activity in the presence of recombinant NS5A. Incubation of purified native PKR with recombinant GST-NS5A from HCV-1a resulted in the specific inhibition of both PKR autophosphorylation and phosphorylation of an exogenous histone substrate (Fig. 3A). It is worth noting that as little as 0.2 pmol of NS5A can inhibit 1.8 pmol of PKR. At present we cannot determine whether this results from a relatively small percentage of PKR being active or whether NS5A may function at low levels due, perhaps, to disruption of higher order PKR dimers. We confirmed that the loss of PKR activity was not due to degradation of PKR or NS5A-mediated hydrolysis of ATP (data not shown). Furthermore, while it is possible that the inhibition of PKR and histone phosphorylation in our *in vitro* assay could be due to a GST-NS5A-mediated phosphatase activity, we believe this to be unlikely since NS5A does not possess any structural attributes indicative of phosphatase function. Equal molar levels of GST had no effect upon PKR activity (Fig. 3A, lanes 6–9). Thus, similar to the action of other virally encoded PKR inhibitory proteins, NS5A can specifically

inhibit the function of PKR *in vitro*, likely through a direct effect of the NS5A–PKR interaction.

Expression of PKR in yeast provides an *in vivo* functional assay for direct measurement of PKR activity (Romano *et al.*, 1995). Through its eIF-2 α phosphorylating activity, PKR is growth suppressive when expressed in yeast. Coexpression of PKR with transdominant inhibitory PKR mutants or virally encoded PKR inhibitory proteins, such as HIV Tat or vaccinia virus K3L, reverses the PKR-mediated slow growth phenotype in yeast due to *in vivo* inhibition of PKR and a concomitant decrease in eIF-2 α phosphorylation (Romano *et al.*, 1995; McMillan *et al.*, 1995; M. Kobayashi, E. Locke, J. Silverman, T. Ung, and T. Dever, manuscript submitted for publication). To assess the effects of NS5A on PKR function *in vivo*, we expressed NS5A under control of the *GAL1* galactose-inducible promoter in yeast strain RY1-1, developed by Romano *et al.* (1995). RY1-1 possesses two integrated copies of human PKR under control of the *GAL1-CYC1* galactose-inducible promoter and maintains a PKR-mediated growth-suppressive phenotype when grown on galactose medium. For comparison, we included a parallel analysis of RY1-1 harboring the well-characterized vaccinia virus PKR inhibitor, K3L, also under control of the *GAL1-CYC1* promoter (M. Kobayashi, E. Locke, J. Silverman, T. Ung and T. Dever, manuscript submitted for publication). We began our examination of NS5A function by determining the growth properties of the RY1-1 transfectants. While all transfectants maintained efficient growth on noninducing dextrose (SD) medium (Fig. 3B, left), only those harboring NS5A or K3L were able to sustain growth under conditions of PKR expression (Fig. 3B, right). Vector control transfectants exhibited a severe slow growth phenotype consistent with the growth suppressive activity associated with high levels of PKR expression (Fig. 3B) (Romano *et al.*, 1995). These results demonstrate that NS5A is sufficient to reverse PKR-mediated growth suppression in yeast, indicating that NS5A can directly repress PKR function *in vivo*. To confirm that PKR function was repressed in the presence of NS5A, we determined the level of eIF-2 α phosphorylation in the RY1-1 transfectants. Similar to K3L, expression of NS5A resulted in approximately an 11-fold increase in the level of unphosphorylated eIF-2 α over that observed in control transfectants harboring the vector alone (Fig. 3C). While the majority of the eIF-2 α in these cells remained in the hyperphosphorylated state, these results suggest that relatively small changes in the overall level of eIF-2 α phosphorylation can nevertheless have dramatic effects on cell growth. Taken together, these observations indicate that NS5A, through a direct interaction with PKR, can repress PKR function *in vivo*, resulting in alterations in the phosphorylation state of the PKR substrate, eIF-2 α .

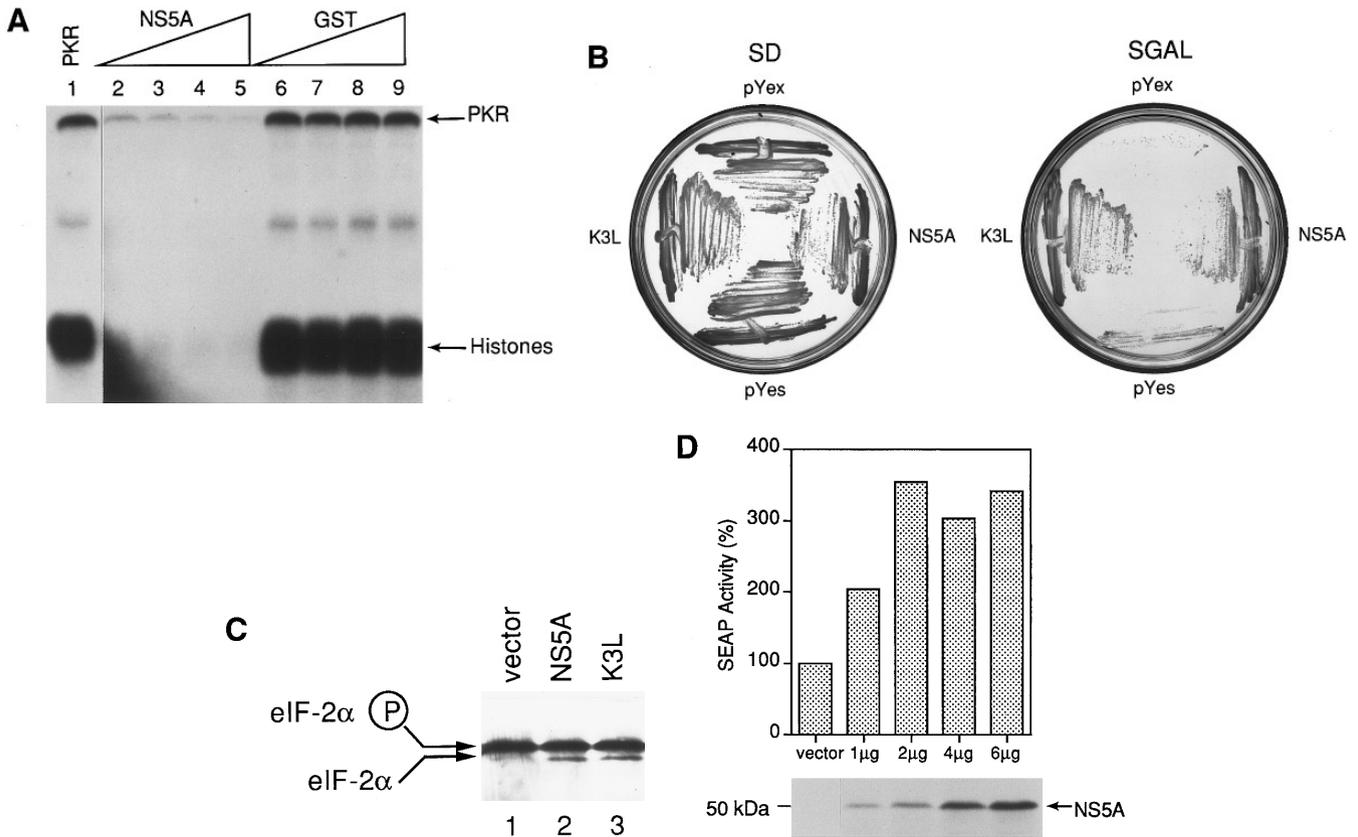


FIG. 3. NS5A represses PKR function. (A) Recombinant NS5A inhibits PKR activity *in vitro*. Purified native PKR (1.8 pmol) was preincubated with buffer (lane 1) or increasing amounts of recombinant purified GST-NS5A (HCV 1a, lanes 2–5) or GST (lanes 6–9) and assayed for *in vitro* kinase activity in the presence of histone H2a substrate. After termination of kinase reactions, phosphorylated products were separated by 12.5% SDS-PAGE and visualized by autoradiography of the dried gel. Arrows indicate the positions of PKR and histones. The molar amounts of input GST-NS5A or GST proteins were 0.2 pmol (lanes 2 and 6), 0.4 pmol (lanes 3 and 7), 0.8 pmol (lanes 4 and 8), and 1.2 pmol (lanes 5 and 9). (B) NS5A reverses PKR-mediated growth suppression in yeast. Yeast strain RY1-1 was transfected with the 2 μ yeast expression constructs pYES2-NS5A (NS5A; from HCV-1a) or pEMBLYex4-K3L (vaccinia virus K3L; positive control) or the negative control vectors pEMBLYex4 (pYex) and pYes2 (pYes). Transfectants were streaked onto minimal synthetic medium containing 2% dextrose (SD, left) or 10% galactose/2% raffinose (SGAL, right) as the carbon source and scored for growth. SGAL plate represents 5 days of growth. (C) eIF-2 α phosphorylation analysis. Extracts from RY1-1 yeast harboring the vector control plasmid pYes2 (lane 1), NS5A (lane 2), or K3L (control; lane 3) were separated by isoelectric focusing and subjected to immunoblot analysis with an antiserum to yeast eIF-2 α . Arrows indicate the positions of eIF-2 α phosphorylated on basal sites only (lower band) and eIF-2 α phosphorylated on Ser 51, the site of phosphorylation by PKR (upper band). The levels of basally phosphorylated eIF-2 α relative to total were quantitated by scanning laser densitometry and are: vector, 0.9%; NS5A, 11.2%; and K3L, 11.7%. (D) NS5A stimulates protein synthesis in mammalian cells. COS1 cells expressing the SEAP reporter protein were cotransfected with 10 μ g of the control vector, pcDNA3.1/His (vector), or 10 μ g plasmid DNA containing pcDNA3.1/His and titrating amounts (indicated in μ g) of pcDNA3.1/His-NS5A. SEAP activity was measured from the supernatant of each transfection culture and is shown as the percentage of SEAP activity relative to that of control cells harboring the vector alone, which was normalized to 100%. (Bottom) Expression of NS5A from COS1 cells transfected with vector DNA (vector) or 1, 2, 4, and 6 μ g of pcDNA3.1/His-NS5A, as detected by immunoblot analysis using α -NS5 sera. Each lane represents 50 μ g of extract electrophoresed from 10^6 cells.

We next sought to determine if NS5A could similarly regulate PKR within mammalian cells. Through repression of the eIF-2 α -phosphorylating activity of PKR, transient inhibition of PKR in mammalian cells results in a net increase in the level of protein synthesis. Reflecting this effect of PKR inhibition, negative regulators of PKR can stimulate protein synthesis above basal levels when introduced into mammalian cells (Svensson and Akusjarvi, 1985; Seliger *et al.*, 1992; Kaufman and Murtha, 1987; Giantini and Shatkin, 1989; Tang *et al.*, 1996). To determine if NS5A expression could alter the level

of protein synthesis in mammalian cells, we tested the ability of NS5A to stimulate protein synthesis in transfected COS1 cells using a secreted embryonic alkaline phosphatase (SEAP) reporter assay (Berger *et al.*, 1988). Coexpression of SEAP and NS5A (HCV-1a) in COS1 cells resulted in greater than a 300% stimulation of protein synthesis over SEAP/vector cotransfectants (Fig. 3D). Thus, similar to other PKR-inhibitory molecules, including adenovirus VAI RNA, P58^{IPK} and reovirus σ 3 protein (Svensson and Akusjarvi, 1985; Seliger *et al.*, 1992; Kaufman and Murtha, 1987; Giantini

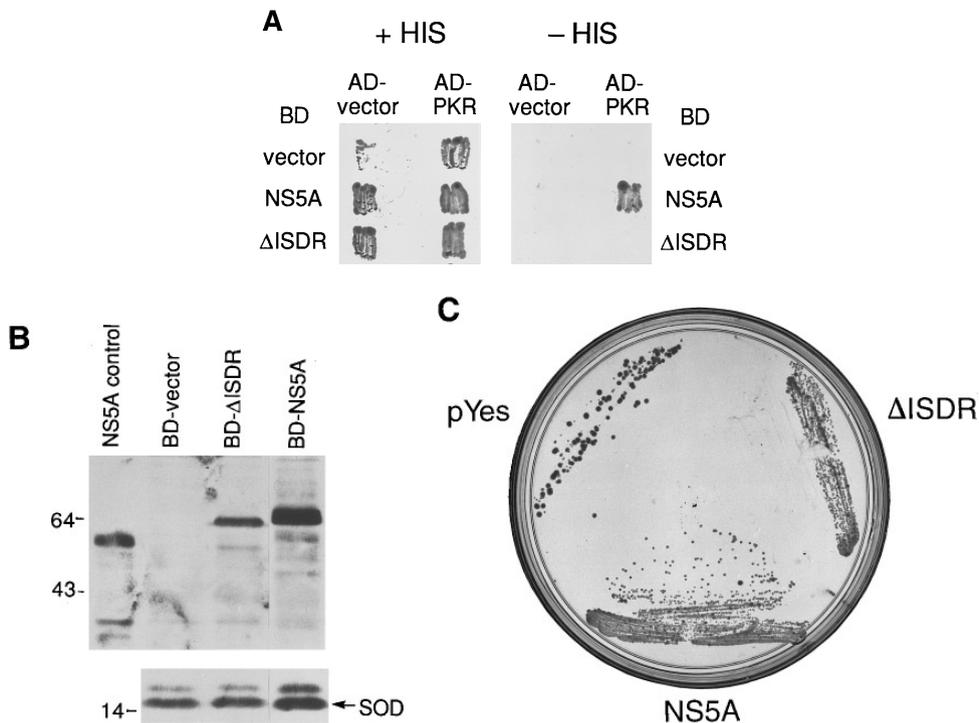


FIG. 4. Requirement of the ISDR region of NS5A for PKR interaction and repression of PKR function. (A) The ISDR of NS5A is required for interaction with PKR *in vivo*. Yeast expressing the AD (AD-vector) or AD-PKR K296R (AD-PKR) were transfected with constructs expressing BD-NS5A; BD- Δ ISDR, which lacks the entire NS5A ISDR region (Fig. 1A); or BD-vector (bottom row). Both NS5A constructs were derived from HCV-1a. Transfected yeast were replica plated onto +His (left panel) and -His medium (right) and assayed for growth as described under Materials and Methods. Two-hybrid protein interaction was defined as specific growth on -His medium. (B) Expression of NS5A GAL4-fusion constructs. Extracts from COS1 cells transfected with pcDNA3.1/His-NS5A (NS5A control; lane 1) or yeast cells with pBD (BD-vector; lane 2), pBD- Δ ISDR (Δ ISDR; lane 3), or pBD-NS5A (BD-NS5A; lane 4) were subjected to immunoblot analysis using α -NS5 sera. (Bottom) The corresponding expression of yeast superoxide dismutase (SOD) using α -SOD sera as determined from the same blot and included to control for variations in extract protein concentrations. By this method we determined that both BD- Δ ISDR and BD-NS5A were expressed to the same levels. The apparent differences seen on the blot reflect a threefold difference in protein concentration between the two extracts. Gel standards are shown in kDa. (C) The ISDR of NS5A is required for reversal of PKR-mediated growth suppression in yeast. Yeast strain RY1-1 harboring the expression constructs pYES2-NS5A (NS5A), pYES2- Δ ISDR (Δ ISDR) or the negative control vector pYES2 (pYes), were streaked onto inducing medium containing 10% galactose/2% raffinose (SGAL) and scored for growth. SGAL plate shown represents 6 days of growth. All transfectants grew when streaked onto noninducing SD medium (data not shown).

and Shatkin, 1989; Tang *et al.*, 1996), NS5A can stimulate protein synthesis in mammalian cells, likely through a direct inhibitory effect upon PKR.

The NS5A ISDR is required for interaction with PKR and repression of PKR function *in vivo*

Inhibition of PKR in infected mammalian cells may be one mechanism by which HCV mediates resistance to IFN. Correlation of IFN sensitivity with mutations within the ISDR of NS5A suggested that the ISDR may play a role in mediating the IFN-resistant phenotype of HCV. A key question remaining was whether or not the ISDR contributed to the ability of NS5A to bind to and inhibit PKR. To begin to determine the requirement for the ISDR in the NS5A-PKR interaction, we compared the ability of BD-NS5A and of a BD-NS5A ISDR-deletion mutant derived from HCV-1a (BD- Δ ISDR; Fig. 1A) to bind to AD-PKR K296R *in vivo*. BD- Δ ISDR failed to mediate interaction with AD-PKR K296R in the yeast 2-hybrid assay (Fig. 4A). We also found that the N-terminal 236 aa of NS5A,

which similarly lack the ISDR, failed to interact with PKR in this assay (data not shown). In contrast, BD-NS5A specifically interacted with AD-PKR K296R (Fig. 4A). Deletion of the ISDR from NS5A could conceivably render the mutant protein unstable and unavailable to interact with PKR. However, immunoblot analysis demonstrated that both BD-NS5A and - Δ ISDR constructs were efficiently expressed in yeast transfectants (Fig. 4B). By standardizing protein loading, using antibody to yeast superoxide dismutase, we quantified NS5A levels and determined that both BD- Δ ISDR and BD-NS5A were expressed to approximately the same levels (see the legend to Fig. 4B). The possibility remains, however, that deletion of the ISDR may render NS5A conformationally constrained, thus precluding interaction with PKR. Collectively, these results suggest that the ISDR of NS5A is necessary for interaction with PKR and that deletion of the ISDR may render NS5A nonfunctional *in vivo*. We thereby compared the ability of NS5A and the Δ ISDR mutant to reverse the PKR-mediated slow growth phenotype in yeast. Coex-

pression of Δ ISDR with PKR in yeast strain RY1-1 failed to reverse PKR-mediated growth repression while expression of NS5A specifically restored growth in the transfected yeast clones (Fig. 4C). These results, taken together, indicate that the NS5A ISDR is required for interaction with and inhibition of PKR activity *in vivo*.

DISCUSSION

The cellular IFN response is one of the body's first defense mechanisms to prevent the establishment of viral infection (Sen and Lengyel, 1992; Sen and Ransohoff, 1993). The ability of a given animal virus to establish a productive infection depends in part on the ability of the virus to circumvent the IFN response, which includes the transcriptional induction and functional activation of PKR. Indeed, we have found in many cases that the ability of a virus to establish a productive infection correlates well with its ability to repress PKR function (Katze, 1993, 1995). Results presented in this study demonstrate that HCV similarly encodes a mechanism to repress PKR. We propose that the HCV NS5A protein functions to directly repress PKR, thus preventing, at least in part, the antiviral effects of IFN on HCV replication.

In addition to its antiviral properties, PKR plays a functional role in signal transduction pathways leading to the transcriptional regulation of gene expression and the induction of apoptosis (reviewed by Proud, 1995). Thus, inhibitors of PKR can have profound effects on gene expression which may ultimately be detrimental to the cell. Indeed, previous studies from our laboratory and others have shown that inhibitors of PKR can induce oncogenesis (Koromilas *et al.*, 1992; Meurs *et al.*, 1993; Barber *et al.*, 1995). While other mechanisms of oncogenesis may exist within an HCV-infected cell, including those specific to the transforming properties of the NS3 and viral core proteins (Sakamuro *et al.*, 1995; Ray *et al.*, 1996), it is tempting to speculate that the NS5A-mediated inhibition of PKR may lead to malignant transformation of the infected hepatocyte, perhaps reflecting the high frequency of hepatocellular carcinomas within the HCV-infected population.

Molecular epidemiological studies have revealed that the HCV genome exhibits a significant level of heterogeneity within HCV infected individuals as a result of the high frequency of mutations that occur during viral replication (Bukh *et al.*, 1995). Referred to as viral quasispecies, the population of HCV genomes within an infected individual may influence the outcome of IFN therapy. Indeed, this is supported by the recent studies which correlate mutations in the ISDR of the HCV-1b NS5A protein with IFN sensitivity (Enomoto *et al.*, 1995, 1996). Importantly, our data have indicated that the ISDR was required for both PKR binding and functional PKR repression, possibly defining a critical role for this region in mediating *in vivo* function

of NS5A. It is tempting to speculate that specific mutations in the ISDR, which may exist within an HCV quasispecies population, may compromise the ability of NS5A to repress PKR. Though beyond the scope of the current report, a broader analysis of NS5A proteins from IFN-resistant and IFN-sensitive HCV strains is required to correlate ISDR structure with the ability to repress PKR. NS5A from both HCV-1a and -1b possessed the ability to bind to PKR, suggesting the possibility that PKR regulation may be a common mechanism by which both genotypes may mediate IFN resistance. This is supported by the high response rate to IFN therapy exhibited in HCV genotypes 2–4, which lack a consensus ISDR (S. Polyak and D. Gretch, unpublished observations). We suggest that combinatorial therapeutic strategies designed to block the NS5A–PKR interaction may increase the efficacy of IFN treatment of an HCV-1a or -1b infection.

We have shown that HCV NS5A from both IFN-resistant HCV-1a and HCV-1b strains can specifically interact with and inhibit the activity of PKR, also raising the possibility that NS5A could be a PKR substrate. However, our preliminary results argue against this as we have not found that PKR can phosphorylate NS5A in our *in vitro* assay. Moreover, while NS5A does reside in the cell as a phosphoprotein (Tanji *et al.*, 1995; Kaneko *et al.*, 1994), we currently do not know what role, if any, that NS5A phosphorylation plays in mediating NS5A function or interaction with PKR. Importantly, NS5A binds to a region of PKR (aa 244–366; Fig 1B) which participates in the formation of active PKR dimers (Patel *et al.*, 1995). This region includes a regulatory autophosphorylation site, T-258, followed by protein kinase catalytic domain conservation regions I–V (Taylor *et al.*, 1996; Hanks *et al.*, 1988). Based upon the known crystal structure of the catalytic domain of related protein kinases, NS5A would occupy a critical region within the small lobe and catalytic cleft of the PKR catalytic domain that is involved in nucleotide binding and catalysis (Bossemeyer, 1995; Taylor *et al.*, 1993). Significantly, among various protein kinases this region of the catalytic domain appears to be the target for interaction by kinase-regulatory proteins, including that between the cyclic AMP-dependent protein kinase and its RI regulatory subunit, as well as the cyclin/cyclin-dependent kinase interactions (reviewed in Bossemeyer, 1995; Taylor *et al.*, 1993). Unlike the vaccinia virus K3L and adenovirus VAI gene products, which inhibit PKR by functioning as a pseudosubstrate and competitive inhibitor of activator–dsRNA binding, respectively (Katze, 1993, 1995), we propose that NS5A may interfere with events leading to activation and catalysis by directly interfering with dimerization, autophosphorylation, and/or steps within the catalytic process. It is worth noting that the NS5A-interactive region of PKR includes the domain which binds the cellular PKR inhibitor, P58^{IPK} (Fig. 1B) (Gale *et al.*, 1996). The localization of both the P58^{IPK}-

and the NS5A-interactive sites to overlapping domains of PKR reflects the importance of this region for kinase function and suggests that these divergent inhibitors may repress PKR by similar mechanisms.

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