The hepatitis C virus (HCV) RNA-dependent RNA polymerase (RdRp), represented by nonstructural protein 5B (NS5B), has recently emerged as a promising target for antiviral intervention. Here, we describe the isolation, functional characterization, and molecular cloning of a monoclonal antibody (mAb) inhibiting the HCV RdRp. This mAb, designated 5B-12B7, binds with high affinity to a conformational epitope in the palm subdomain of the HCV RdRp and recognizes native NS5B expressed in the context of the entire HCV polyprotein or subgenomic replicons. Complete inhibition of RdRp activity in vitro was observed at equimolar concentrations of NS5B and mAb 5B-12B7, whereas RdRp activities of classical swine fever virus NS5B and poliovirus 3D polymerase were not affected. mAb 5B-12B7 selectively inhibited NTP binding to HCV NS5B, whereas binding of template RNA was unaffected, thus explaining the mechanism of action at the molecular level. The mAb 5B-12B7 heavy and light chain variable domains were cloned by reverse transcription-PCR, and a single chain Fv fragment was assembled for expression in Escherichia coli and in eukaryotic cells. The mAb 5B-12B7 single chain Fv fragment bound to NS5B both in vitro and in transfected human cell lines and therefore may be potentially useful for intracellular immunization against HCV. More important, detailed knowledge of the mAb 5B-12B7 contact sites on the enzyme may facilitate the development of small molecule RdRp inhibitors as novel antiviral agents.

The hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (1). A protective vaccine does not exist to date, and therapeutic options are limited (2, 3). HCV contains a single-strand RNA genome of positive polarity and ~9600 nucleotides in length that encodes a polyprotein precursor of ~3000 amino acids (aa) (see Refs. 4 and 5 for recent reviews). The polyprotein precursor is co- and post-translationally processed by cellular and viral proteases to yield the mature structural and nonstructural proteins. HCV RNA replication proceeds via synthesis of a complementary (−) strand RNA using the genome as a template and the subsequent synthesis of genomic RNA from this (−) strand template. The key enzyme responsible for both of these steps is the virally encoded RNA-dependent RNA polymerase (RdRp), represented by nonstructural protein 5B (NS5B).

The HCV RdRp has been shown to be essential for viral replication in vitro (6) and in vivo (7, 8), and it has been extensively characterized both at the biochemical (9–12) and structural (13–16) levels. HCV NS5B contains motifs shared by all RdRps and possesses the classical fingers, palm, and thumb subdomains. As a unique feature of the HCV RdRp, extensive interactions between the fingers and thumb subdomains result in a completely encircled active site. The HCV RdRp has emerged as a promising target for antiviral drug development. In this context, it has recently been validated as an antiviral target in the related pestiviruses (17).

In this study, we describe the isolation, functional characterization, and molecular cloning of a mAb that specifically and efficiently inhibits the HCV RdRp. The mechanism of enzyme inhibition was elucidated at the molecular level. Hence, this mAb may serve as a unique molecular probe for future mechanistic studies toward the elucidation of the HCV RdRp reaction pathway and may provide a new framework for the development of small molecule RdRp inhibitors as novel antiviral agents.

**EXPERIMENTAL PROCEDURES**

Production and Purification of RdRps—Recombinant HCV and classical swine fever virus (CSFV, kindly provided by Jon-Duri Tratschin, Institute of Virology and Immunophylaxis, Mittelhaeusern, Switzerland) NS5B were produced in a recombinant baculovirus system and purified by affinity chromatography as described (10, 18). Purified poliovirus 3D polymerase was a gift of Eckard Wimmer and Aniko Paul (State University of New York, Stony Brook, NY).

Establishment of mAbs—Eight-week-old female BALB/c mice were immunized with recombinant HCV NS5B in its functionally active, native conformation. Spleen cells from immunized mice were fused with the X63-Ag8.653 myeloma cell line (American Type Culture Collection, Manassas, VA). Hybridomas were selected, and supernatants were screened by ELISA essentially as described (19). Hybridomas immunoreactive with recombinant and celluloarly expressed NS5B protein were cloned twice by limiting dilution. mAb isotypes were determined with the X63-Ag8.653 myeloma cell line (American Type Culture Collection, Manassas, VA). Hybridomas were selected, and supernatants were screened by ELISA essentially as described (19). Hybridomas immunoreactive with recombinant and celluloarly expressed NS5B protein were cloned twice by limiting dilution. mAb isotypes were determined with the X63-Ag8.653 myeloma cell line (American Type Culture Collection, Manassas, VA). Hybridomas were selected, and supernatants were screened by ELISA essentially as described (19). Hybridomas immunoreactive with recombinant and celluloarly expressed NS5B protein were cloned twice by limiting dilution. mAb isotypes were determined with the X63-Ag8.653 myeloma cell line (American Type Culture Collection, Manassas, VA). Hybridomas were selected, and supernatants were screened by ELISA essentially as described (19). Hybridomas immunoreactive with recombinant and celluloarly expressed NS5B protein were cloned twice by limiting dilution. mAb isotypes were determined with the X63-Ag8.653 myeloma cell line (American Type Culture Collection, Manassas, VA). Hybridomas were selected, and supernatants were screened by ELISA essentially as described (19). Hybridomas immunoreactive with recombinant and celluloarly expressed NS5B protein were cloned twice by limiting dilution. mAb isotypes were determined with the X63-Ag8.653 myeloma cell line (American Type Culture Collection, Manassas, VA). Hybridomas were selected, and supernatants were screened by ELISA essentially as described (19). Hybridomas immunoreactive with recombinant and celluloarly expressed NS5B protein were cloned twice by limiting dilution. mAb isotypes were determined with the X63-Ag8.653 myeloma cell line (American Type Culture Collection, Manassas, VA).
lyzed for 16 h at 4 °C against buffer containing 100 mM NaCl and 10 mM Tris-HCl (pH 7.2). Biotinylation was performed using the FluoroReporter Biotin-XX labeling kit (Molecular Probes, Inc., Eugene, OR). Reactivity of biotinylated mAbs was revealed with horseradish peroxidase-conjugated streptavidin (Molecular Probes, Inc.).

**Expression Constructs**—Plasmids pGEM-11-BX-NS5Bcon, pCMVNS5Bcon, and pCMVNS5BconAC21, containing NS5B sequences derived from a functional HCV H strain consensus cDNA (7), were described previously (20). Plasmids pCMVNS5Bcon392, pCMVNS5Bcon299, and pCMVNS5Bcon139, allowing expression of NS5B aa 1–299, 1–299, and 1–139, respectively (see Fig. 3A), were constructed by ligation of the BamHI-SmaI fragments of pCMVNS5Bcon into the BamHI-EcoRI sites of pcDNA3.1 (Invitrogen, San Diego, CA). Plasmids pCMVNS5Bcon203, allowing expression of NS5B aa 1–203, was constructed by ligation of the EcoRI-EcoRI fragment of pGEM-BX-NS5Bcon into the EcoRI site of pcDNA3.1. To construct pCMVNS5Bcon299–392, a fragment representing aa 299–392 was amplified by PCR from pBRTM/HCV1-3011con (7) using primers 5′-GAGTACCCATTGCTAGATCTCTAGAAKCTCGAGYTTKGTSo-3′ and 5′-GACATTCAGCTGACCCAGWCTSC-3′, respectively (see Table I). The excitation wavelength was 330 nm when it is excited at 295 nm or an 8-mer RNA template (GR-1, 5′-ACCGAACGTCCACGGAACCTCo-3′) by following the manufacturer's recommendations. Reactions were routinely performed in 2× sample loading buffer, boiled for 5 min, and analyzed by SDS-PAGE. In some experiments, cells were homogenized in a Dounce homogenizer in hypotonic buffer containing 10 mM Tris-HCl (pH 7.5) and 2 mM MgCl2, and immunoprecipitation was performed from post-nuclear supernatants as described above.

**Epitope Mapping**—The mAb 5B-3B1 epitope was mapped by random DNaSe F fragment expression library screening using the NovaTape system (Novagen, Madison, WI).

**In Vitro RdRp Assays**—Polymerase assays contained 500 ng of HCV-specific in vitro transcript corresponding to a functional replicon RNA (5); 200 ng of HCV NS5B, 400 ng of CSFV NS5B, or 20 ng of poliovirus 3D polymerase; various concentrations of mAbs as specified under "Results"; 1 μCi of [α-32P]CTP adjusted to a 10 μM final concentration; a 500 μM concentration of each of the remaining NTPs; 10 units of RNase (Promega); and assay buffer (5 mM dithiothreitol, 20 mM Tris-HCl (pH 7.5), 12.5 mM MgCl2, 10 mM KCl, and 1 mM EDTA) in a total volume of 25 μl. After a 5-min incubation of the enzyme with the mAb at 4 °C, the RdRp reaction was initiated by the addition of NTPs and RNA, followed by a 1-h incubation at 27 °C. The reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid and 0.5% tetrasodium pyrophosphate and 100 μg of salmon sperm DNA. After a 30-min incubation at 4 °C, samples were filtered through glass microfiber GF/C filters (Whatman, Kent, United Kingdom). Filters were washed five times with 150 mM trichloroacetic acid and 0.1% tetrasodium pyrophosphate, and bound radioactivity was measured after the addition of 1 ml of scintillation fluid (Micro-Scint; Packard Instruments, Meriden, CT). Background values obtained with an inactive RdRp mutant were subtracted.

**Determination of Equilibrium Dissociation Constants (Kd)**—Using the carboxyl-terminal 21 aa (NS5BAC21) was expressed in Escherichia coli and purified to homogeneity as described previously (12). This recombinant protein has nine tryptophan residues and thus has strong protein fluorescence at 330 nm when it is excited at 283 nm. This property was utilized to determine the equilibrium dissociation constant (Kd) of NS5B for a nucleotide (GTP, CTP, ATP, or UTP) or an 8-mer RNA template (GR-1, 5′-AGAGAGCC-3′) by following the quenching of intrinsic protein fluorescence. Measurements were performed at 23 °C on a PTFU spectrofluorometer (Photon Technology International, Lawrenceville, NJ). The excitation wavelength was set at 283 nm, and the emission wavelength was set at 330 nm. The binding buffer included 20 mM Tris-HCl (pH 7.5), 10 mM KCl, 50 mM NaCl, 20 mM MgCl2, and 5 mM dithiothreitol. The experiments involved successive titration of the protein (53 nM) with a ligand or substrate (0.2–200 μM). The measured fluorescence intensity (I) at a given ligand concentration (IL) was fitted to Equation 1 to calculate the dissociation constant (Kd).

\[
I = I_0 - I_{max} \times \frac{[L]_{tot}}{K_d + [L]_{tot}}
\]

(1)

where I0 is the fluorescence of free protein and Itotal is the fluorescence loss due to the full occupation of all the binding sites by a ligand or substrate.
substrate. Nonlinear least-squares fit of the data was performed using Kaleidagraph (Synergy Software, Reading, PA). In the experiments, a control sample containing fluorescence intensity similar to that of tryptophan was titrated in the same way as the protein sample. The fluorescence loss due to the addition of free ligand was determined. The percentage fluorescence loss was used to compensate for the measured protein fluorescence intensity in determining the real f value. To study the effect of mAb 5B-12B7 on the NS5B/substrate (RNA/NTP) interaction, the mAb (54 nm; the NS5B-binding site concentration is 108 nm) was mixed with NS5B (53 nm) before the addition of a substrate. The Kd of NS5B for the substrate in the presence of mAb 5B-12B7 was calculated as described above.

Cloning of mAb Variable Domains and Assembly of Single Chain Variable Domain Fragment (scFv) Constructs—Total cellular RNA was extracted from early passage 5B-12B7.54.1 hybridoma cells using RNAzol (Biotex Laboratories, Houston, TX). First-strand cDNA synthesis with an oligo(dT) primer was performed using the first-strand cDNA synthesis kit (Amer sham Biosciences, Inc.). The heavy chain variable domain (VH) was amplified by PCR using the degenerate primers VH-1B and VH-1F (Table I). The VH amplification product was digested with HindIII and BstEII and ligated into the HindIII-BstEII sites of pWW152 (23, 24) to yield plasmid pWW12B7VH. The VH amplification product was digested with PvuII and XbaI and ligated into the PvuI-XbaI sites of pWW152 to yield plasmid pWW12B7VHPvuII. The PvuII vector contains HindIII and BstEII sites for the subcloning of murine VH cDNA fragments, followed by a synthetic sequence encoding the 15-aa linker (GGGGS)3, and PvuII and XbaI sites for the subcloning of murine VK cDNA fragments. Eight clones each of the VH and VK domains were sequenced. Subsequently, the HindIII-BstEII fragment of pWW12B7VH-6 was ligated into the HindIII-BstEII sites of pWW12B7VK-2 to yield the scFv construct pWW12B7.

For bacterio expression, the 5B-12B7 scFv sequence was isolated from pWW12B7 as a HindIII-XbaI fragment and fused in frame to the E. coli phoA alkaline phosphatase gene in plasmid pSW602, which is derived from the expression vector pFLAG-1 (IBI Biochemicals, New Haven, CT). The resulting pSW602-12B7 construct encodes a fusion alkaline phosphatase gene in plasmid pSW602, which is under PhoA control. A synthetic sequence encoding the 15-aa linker (GGGGS)3, and PvuII and XbaI sites for the subcloning of murine VK cDNA fragments. Eight clones each of the VH and VK domains were sequenced. Subsequently, the HindIII-BstEII fragment of pWW12B7VH-6 was ligated into the HindIII-BstEII sites of pWW12B7VK-2 to yield the scFv construct pWW12B7.

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Fig. 1. Characteristics of mAbs 5B-12B7 and 5B-3B1. A, indirect immunofluorescence microscopy. U-2 OS cells were transiently transfected with pCMVNS5Bcon (NS5B) or pCMVNS5Bcon392 (NS5B392), encoding a carboxyl-terminally truncated protein comprising NS5B aa 1–392, followed by indirect immunofluorescence microscopy using mAb 5B-12B7 as described under “Experimental Procedures.” B, immunoprecipitation/Western blot analysis. In lanes 1 and 3, UHCVcon-57.3 cells, which inducibly express the entire polyprotein derived from a functional HCV genotype 1a consensus cDNA, were cultured for 24 h in the presence (57.3 + tet) or absence (57.3 − tet) of tetracycline. In lanes 3–5, U-2 OS cells were not transfected (U-2 OS) or were transiently transfected with pCMVNS5Bcon (NS5B) or pCMVNS5Bcon392 (NS5B392). Cells were lysed in buffer containing 1% Nonidet P-40 and subjected to immunoprecipitation with mAb 5B-12B7 as described under “Experimental Procedures.” In lanes 6 and 7, HuH-7 cells or an HuH-7 cell line harboring a selectable subgenomic HCV replicon (9-13) was lysed in hypotonic buffer without the use of detergents as described under “Experimental Procedures,” followed by immunoprecipitation with mAb 5B-12B7. Immunoprecipitates were separated by 12% SDS-PAGE and analyzed by Western blotting using biotinylated mAb 5B-12B7 and streptavidin. Molecular mass standards are indicated on the left. C, competitive inhibition experiments. The reactivity of 0.1 μg of biotinylated mAb 5B-3B1 (5B-3B1∗) or 5B-12B7 (5B-12B7∗) per 96-well with NS5B bound to the solid phase was examined in the presence of 0, 1, 5, or 10 μg of the indicated mAb (corresponding to a 0-, 10-, 50-, or 100-fold molar excess). ELISA reactivity revealed by horseradish peroxidase-conjugated streptavidin is expressed as percent inhibition (percent of reactions performed in the absence of competing mAb). Values represent the means of duplicate determinations.

The observation that mAb 5B-12B7 did not react in immunoblot assays suggested that it recognizes a conformation-sensitive epitope on NS5B. Such epitopes are difficult to map, particularly in the case of proteins with a complex three-dimensional structure such as HCV RdRp. Using a comprehensive set of amino- and carboxy-terminal NS5B deletion constructs (Fig. 3A), we examined the reactivity of this mAb by immunoprecipitation analyses. As shown in Fig. 3B, all constructs yield stable proteins by in vitro transcription-translation. All truncated proteins were efficiently immunoprecipitated by a mouse polyclonal antiserum raised against recombinant NS5B (data not shown). mAb 5B-12B7 efficiently immunoprecipitated the full-length NS5B protein, carboxy-terminal deletions to aa 392 (NS5BΔC21 and NS5B392), but not NS5B299), and amino-terminal deletions to aa 139 (NS5B11–591, NS5B46–591, and NS5B139–591, but not NS5B299–591) (Fig. 3C). These results allowed us to map the mAb 5B-12B7 epitope to HCV aa 139–392. Accordingly, this mAb efficiently immunoprecipitated a NS5B fragment corresponding to aa 139–392, which constitute the entire palm subdomain and a small portion of the fingers subdomain. mAb 5B-12B7 efficiently immunoprecipitated recombinant NS5B proteins with amino acid substitutions at residues important for enzymatic activity, viz. D220G, D225G, G283R, T286V, T287K, N291K, G317A, D318H, D319E, and R345K (10), indicating that these amino acid residues are not critical for mAb binding (data not shown).

Inhibition of HCV RdRp Activity by mAb 5B-12B7—Given the highly specific interaction of mAb 5B-12B7 with NS5B under native conditions, we reasoned that this mAb might inhibit the RdRp activity. To explore this possibility, constant amounts of highly purified HCV NS5B were used for an in vitro RdRp assay in the presence of various concentrations of mAb 5B-12B7 that was purified from ascites fluid by protein G affinity chromatography. Incorporation of radioactivity into newly synthesized RNA was measured after trichloroacetic acid precipitation onto glass fiber filters by liquid scintillation counting, and background values as determined by analogous assays with a purified inactive HCV RdRp (~1200 cpm) were subtracted. Control reactions without mAbs routinely yielded 80,000–90,000 cpm. As shown in Fig. 4, RNA synthesis was efficiently blocked by very low concentrations of mAb 5B-12B7. Assuming that the majority of the antibodies isolated from ascites fluid correspond to mAb 5B-12B7, we calculated a virtually complete inhibition of HCV RdRp at a 1:1 molar ratio of mAb to enzyme. This effect was specific because no inhibition was found with mAb 5B-3B1 (directed against the linear NS5B epitope) and mAb 1B6 (directed against the HCV nonstructural protein 3 serine protease domain) (26).
residues and thus has strong protein fluorescence at 330 nm when it is excited at 283 nm. This property was utilized to determine the equilibrium dissociation constant ($K_d$) of NS5B for nucleotide or 8-mer RNA ligands. We performed fluorescence quenching experiments by titration of the intrinsic protein fluorescence with successive addition of a ligand. In the experiments performed in the absence of mAb 5B-12B7, NS5B fluorescence was quenched by all five ligands tested, including four nucleotides (GTP, CTP, ATP, and UTP) and the RNA template GR-1 (Fig. 5A), but it was not quenched by a 5' - unphosphorylated dinucleotide, GG (data not shown). The $K_d$ values calculated from the quenching curves shown in Fig. 5 are summarized in Table III.

Fluorescence quenching experiments were then performed to investigate the effect of mAb 5B-12B7 on the NS5B/RNA/NTP interaction. In the experiments performed in the presence of mAb 5B-12B7 (Fig. 5B), the molar ratio of NS5B to mAb-binding site concentration was set at 1:2 to assure that all the NS5B bound to mAb 5B-12B7. In this context, the apparent inhibition constant ($K_i$) of mAb 5B-12B7 for NS5B polymerase activity was found to be $<2$ nM (data not shown). Thus, NS5B and mAb 5B-12B7 should be in a fully associated form in our fluorescence quenching experiments. The addition of mAb 5B-12B7 to the NS5B solution increased the total protein fluorescence by 1.7 – 2-fold. Control experiments showed that the mAb 5B-12B7 fluorescence was not quenched by the addition of GTP or CTP at the concentrations used (data not shown). Therefore, the fluorescence quenching experiments could be used to probe the effect of the mAb on the interaction between NS5B and nucleotide or RNA ligands and therefore to deduce the molecular mechanism of action and to functionally map mAb 5B-12B7.

Among the five ligands tested for NS5B bound to mAb 5B-12B7, protein fluorescence was quenched by all except CTP at the concentrations used (Fig. 5B). The $K_i$ values of NS5B for ligands in the presence of mAb 5B-12B7 were calculated from
The fluorescence intensity of NS5B was bound to mAb 5B-12B7. Judged from the fluorescence intensity, the RNA oligonucleotide still bound to NS5B at submicromolar concentrations, suggesting that the mAb did not interfere with the NS5B/GTP interaction. This construct was then subcloned into the appropriate vectors for expression in E. coli and in eukaryotic cells. The nucleotide sequence determined by sequencing eight clones each of the VH and VK domains were cloned, and a scFv was assembled to assess the potential of mAb 5B-12B7 to bind to NS5B intracellularly. To this end, total cellular RNA was prepared from early passage 5B-12B7.54.1 hybridoma cells. Subsequently, reverse transcription was performed using an oligo(dT) primer, and PCR was performed using degenerate primers designed to hybridize to the partially conserved 5′- and 3′-regions of the heavy and light chain variable domains. PCR products corresponding to the VH and VK domains were inserted into the pWW152 vector, which contains the coding sequence for a flexible (GGGGS)_3 linker. This construct was then subcloned into the appropriate vectors for expression in E. coli and in eukaryotic cells. The nucleotide sequence determined by sequencing eight clones each of the VH and VK domains and the deduced amino acid sequence of the 5B-12B7 scFv are illustrated in Fig. 6.

For bacterial expression, the 5B-12B7 scFv sequence was fused to the E. coli phoA alkaline phosphatase gene. 5B-12B7 scFv-PhoA fusion protein was expressed in E. coli strain CC118 as described under “Experimental Procedures” and enriched by preparation of periplasmic extracts. As a control, a similar fusion protein containing the c-Myc-specific 9E10 scFv construct was produced. Binding of recombinant scFv-PhoA proteins to immobilized NS5B was determined by ELISA. As shown in Fig. 7A, specific binding of bacterially expressed 5B-12B7 scFv-PhoA fusion protein to NS5B was observed, whereas 9E10 scFv-PhoA showed only background binding.

For expression in eukaryotic cells, scFv was subcloned under the control of a cytomegalovirus promoter, and a FLAG tag was fused to the carboxyl terminus by PCR. The potential of this construct to bind to NS5B intracellularly was assessed by cotransfection with full-length and carboxyl-terminally truncated NS5B proteins. In contrast to the full-length protein, the NS5B392 construct was not membrane-associated in the cell and was expressed at very high levels (Fig. 1A). As shown in Fig. 1B, this construct was efficiently immunoprecipitated by parental mAb 5B-12B7. As non-relevant controls, the scFv constructs 9E10 (against c-Myc) and E6 (against HCV non-
structural protein 4A) were used. In addition, a construct in which the E6 sequence was cloned in reverse orientation served as a control for the background of the immunoprecipitation/Western blot procedure. As shown in Fig. 7B, the 5B-12B7 scFv efficiently immunoprecipitated the NS5B392 construct and, to a lesser extent, the full-length protein, whereas the other scFv constructs showed no or only background binding. Taken together, these results clearly demonstrate that mAb 5B-12B7 retains its capacity to specifically bind to its target antigen as a scFv fragment.

**DISCUSSION**

In this study, we have described a mAb, designated 5B-12B7, that specifically and efficiently inhibits the HCV RdRp. This mAb binds with high affinity to a conformational epitope represented by NS5B aa 139–392, which constitute the entire palm subdomain and a small portion of the fingers subdomain. The catalytically active center of the enzyme resides in the palm subdomain. mAb 5B-12B7 completely inhibited HCV RdRp activity in vitro at a 1:1 molar ratio. Inhibition was highly specific since RdRps of related viruses, viz. CSFV NS5B and poliovirus 3D polymerase, were unaffected by this mAb. In addition, the activity of the structurally closely related bacteriophage φ6 RdRp (28) was not affected by mAb 5B-12B7, which further demonstrates the specificity of inhibition. Interestingly, mAb 5B-12B7 was found to block NTP binding to NS5B selectively, whereas binding of an RNA substrate was not affected. These observations suggest that the mAb 5B-12B7 epitope overlaps with the NTP (but not the RNA)-binding site.

By contrast, mAb 5B-3B1, which recognizes a distinct linear epitope located at NS5B aa 372–382, did not inhibit RdRp activity. As shown in Fig. 2B, the mAb 5B-3B1 epitope is located far away from the tunnel for nucleotide entry and the RNA-binding groove. Interestingly, mAb 5B-3B1, which reacts very strongly in ELISA and immunoblot assays, failed to stain full-length NS5B in immunofluorescence analyses. This property was independent of the coexpression of other HCV structural and nonstructural proteins, suggesting that in cells the mAb 5B-3B1 epitope is masked by homo- or heterotypic protein/protein interactions. Consistent with this notion, mAb 5B-3B1 reacts well in immunofluorescence analyses of carboxyl-terminally truncated NS5B (NS5B392, consisting of aa 1–392), which must somehow expose the linear epitope (data not shown). Interestingly, three different mAbs directed against the palm subdomain of HCV RdRp have recently been reported to lack polymerase inhibitory activity (29). This underscores the unique properties of mAb 5B-12B7.

*De novo* RNA synthesis is often associated with RNA polymerase-catalyzed reactions (30, 31). It involves two discrete nucleotide-binding sites: one for the initiating nucleotide and the other for the elongating nucleotide. Consistent with this notion, mAb 5B-3B1 reacts well in immunofluorescence analyses of carboxyl-terminally truncated NS5B (NS5B392, consisting of aa 1–392), which must somehow expose the linear epitope (data not shown). Interestingly, three different mAbs directed against the palm subdomain of HCV RdRp have recently been reported to lack polymerase inhibitory activity (29). This underscores the unique properties of mAb 5B-12B7.

3 E. V. Makeyev and D. H. Bamford, unpublished data.
must serve as a primer and bind to a site different from that of an elongating nucleotide. It was recently reported that a conformational change of the thumb subdomain was deleted (15, 27). It was found that both were inhibited equally by the mAb, suggesting that the binding site of mAb 5B-12B7 is not in the proximity of the α-hairpin, which is on the opposite site of the tunnel for NTP entry (data not shown).

Cloning of the mAb 5B-12B7 VH and VK domains allowed the assembly of a scFv that retained NS5B binding activity in vitro and in transfected cells. This construct may therefore be useful for intracellular immunization strategies against HCV. A similar approach has been successfully explored, for example, for the inhibition of human immunodeficiency virus (34) and hepatitis B virus replication (35). More important, identification of the exact mAb 5B-12B7 contact sites on NS5B may facilitate the development of small molecule RdRp inhibitors as novel antiviral agents. Finally, the well characterized mAbs described here represent highly valuable tools to further investigate the HCV RdRp and its role during formation of the viral replication complex.

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Fig. 7. Binding of 5B-12B7 scFv to NS5B in vitro and in transfected cells. A, binding of bacterially expressed scFv. Immobilized NS5B (black bars) or bovine serum albumin (BSA) (white bars) was incubated with periplasmic extracts containing 5B-12B7 or c-Myc-specific 9E10 scFv-PhoA fusion proteins as indicated. Bound scFv proteins were detected after incubation with rabbit anti-PhoA serum, followed by alkaline phosphatase-conjugated anti-rabbit IgG and conversion of the phosphatase substrate p-nitrophenyl phosphate by measuring the absorbance at 405 nm. Values are the means ± S.D. (error bars) of triplicate determinations. B, co-immunoprecipitation experiments. Plasmids pCMV12B7FLAG (12B7), pCMV9E10FLAG (9E10), pCMV6E6FLAG (E6), and pCMV6E6FLAGrev (E6rev) were cotransfected with pCMVN5Bcon392 (5B) or pCMVN5Bcon392 (392) into U-2 OS cells. Thirty-six hours post-transfection, cells were lysed as described under “Experimental Procedures,” and lysates were subjected to immunoprecipitation with anti-FLAG mAb M2. Immunoprecipitates were separated by 12% SDS-PAGE. Western blotting was performed using biotinylated mAbs 5B-3B1 and M2, followed by detection with horseradish peroxidase-conjugated streptavidin. Molecular mass standards are indicated on the right. The positions of full-length NS5B, the fragment representing NS5B aa 1–392, and the scFv constructs are shown on the left.

In a different set of experiments, we compared the inhibitory effect of mAb 5B-12B7 on wild-type NS5B and an enzymatically active derivative of NS5B in which the unique β-hairpin in the thumb subdomain was deleted (15, 27). It was found that both were inhibited equally by the mAb, suggesting that the binding site of mAb 5B-12B7 is not in the proximity of the β-hairpin, which is on the opposite site of the tunnel for NTP entry (data not shown).
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