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Antiviral Research 46 (2000) 181–193

Antiviral Research

www.elsevier.com/locate/antiviral

A novel high throughput screening assay for HCV NS3 helicase activity

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Received 23 September 1999; accepted 24 February 2000

Abstract

A novel assay for measurement of Hepatitis C virus (HCV) NS3 helicase activity was developed using Flashplate™ technology. This assay involves the use of a DNA duplex substrate and recombinant HCV NS3 produced in *Escherichia coli*. The DNA duplex consisted of a pair of oligonucleotides, one biotinylated, the other radiolabeled at their respective 5′ termini. This DNA duplex was immobilized, via the biotin molecule, on the surface of a neutravidin-coated SMP103 Flashplate™ (NEN Life Science Products). Helicase activity results in the release of the radiolabeled oligonucleotide, which translates in signal reduction with respect to control wells. Biochemical characterization of the HCV NS3 helicase activity was performed using this assay. We demonstrated that the NS3-mediated unwinding is proportional to both the amount of DNA substrate in the well, and to the NS3 concentration in the reaction. Most of the NS3-mediated unwinding was achieved in the initial 60 min of incubation. As expected the reactions were ATP-dependent and found to be affected by the concentration of $MgCl_2$, $MnCl_2$, KCl, EDTA, and by pH. We found this assay to be highly reproducible since only slight variation was observed when a total of 68 helicase reactions were performed on one plate. Therefore, this Flashplate™ helicase assay is fast, convenient and reproducible. These criteria make it suitable for high throughput screening of potential NS3 helicase inhibitors.

Keywords: HCV NS3 helicase; Flashplate™; High throughput screening assay

1. Introduction

Hepatitis C virus (HCV) infection is now recognized as a major health problem with an estimated 100 million afflicted individuals and a possibility of 500 million chronic carriers worldwide (Dhillon and Dusheiko, 1995). HCV is the major ethiological pathogen of blood transmitted non-A non-B hepatitis (NANBH; Choo et al., 1989; Kuo et al., 1989). This virus is enveloped and has been assigned as a separate genus within the Flaviviridae family (Rice, 1996). Phylogenetic analyses lead to the assignation of six different types of HCV with more than 30 subtypes

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0166-3542/00/$ - see front matter
PII: S0166-3542(00)00085-1

(Clarke, 1997). Genotypes 1a and 1b are the most common and account for 60% of the HCV infections worldwide (Bartenschlager, 1997a).

Infections with HCV can manifest in a wide range of clinical outcomes from an asymptomatic carrier state to acute or chronic hepatitis. In approximately 20% of chronically infected patients, the disease progresses to a liver cirrhosis which is highly associated with the subsequent development of hepatocellular carcinoma (HCC; Saito et al., 1990; Iwarson, 1994; Kew, 1994; Houghton, 1996). Alpha-interferon alone or in combination with ribavirin is the only approved therapy against HCV, but is only effective in a minority of individuals (Weiland, 1994; Bartenschlager, 1997b). It is therefore urgent to identify novel and effective anti-HCV drugs.

The HCV genome consists of a positive strand RNA molecule of approximately 9600 nucleotides with an open reading frame encoding a single polyprotein of 3010–3030 amino acids. This polyprotein is thereafter processed co- and post-translationally into ten distinct mature proteins both structural and non-structural (NS) by host and viral proteinases (Rice, 1996). Viral proteins are believed to be part of a membrane-associated replication complex in which NS3 and NS5B, and possibly cellular proteins, are implicated (Bartenschlager, 1997a). The enzymatic activities of some components of the HCV replicase complex have recently been elucidated. Helicase and nucleotide triphosphatase (NTPase) enzymatic activities have been assigned to the C-terminal two-thirds of the NS3 protein (Kim et al., 1997) whereas a protease function is associated with the amino portion of this protein (Tomei et al., 1993; Faila et al., 1994). Helicases are enzymes capable of unwinding nucleic acid duplexes during the process of replication. The disruption of the hydrogen bonds involved in base pairing requires energy and therefore, the unwinding of double stranded nucleic acids is coupled with hydrolysis of nucleotide triphosphates (Kadare and Haenni, 1997). It is generally accepted that these enzymes play a key role in the modulation of biological processes such as DNA or RNA synthesis, splicing, replication, and translation by inducing conformational changes in the DNA (chromatin) or RNA structures (Kadare and Haenni, 1997; Nakajima et al., 1997; Lüking et al., 1998). Based on sequence homology, three superfamilies have been attributed to RNA helicases with HCV NS3 protein belonging to the superfamily II, which also includes helicases from the bovine viral diarrhea pestivirus, plum pox potyvirus, and vaccinia virus. Despite having little sequence homology with the members of superfamily I, HCV NS3 shares some structural similarities with this helicase family (Korolev et al., 1998) implying the conservation of structural scaffolds among these two families. Several amino acid sequence motifs are found conserved among RNA helicases of both superfamily I and II. These consist of GSGKST, DECH, TAT, and QRRGRTGRGRRG motifs. The GSGKST consensus sequence is responsible for NTP binding, the DECH domain is involved in chelating the Mg^{2+} in the Mg^{2+}-NTP complex, the TAT box has been shown to be critical for helicase function, and the ATP hydrolysis and unwinding of RNA biochemical functions have both been attributed to the QRRGRTGRGRRG conserved element (Bartenschlager, 1997b; Lüking et al., 1998; Yao et al., 1997). Viral RNA helicases reported so far, including the HCV NS3 protein, are characterized by a 3′ to 5′ polarity of unwinding (Kadare and Haenni, 1997). During the past years, efforts have been devoted to understanding the precise role and importance of these RNA helicases in virus replication. It has been proposed that unwinding of base pairing, occurring within the template RNA or between the template RNA and the newly synthesized complementary RNA strand, is required for replication of viral single-stranded (ss) RNA genomes (Kadare and Haenni, 1997). Recently, the crystal structures of the PcrA DNA helicase, Rep DNA helicase, and the RNA helicase domain of HCV NS3 have been solved (Yao et al., 1997; Bird et al., 1998; Cho et al., 1998; Kim et al., 1998) providing some insights on the mechanism of catalytic activities of these enzymes.

In the past, due to the lack of effective and reliable in vitro systems for the propagation of HCV, it was difficult to perform molecular studies of the mechanism of replication of this virus. However, a cell culture system using a functional

subgenomic HCV replicon has been recently established (Lohmann et al., 1999). Several HCV enzymes such as the NS3 helicase, NS3-4A proteinase complex, and the NS5B RNA-dependent RNA polymerase (RdRp) can be viewed as potential targets for the discovery and development of anti-HCV agents (Bartenschlager, 1997a). In this report, we describe the expression, purification and characterization of full-length HCV NS3 and the development of an assay for measurement of HCV NS3 helicase activity suitable for high throughput screening (HTS). We investigated the effect of time and pH, as well as substrate, enzyme, EDTA (ethylenediaminetetraacetic acid), mono and divalent cation concentrations on the enzymatic activity of this protein using a Flashplate™ assay format.

2. Material and methods

2.1. Expression and purification of HCV full-length NS3 protein

A near full length HCV cDNA clone encompassing nucleotides 38-9416 was used as a template to amplify, by polymerase chain reaction (PCR), the full-length NS3 coding region (positions 3414–5303) using recombinant Taq DNA polymerase (Gibco-BRL, Burlington, ON, Canada) and the primer oligonucleotides 5′-GCTAGCGCTAGCCCCATCACGGCCTAC-3′ and 5′-CTCGAGCTCGAGAGTGACGACCTCCAG-3′. The PCR product was cleaved using *Nhe*I/*Xho*I and ligated into the prokaryotic expression vector pET-21b (Novagen, Madison, WI, USA). The difference between this recombinant protein and the native HCV NS3 protein consists of the presence of a methionine, alanine, and serine amino acid at the N-terminus in addition to a leucine, glutamic acid, and a six-histidine residues at the C-terminus of the expressed protein. Nucleotide sequence was verified prior to protein production. The recombinant *Escherichia coli* strain BL21(DE3) (Novagen) was used for protein expression. Cultures were induced at an optical density (600 nm) of 0.6, upon addition of isopropyl β-D-thiogalactopyranoside (IPTG, Sigma, St-Louis, MO, USA) at a concentration of 0.4 mM in Luria Bertani medium containing 100 μg ampicillin/ml. Protein expression was allowed to proceed for 2.5 h at 30°C. All subsequent steps were performed at 4°C. The pellet from a 1-l culture was resuspended in 30 ml of 20 mM sodium phosphate pH 7.5, 300 mM NaCl, 100 μg/ml lysozyme and Triton X-100 was added to 0.1% final concentration. Following a 30-min incubation on ice, the suspension was sonicated four times for 15 s with 15 s intervals (Branson sonifier 450, Branson Ultrasonics, Danbury, CT, USA) and then centrifuged at $15\,000 \times g$ for 20 min. Clarified homogenates were adjusted to 10 mM imidazole (Sigma), filtered through 0.45 μm membrane and loaded twice on a nickel affinity column (5 cc, Hi-Trap chelating, Amersham Pharmacia Biotech, Baie d'Urfée, QC, Canada). After washing the column with five column volumes of buffer S (20 mM sodium phosphate pH 7.4, 500 mM NaCl) containing 10 mM imidazole, NS3 was eluted with buffer S containing 300 mM imidazole. To avoid precipitation, and immediately after elution, the buffer in helicase-containing fractions was exchanged for 25 mM Tris–HCl pH 7.5, 0.05% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate), 20% Glycerol, 5 mM DTT(dithiothreitol) (buffer P) using pre-packed Sephadex G-25 columns (PD-10, Amersham Pharmacia Biotech). Further purification was achieved by loading the protein onto a poly U Sepharose column (Amersham Pharmacia Biotech) equilibrated in 20 mM Tris–HCl pH 7.4, 100 mM NaCl, 1 mM EDTA (TNE). To do this, recombinant NS3 in buffer P was diluted five times with TNE prior to loading at a ratio of about 1 mg of NS3 per ml of resin bed. After washing the column with ten column volumes of TNE, then with three column volumes of TNE containing 500 mM NaCl, NS3 was eluted as a pure protein, using three column volumes of 20 mM Tris–HCl pH 7.4, 1 M NaCl, 1 mM EDTA, 0.1% Triton X-100, 20% Glycerol, and 10 mM DTT. Immediately after elution, helicase-containing fractions were pooled and the buffer was exchanged for buffer P. Protein concentration was evaluated using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with

bovine serum albumin (BSA) as standard. Purified NS3 protein was aliquoted and stored at −80°C. This protein preparation was estimated to be greater than 90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining. The purification procedure yields approximately 1.5 mg of HCV NS3 per liter of *E. coli* cultures.

2.2. Helicase Flashplate™ assay

The DNA oligonucleotides 5′-biotin- GCTGACCCTGCTCCCAATCGTAATCTATAGTGTCACCTA (39-mer template strand) and 5′-CGATTGGGAGCAGGGTCAGC (20-mer release strand) were obtained from Gibco-BRL. The release strand was radiolabeled at the 5′-end. Briefly, the transfer of the terminal radiolabeled phosphate group of ATP (adenosine 5′-triphosphate) to the 5′-end of the 20-mer oligonuceotide was performed by incubating 30 pmol of oligonucleotides in a One-Phor-All Buffer PLUS (Amersham Pharmacia Biotech) supplemented with 50 mM dithiothreitol, 50 μCi [γ-^{35}S]ATP (10 mCi/ml, >1000 Ci/mmol, Amersham Pharmacia Biotech) and 9.5 units of T4 polynuceotide kinase (Amersham Pharmacia Biotech) for 45 min at 37°C. Thereafter, the reaction was stopped upon addition of EDTA and NH_4Ac at final concentrations of 1 mM and 3 M, respectively. Residual [γ-^{35}S]ATP was removed by applying the reaction mix to a microspin G-25 column (Amersham Pharmacia Biotech) followed by a centrifugation at $735 \times g$ for 2 min. The helicase substrate was prepared by annealing the template to the release strands. Both strands were mixed in a 1:1 molar ratio in 2 mM HEPES (*N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]), 0.05 M NaCl, 0.1 mM EDTA, and 0.01% SDS and subjected to a denaturation–renaturation process in which the oligonucleotides were heated at 100°C for 5 min., followed by a 30-min incubation at 65°C and then a slow renaturation step at 22°C for 4 h. The hybridized NS3 helicase substrate was kept at −20°C. A stock solution of neutravidin (Pierce, Rockford, IL, USA) was prepared at a final concentration of 1 mg/ml in phosphate buffered saline (PBS). The Flashplate™ (NEN Life Science Products Inc., Boston, MA, USA) was coated overnight at 4°C with a 100-μl/well of a 5-μg/ml neutravidin solution in a 0.5-M carbonate buffer pH 9.3. Wells were subsequently blocked upon addition of 100 μl of a 0.1% BSA solution followed by an incubation at 22°C for 2 h. Plates were then washed three times with 200 μl/well of PBS, air-dried at room temperature and stored at 4°C with desiccant. For standard assays, 75 μl of 1 M phosphate buffer pH 7.0, containing 1 M NaCl and 2.5 ng of the partially annealed DNA duplex was applied to each well, followed by an incubation at 22°C for 4 h. The wells were then washed twice with 200 μl PBS and once with 200 μl of 50 mM Tris pH 7.5, 50 mM NaCl solution, pre-warmed at 37°C.

Helicase reactions were initiated upon addition of 90 μl of a reaction mix consisting of 11 nM of purified full-length HCV NS3 protein, 25 mM 4-Morpholine-propanesulfonic acid (MOPS) pH 7.0, 5 mM ATP, 2 mM DTT, 3 mM $MnCl_2$, and 100 μg/ml of BSA to the wells in which 2.5 ng of DNA substrate was previously applied. For negative controls, the reaction mix contained no ATP. Moreover, in experiments where the effect of metal cations was investigated, either $MgCl_2$ or $MnCl_2$ was used as the metal co-factor. Reactions were allowed to proceed for 60 min at 37°C. Wells were then washed twice with 200 μl of a 150-mM NaCl solution and dried at room temperature for 15 min. Bound radioactivity was measured as counts per minute (CPM), using a liquid scintillation counter (1450-Microbeta, Wallac Oy, Turku, Finland). In this assay, the background counts correspond to wells devoid of DNA duplexed substrates. The percentage of unwinding catalyzed by the HCV NS3 protein was calculated according to the following formula:

$$\% \text{ Unwinding} = 100 \times [(a-c)-(b-c)]/(a-c)$$

where a is the CPM average of wells lacking ATP; b is the CPM average of reactions performed in the presence of ATP; and c is the CPM average of background sample (lacking ATP and DNA oligonucleotides).

2.3. Helicase gel-shift assay

The same set of oligonucleotides as the ones described for the Flashplate™ assay were used here, except that the short 20-mer oligonucleotide was 5′ end-labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech). Annealing was performed as described above, with the exception that both DNA strands were mixed in a 3:1 (template/release) molar ratio. Helicase reactions were allowed to proceed for 30 min at 37°C in a mixture containing 25 mM MOPS pH 7.0, 5 mM ATP, 2 mM DTT, 3 mM $MnCl_2$, 100 μg/ml of BSA, 1 nM duplex DNA substrate, and purified full length HCV NS3 protein at a final concentration of 270 nM. Reactions were stopped upon addition of 100 mM Tris–HCl pH 7.4, 2 mM EDTA, 0.05% SDS, 0.01% bromophenol blue, 0.01% xylene cyanol, and 5% glycerol final concentration. The reaction mixture was subjected to electrophoresis on a native 12% polyacrylamide–TBE gel at 90 V/cm for 60 min at room temperature. The gel was dried under vacuum and radiolabeled DNA was visualized by autoradiography.

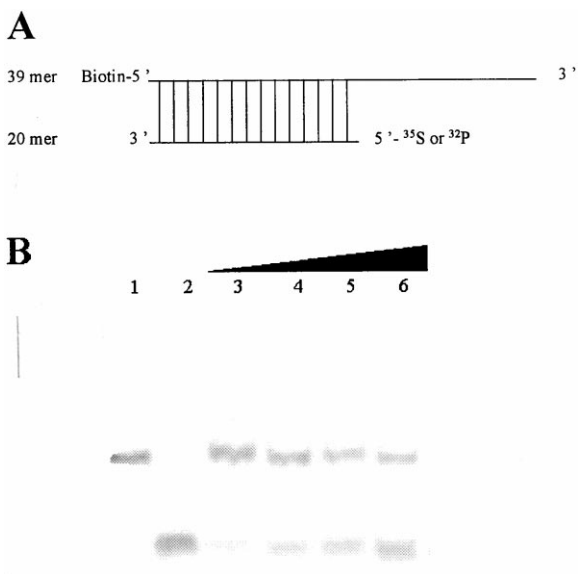

Fig. 1. (A). Structure of the duplex DNA substrate used in both gel the shift and Flashplate™ assays. (B). Time course analysis of HCV NS3 helicase activity using a gel-shift assay. Helicase reactions were carried out at 37°C in a reaction containing 1 nM DNA duplex substrate and 270 nM of HCV NS3 protein. Products were resolved on a native 12% polyacrylamide-TBE gel and detected by autoradiography. Lane 1, negative control corresponding to reactions lacking NS3 protein; lane 2, denatured duplex DNA substrate following heating at 100°C for 10 min. Helicase reactions were carried out for 5 (lane 3), 10 (lane 4), 15 (lane 5), or 30 min (lane 6).

3. Results

3.1. Gel shift assay and confirmation of helicase activity displayed by purified HCV NS3 protein

The coding region for the full-length NS3 protein of HCV genotype 1b (BK strain) was amplified by PCR and cloned in the prokaryotic expression plasmid pET-21b, under the control of the T7 RNA polymerase promoter. The resulting plasmid was used to transform competent BL21 (DE3) cells. Induction of transformed cells with IPTG resulted in a high level expression of HCV NS3. Subsequently, purified recombinant NS3 protein was judged to be greater than 90% pure as described in Section 2. This purified protein was used at a final concentration of 270 nM. The NS3 helicase activity was evaluated using 1 nM of a DNA duplex substrate consisting of a pair of oligonucleotides of 39 (unlabeled template strand) and 20 (5′-^{32}P-labeled release strand) nucleotides, respectively (Fig. 1(A)). Helicase activity displayed by the recombinant HCV NS3 causes unwinding of the DNA duplex and release of the labeled short strand. This unwinding event could be monitored based on the differential mobility of double-strand versus single strand DNA molecules on native polyacrylamide gels (Fig. 1(B)). Using this gel-mobility shift assay, we demonstrated that our recombinant HCV NS3 protein causes, in a time-dependent manner, unwinding of duplex DNA substrates (Fig. 1(B), lanes 3–6). No helicase activity was observed in a mock preparation using the identical purification conditions from *E. coli*, which did not express the HCV NS3 protein (data not shown), suggesting that the observed activity is not due to contaminating *E. coli* proteins. Similar results were obtained when duplex RNA oligonucleotides were used as substrate (data not shown). In either case, only a fraction of duplexed oligonucleotides was

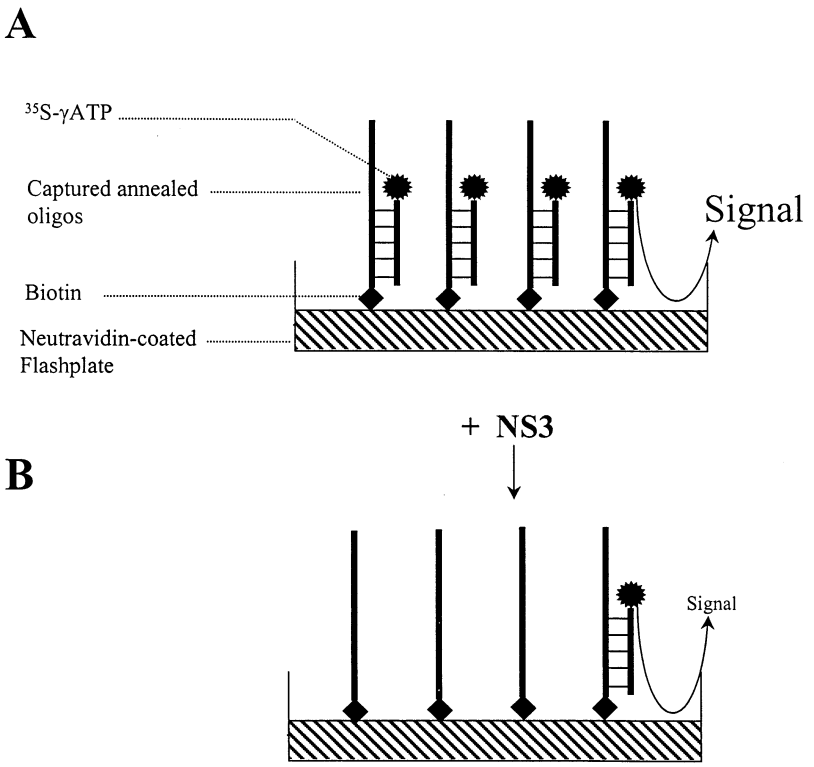

Fig. 2. Schematic diagram of the HCV NS3 Flashplate™ helicase assay. (A). A partially duplex DNA substrate is immobilized, on the surface of a neutravidin-coated well, via a biotin molecule attached to the 5′ end of the template DNA strand. (B). Upon addition of the purified HCV NS3 protein, unwinding of the duplex DNA substrate occurs, resulting in the release of the 5′-end radiolabeled short DNA strand which translates in a reduction of the recorded signal.

successfully unwound following NS3-mediated helicase reactions. The reason behind this low apparent unwinding is not clear; a higher enzyme concentration could be required to achieve complete unwinding.

3.2. Analysis of NS3 helicase activity on immobilized DNA duplex substrate

With the goal of performing high throughput screening (HTS) of large compound libraries for potential NS3 helicase inhibitors, a helicase assay adapted to a 96-well plate format was needed. We took advantage of the Flashplate™ technology (NEN) to develop a simple and practical assay, which addresses special criteria needed for HTS. Flashplates™ contain scintillant already incorporated in the plastic coat of each well, eliminating the need for addition of liquid scintillant prior to reading. The assay design is illustrated in Fig. 2. Briefly, we used a helicase substrate consisting of two oligonucleotides (20-mer and 39-mer) identical to the ones used in the gel-shift assay described above (Fig. 1(A)), with the exception that the template strand is biotinylated at its 5′ end, and the release strand is labeled with ^{35}S instead of ^{32}P. Following annealing, double stranded DNA molecules are captured on neutravidin-coated Flashplates™. Unbound material is washed off and wells are further washed twice with PBS then once with a 50 mM Tris–HCl solution (Fig. 2(A)). Preliminary experiments were conducted in order to determine the variation in counts recorded in each well after coating with the DNA duplex. Counts were found to be very consistent since the standard deviation was less than

10% (data not shown). Standard helicase reactions are initiated upon addition of the enzyme mixture described in Section 2. In the final assay format, helicase reactions were carried out at 37°C for 60 min in a reaction mixture containing 2.5 ng/well of DNA duplex substrate and 11 nM of HCV NS3 protein. Plates were then washed twice and the remaining bound ^{35}S-labeled oligonucleotides were quantified (Fig. 2(B)). In this assay, helicase activity results in a decrease of radioactive CPMs. This difference in CPM, with respect to the wells devoid of ATP, is used to determine the percentage of NS3-mediated unwinding. In preliminary experiments, we determined the effect of repeated washing on the total amount of DNA duplex coated in each well. To do this, the CPMs produced by the addition of 2.5 ng/well of duplex DNA was determined after which the wells were washed and the CPMs determined again in order to evaluate the residual amount of DNA substrate immobilized prior to enzyme addition. Overall, the amount remaining after the washing steps represented 90% of the original amount applied. In our assays, the term amount of DNA applied per well refers to the DNA substrate concentration.

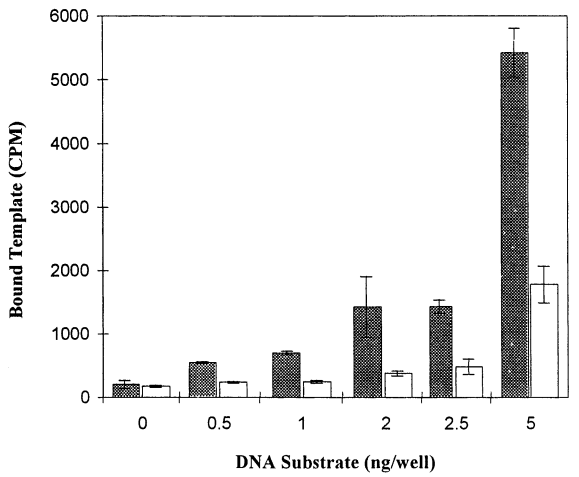

Fig. 3. Effect of the DNA substrate concentration on the helicase activity. HCV NS3 helicase reactions were performed at 37°C for 30 min in the absence (shaded bars) and in the presence (open bars) of 5 mM ATP, and various amounts (0–5 ng/well) of immobilized DNA substrate (*X*-axis). The final concentration of the enzyme used in the reaction mixture was 11 nM. Experiments were performed in triplicates and the measured CPM for each set of wells is shown (*Y*-axis). The data are presented as the mean ± standard deviation for each substrate concentrations.

3.3. Effect of DNA duplex concentration on HCV NS3 helicase activity

In order to optimize the signal-to-noise ratio of the assay, various amounts (0, 0.5, 1, 2, 2.5 and 5 ng) of duplex DNA substrate were immobilized per well. For each of these concentrations of duplex, helicase reactions were performed at 37°C, using 11 nM of HCV NS3, in the presence or absence of 5 mM ATP. Following a 30-min incubation, samples were processed as described above and counted (Fig. 3). As expected, the signal obtained was directly proportional to the concentration of DNA substrate used. At 5 ng of duplex DNA/well, the signal (helicase activity was determined as the percentage of unwinding, in the presence of ATP) -to-noise (background counts in the absence of DNA) ratio was over 20. The overall activity value however, was found to decrease slightly with increasing substrate concentrations. The values observed were 90, 91, 86, 77 and 70% unwinding for 0.5, 1, 2, 2.5, and 5 ng of substrate per well, respectively (data not shown). In subsequent experiments, 2.5 ng/well of duplex DNA was hence selected for our optimal assay conditions since at this concentration both the signal to noise ratio, and unwinding activity were in a robust range.

3.4. Characterization of the HCV NS3 helicase activity

Next, we sought to assess the degree of NS3-mediated unwinding as a function of incubation time. Helicase reactions were carried out at 37°C using 2.5 ng/well of duplex DNA substrate and 11 nM of purified HCV NS3 protein. Reactions were stopped at various times by removing the reaction mix and washing the wells three times with a 0.15-M NaCl solution, and percentage of unwinding was determined for each time point (Fig. 4(A)). Using these assay conditions, maximum unwinding was never found to be greater than

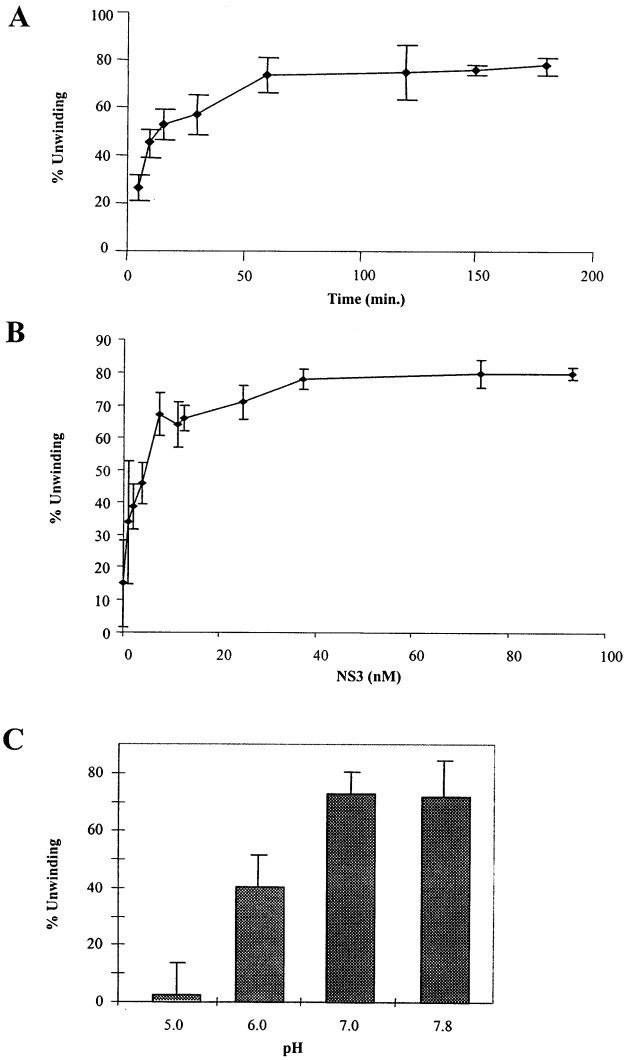

Fig. 4. Characterization of HCV NS3 helicase activity using the Flashplate™ assay. (A). Time course analysis of the NS3 helicase activity. Helicase reactions were performed at 37°C using 2.5 ng/well of substrate and HCV NS3 protein at a final concentration of 11 nM. Reactions were subsequently stopped at various time points (5, 10, 15, 30, 60, 120, 150 and 180 min). (B). Effect of enzyme concentration. Reactions were carried out at 37°C for 60 min using 2.5 ng/well of substrate in the presence of increasing concentrations (0, 1, 2, 4, 7, 11, 12, 25, 37, 74 and 93 nM) of purified HCV NS3 protein. (C). The effect of pH on the enzymatic activity was determined by performing helicase reactions at 37°C for 60 min at pH 5.0, 6.0, 7.0, or 7.8, in the presence of 2.5 ng/well of substrate and 11 nM of purified HCV NS3 protein. Experiments were performed in triplicates and data are presented as the mean of the percentage of unwinding ± standard deviation.

80% even after a 3-h incubation period. In fact, most (about 60%) of the observed unwinding activity occurred in the initial 30 min, after which the activity reached a plateau at approximately 73% unwinding (Fig. 4(A)). It is probable that this plateau reflects a molecular equilibrium between released and annealed labeled oligonucleotides.

The effect of enzyme concentration on the NS3 helicase activity was also investigated. Reactions containing increasing concentrations of recombinant NS3 and 2.5 ng/well of substrate were incubated for 1 h at 37°C. Wells were processed as described in Section 2 and counted. The percentage of unwinding was calculated for each protein concentration (Fig. 4(B)). For concentrations up to 5.5 nM of NS3 protein, a linear correlation exists between the enzyme concentration and the percentage of unwinding. NS3 concentrations beyond 7 nM do not result in a significant increase in measurable helicase activity. In fact, at these concentrations, percentage of unwinding reaches a plateau and remains unchanged.

With the goal to optimize the conditions of this helicase assay, the effect of pH of the reaction buffer was also investigated. Helicase reactions were performed using 2.5 ng/well of DNA substrate and 11 nM of HCV NS3 in MOPS-containing buffers (Section 2) with pH ranging from 5.0 to 7.8. The percentage of unwinding was calculated for each of these conditions (Fig. 4(C)). While no helicase activity was observed at pH 5, optimal unwinding was obtained at pHs between 7 and 7.8. Reactions with a pH above 7.8 were not performed. The effect of temperature was also investigated and it was found that 37°C represents the optimal temperature for this enzymatic activity (data not shown).

Based on these results, NS3 helicase assays in the Flashplate™ optimize reaction conditions called for with a 1-h incubation at 37°C, using 11 nM of enzyme in a MOPS-containing reaction buffer with a pH of 7.0.

3.5. Effects of cations on the NS3 helicase activity

The HCV NS3 protein, as seen with other helicases, contains an NTPase domain (Kadare

and Haenni, 1997). The NTP binding site is located at the periphery of the NTPase domain (Cho et al., 1998). Upon superimposing the NTPase domains of the HCV NS3 RNA helicase and the *Bacillus stearothermophilus* (Pcr A) DNA helicase, Asp223 and Glu224 of HCV NS3 have been proposed to coordinate the magnesium ion with ATP (Cho et al., 1998). The importance of this metal cation in the catalytic activity of the HCV RNA helicase was investigated by performing helicase reactions in the presence of various concentrations (0–2.5 mM) of either $MgCl_2$ or $MnCl_2$. Helicase activity was significantly increased with the addition of either $MnCl_2$ or $MgCl_2$ (Fig. 5(A)). $MnCl_2$ was slightly more efficient than $MgCl_2$ (Fig. 5(A)). Less than 8% unwinding was observed in the absence of $MgCl_2$ or $MnCl_2$ consistent with the requirement of divalent cations for unwinding activity (Fig. 5(A)). Similarly, the effect of varying the concentration of KCl was also evaluated. Helicase reactions containing increasing concentrations of KCl showed that potassium interferes, in a concentration-dependent manner, with the unwinding activity of the HCV NS3 protein (Fig. 5(B)). The dependence of the HCV NS3 helicase activity on metal co-factors is also demonstrated, in this assay, by the inhibitory effect of EDTA on this enzymatic reaction. Helicase reactions were performed in the presence of Mn^{2+} and increasing concentrations of EDTA. At concentrations beyond 1 mM, EDTA interferes with the helicase activity of the NS3 protein while at 5 mM, more than 80% of this activity is inhibited (Fig. 5(C)). In conclusion, these results indicate that either Mg^{2+} or Mn^{2+}, with an optimal concentration of 1 mM, could be used as metal cofactors in this helicase assay.

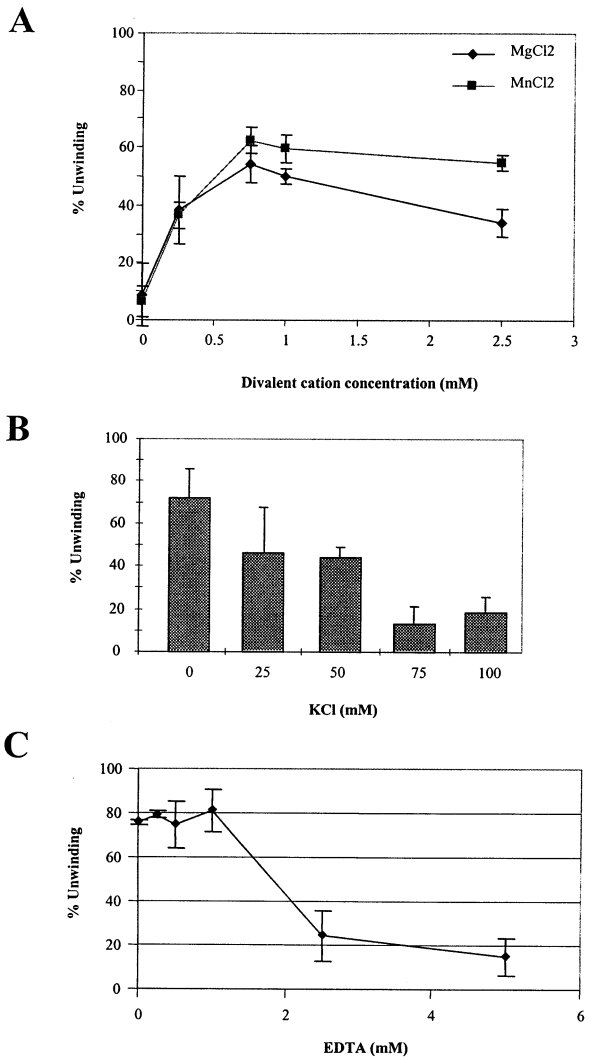

Fig. 5. Effect of cation concentration on the HCV NS3 helicase activity. (A). Effect of $MnCl_2$ and $MgCl_2$. (B). Effect of KCl. (C). Effect of EDTA. Helicase reactions were performed at 37°C for 60 min using 2.5 ng/well of DNA substrate in the presence of HCV NS3 protein at a final concentration of 11 nM. The percentage of unwinding was determined for each concentration. Each condition was tested in triplicates and data is represented as the mean ± standard deviation.

3.6. HCV NS3 helicase high throughput screening assay format

With the goal to perform high throughput screening of a large library of compounds for potential NS3 helicase inhibitors, we sought to determine the reproducibility of the Flashplate™ assay described above. NS3 helicase reactions using 2.5 ng of duplex DNA substrate applied per well and 11 nM HCV NS3 were set up in 66 different wells on the same plate. In addition, six wells containing reaction mixtures lacking ATP were used as negative controls, and three additional wells containing no duplexed DNA were

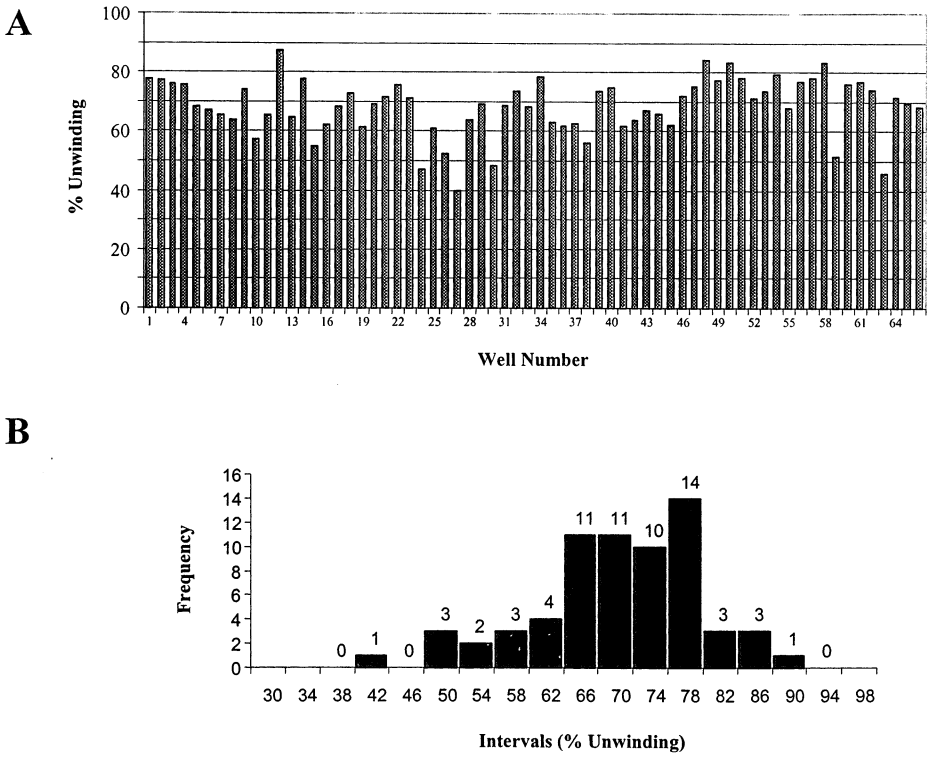

Fig. 6. Signal variation in the HCV NS3 helicase Flashplate™ assay. NS3-mediated helicase reactions were performed at 37°C for 60 min using 2.5 ng/well of DNA substrate in the presence of HCV NS3 protein at a final concentration of 11 nM in 68 different wells on the same neutravidin-coated plate. (A). The percentage unwinding for each sample was determined as described in Section 2, using, as negative controls, signals measured from six different reactions which were performed in the absence of ATP. In addition, three wells where no DNA substrate was applied, were used to calculate the background. (B). Different intervals of four percentage units (34 = 31–34%, 38 = 35–38% and so forth), covering the entire set of results obtained in (A) were determined (*X*-axis). The frequency of samples with a percentage of unwinding falling into one of these intervals (*Y*-axis) was determined using Excel (Microsoft), and is shown on the top of the corresponding bar.

considered as background controls. Following a 1-h incubation at 37°C, reactions were stopped and the percentage of unwinding was calculated for each of the 66 wells (Fig. 6(A)). The average percentage of unwinding in this experiment was determined to be 68.5%, with a standard deviation of 9.6%. With an average of 68.5% unwinding, no wells would have scored as false positives if 50% inhibition of enzyme cut off for screening was used. The frequency of wells with various percentages of unwinding was also calculated using Excel (Microsoft). Values were grouped by intervals with 4% unwinding increments (Fig. 6(B)). In a large proportion of wells (46), the percentage of unwinding ranged between 66 and 78%. The other wells were evenly distributed on either side of this window. These results illustrate the good reproducibility of this assay, which constitutes a prerequisite for high throughput screening.

4. Discussion

Viral helicases are potential molecular targets for new antiviral therapies. Detection of helicase activity can be achieved using a number of different methods, one of which is a standard gel-shift assay. In this assay, double stranded DNA or RNA duplexes are distinguished from single strand molecules based on their relative mobility

on native polyacrylamide gels (Gallinari et al., 1998). However, this method is time-consuming and is not practical when a large number of samples have to be processed simultaneously. Therefore, novel assays are needed to allow high throughput screening of large libraries of potential inhibitors. Such assays must be reliable, fast, and amenable to automation. Recently, a number of higher-throughput helicase assays have been reported in the literature. The most noteworthy were a scintillation proximity assay (Kyono et al., 1998), an ELISA-based assay (Hsu et al., 1998), a fluorescence-based assay (Raney et al., 1994) and a filtration assay (Sivaraja et al., 1998).

In this manuscript, we report the development of a novel and practical HCV NS3 helicase assay suitable for high throughput screening. This assay takes advantage of the convenient Flashplate™ technology. Flashplates™ are 96-well microtiter plates with scintillant already present within each well, eliminating the need for its addition prior to reading. The assay design is based on the fact that the release strand, a 20-mer oligonucleotide, is radiolabeled on its 5′-end, while the template strand, a 39–mer, is immobilized on the plate surface via its 5′-end biotin molecule. Unwinding of the duplex DNA substrate, following the unwinding reaction, releases the radiolabeled oligonucleotide resulting in reduction of signal. A percentage of unwinding is easily determined upon a comparative analysis of signals obtained in the absence (negative control) or presence of ATP.

The major advantage of this assay is the small number of steps required for its completion. Neutravidin-coated plates could be prepared in batch and are stable at 4°C for at least a week (data not shown). Immobilization of DNA duplex substrates is usually performed over a 4-h period, but could also be done overnight at 4°C. With these steps accomplished, the remainder of the assay consists of addition of the reaction mixture, in the presence or absence of compounds, followed by a 1-h incubation, washing and counting. We found this assay less cumbersome than the previously reported ELISA type assay for helicase activity (Hsu et al., 1998). In addition, the assay as described uses standard laboratory equipment with the exception of a 96-well scintillation counter. This method, thus eliminates the need for filtration devices. For example, to reduce the cost of the assay, we decided to optimize the neutravidin coating step on the Flashplate™ instead of acquiring commercially available precoated plates. However, the drawback of the assay described here, is the fact that it requires handling of radioactive material. However, since labeled oligonucleotides are used in minute amounts, we consider this to be a minor disadvantage. Another HCV RNA helicase high-throughput assay using a 96-well FlashplatePLUS system has recently been reported by Kwong and Risano, 1999. The assay utilizes a ^{33}P-labeled double-stranded RNA instead of a DNA substrate for the enzyme. The helicase reaction is initiated by adding the enzyme to the wells precoated with a capture biotinylated DNA oligomer. Once the unwinding reaction is terminated, the displaced radiolabeled single-stranded RNA product is annealed to the capture oligonucleotide, which essentially results in an increase of the signal. The final step consists of washing the wells in order to remove the unbound substrate. Plates are counted in a TopCount scintillation counter. The major difference between the two assays then resides in the fact that in the assay described in this report, there is no need of a capture oligonucleotide since the partially annealed DNA duplex substrate is immobilized to the wells prior the enzyme addition.

The biochemical characteristics of the HCV NS3 helicase activity reported in this paper are similar to those previously published (Morgenstern et al., 1997). Thorough characterization of this enzyme was instrumental in optimizing the Flashplate™ assay conditions reported here. In our system, up to 85% unwinding is achieved following a 1-h incubation. It is very likely that this calculated percentage corresponds to an apparent helicase activity, reflecting an equilibrium between unwinding and reannealing of oligonucleotides. For this particular reason, we chose to wash the wells at the end of the reaction instead of quenching the reaction with EDTA.

Well to well variation is an important criteria to be considered when performing high throughput screening. Using the assay described above, a

relatively low standard deviation was observed when a large number of wells (66) were processed simultaneously.

In summary, the NS3 helicase Flashplate™ assay described here is a convenient method for performing high throughput screening of large compound libraries with the goal of identifying potential anti-HCV chemical leads.

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