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Expression, Purification, and Partial Characterization of HCV RNA Polymerase

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The product of the NS5B gene of Hepatitis C Virus (HCV) has been expressed in Escherichia Coli both as a fusion protein with glutathione-S-transferase (GST) of molecular weight 91KDa and at high level as a single protein of molecular weight 65KDa. The protein was sequestered within inclusion bodies and a variety of procedures designed to minimize inclusion body formation proved unsuccessful. The method finally adopted involved the purification of inclusion bodies followed by the solubilization, purification, and refolding of the expressed protein. A good recovery and protein purity of the order of 80-90% were achieved. The purified protein was shown to possess RNA polymerase activity in an assay using polyA/oligoU as template. The enzymatic activity is rifampicin resistant, poly A dependent, and requires Mg^{++}. The availability of purified HCV RNA polymerase will allow the study of viral replication and constitute the basis for testing new anti-viral drugs.

Hepatitis C Virus (HCV) is a human pathogen associated with the majority of cases of post-transfusion hepatitis and with a large proportion of sporadic forms (Kuo *et al.* 1989) It is a single stranded positive RNA virus distantly related to pestiviruses and flaviviruses. The genome is about 9.500 nucleotides long and contains an Open Reading Frame which extends almost the its whole length and encodes for a 3,011 aminoacid polyprotein (Kato *et al.* 1990). The ORF is flanked at both ends by 5′ and 3′ non-coding regions, the first 341 nucleotides long and well conserved amongst isolates and the 3′ end of variable length and containing a homopolymeric region which is almost invariably poly U followed by a well conserved 98 nucleotide terminal sequence (Tanaka et al. 1995). The ORF contains 10 genes of which those coding for structural proteins are located at the 5′ end, core, envelope 1 and envelope 2, followed by the non-structural genes, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Grakoui *et al.* 1993 *a*). Processing of the polyprotein has been analysed after expression of different genetically engineered recombinant vectors, both procaryotic and eucaryotic, and after *in vitro* translation of synthetic RNA. Structural genes are processed by host cell proteases and non-structural proteins by two virus proteases encoded by the NS3 and NS2 genes. The NS3 enzyme cleaves the NS3/NS4A junction in *cis* and other downstream junctions in *trans* and the NS4A product appears to be required for NS3 processing, although less markedly at the NS5A/NS5B boundary (Grakoui *et al.* 1993*b*; Tomei *et al.* 1993). The NS2 protease on the other hand is responsible for processing at the NS2/NS3 boundary (Hijikata *et al.* 1993).

In addition to the NS3 protease/helicase activity a virus-encoded polymerase of about 65kDa has been predicted to map to the NS5B gene (Grakoui *et al.* 1993 *a*), based on the presence of the GDD motif (aa 2737-2739) which has been recognized as the hallmark of RNA polymerases of RNA viruses. In view of the present inability to propagate the virus in cultured cells further understanding of the replication of the viral genome is mainly dependent on *in vitro* studies using recombinant viral polymerase.

In this report we describe the expression the NS5B gene in a variety of procaryotic expression vectors, purification of the protein and preliminary characterization of the enzymatic activity.

MATERIALS AND METHODS

Plasmids and cells. pUCUKNS5B was derived from a isolate previously reported (Kumar *et al.* 1994). pET 12a and 16b were obtained from *Novagen,* pGEX-3X from *Pharmacia* and *E.Coli* cells were BL21(DE3) and JM101 respectively.

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PCR and cloning of NS5B. NS5B (ncl.7261-9036) was amplified by PCR from pUC18UKHCVNS5B using specific primers P1(*antisense* 5′ CATATGTGGATCCCCACCATGGCAATGTCTTATTCC 3′) and P2 (*sense* 5′ GACTCTAGAGGATCCTCATCGGTTGGGGAGGAGGTAGATGC 3′) over 40 cycles with steps of 95°C, 1 minute; 52°C 3 minutes; and 72°C, 5 minutes. The PCR product was digested with *BamHI* and cloned into the corresponding site in pGX-3X. For cloning into pET-16b both vector and PCR product were digested with *BamHI,* filled in with Klenow and blunt-end ligated.

Sequencing. Sequencing by the dideoxy method was carried out using T7 Sequencing Kit (Pharmacia) and ^{35}S dATP (ICN) either from specific primers within the cloned insert or the vector's flanking regions as indicated. Sequence comparisons were carried out using the Hitachi DNASIS software package.

E.Coli protein expression and lysate preparation. E.Coli cells were grown and induced by addition of IPTG. Temperature, concentration of IPTG and duration of induction were varied as indicated.

After induction cells were collected by centrifugation (6,000 rpm, 10 minutes) and re-suspended in 50mM Tris-HCl, pH:8.0. Sonication was carried out in ice using 20 second bursts with 20 second intervening pauses. The sonicate was cleared by centrifugation at 15,000 rpm at 4° for 20 minutes and both supernatant and pellet saved. Whole cells, and both soluble and insoluble sonnicate fractions were denatured in PAGE sample buffer according to the conventional protocol (Sambrook *et al.* 1989)

PAGE and Western blot analysis. Protein separation was carried out by electrophoresis in 10% acrylamide gels and at 20 volts/cm. Staining with Coomassie Brilliant Blue and de-staining were according to standard protocols (Sambrook *et al.* 1989). Western blot analysis was performed after overnight electrophoretic protein transfer to nitrocellulose non-specific blocking with 5% powdered milk for 4 hrs and overnight incubation with specific antiserum. Bands were finally developed with Protein A gold followed by silver enhancement (*Biorad*). Antibodies against NS5B were generous gifts from Drs. La Monica and Charles Rice.

Purification of inclusion bodies and protein solubilization. Inclusion body purification, solubilization and re-naturing protocols were carried out at 4°C and will be detailed under Results.

RNA polymerase assay. RNA polymerase assay was carried out in a 50ul reaction mixture of 50mM Hepes buffer pH:7.7 containing 4mM DTT, 6 mM magnesium acetate, 6 uM zinc chloride, either 1 ug poly A and 2.5 ug oligoU or natural template and 10uCi of 3H UTP (sp.act.37Ci/mM). Incubations were carried out at 37°C for 60 minutes and the reaction terminated by the addition of 1.0 ml of cold 10% TCA (containing 200mM sodium pyrophosphate). After 10 minutes in ice the solution was filtered through a GM/C *Whatman* cellulose filter previously soaked in 10% TCA containing 200mM sodium pyrophosphate, washed with 10 mls of cold 5% TCA under vacuum filtration, dipped in absolute ethanol and dried under a heat lamp before counting in a liquid scintillation counter. The reactions were started by the addition of different amounts of the enzyme preparation and in the presence of rifampicin (20 ug/ml) to exclude endogenous bacterial RNA polymerase activity.

E.Coli RNA polymerase (Pharmacia) was used as a control both in the absence and in the presence of rifampicin.

The inhibitor ribavirin (kindly supplied by ICN) was used as detailed under Results.

RESULTS

Expression of pGEX-3XHCVPOL. A distinct band was detected with the expected molecular weight of 91kDa (65KDa of HCV polymerase + 26kDa of GST) as shown in Fig.1 and the time course revealed an increased acumulation of the fusion protein reaching a maximum 4.5 hours after induction (not shown).The expressed protein was insoluble as shown after sonication and and centrifugation (FIg.1,lanes PGEX-3X *s* and *is*). The finding of expressed protein within inclusion bodies led us to to try and reduce inclusion body formation. Reduced concentration of IPTG, shorter expression period, lowering expression temperature of to 25°C and increased aeration of the cultures were tried but had no effect.

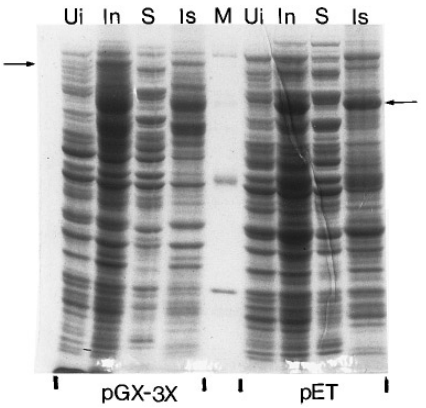


FIG. 1. HCV expressed in pGEX-3X and pET16B. Ui, uninduced; In, induced; S, soluble; Is, insoluble. M, size markers. Arrows indicate positions of expressed proteins, 91kD fusion protein (left) and 65kD protein (right).

One-step purification of the expressed protein solubilized in 2.5M urea pH 10.0 using Glutathione-Sepharose column was also tried but was unsuccessful although the cloning vector showed good expression of GST with efficient chromatographic purification (not shown).

Expression and purification of pET16bHCVPOL. Recloning of HCVPOL gene in pET16b was followed by expression in BL21(DE3) E.Coli cells. Compared to pGEX-3X a high level of expression was achieved in this system (Fig.1, lane pET In) as shown by the very strong band of the predicted size (65KDa), although the protein was again associated with the insoluble fraction (Fig.1, lanes pET *s* and *In*). Changing expression conditions in order to reduce inclusion body formation proved unsuccessful (Figure 2) as did cloning and expression in pET12a for periplasmic expression (not shown). An alternative strategy was then adopted which consisted in the purification of the inclusion bodies followed by solubilization, purification and ultimately refolding of the denatured recombinant NS5B protein.

Purification of the inclusion bodies was carried out by using washing conditions of increasing stringency as shown in Figure 2 and 1% Triton, 5 M urea was

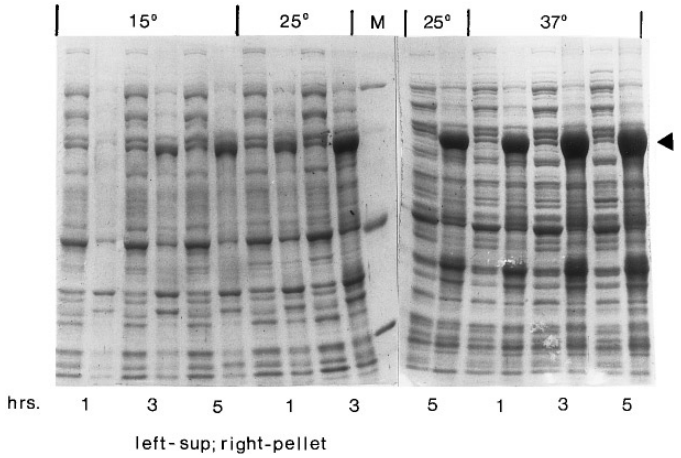


FIG. 2. Effect of temperature and induction time on solubility of HCV NS5B protein. For each time period left lane is soluble fraction and right lane is insoluble (pellet).

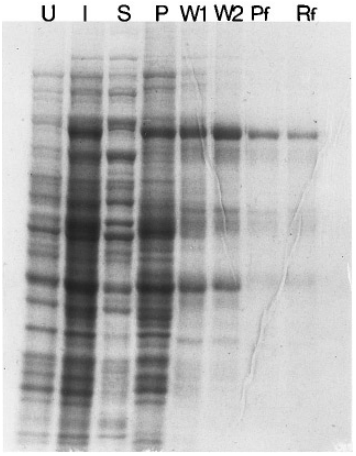


FIG. 4. Sequential steps in HCV NS5B purification. U, uninduced pET16HCVPOL; I, induced pET16HCVPOL; S, soluble fraction; P, pellet; W1, first wash; W2, second wash; Pf, protein purified on zinc column; Rf, protein refolded.

selected (Fig.3, lane l) as providing the best yield of almost pure HCVPOL.

Different protocols were tested for solubilizing the protein from the inclusion bodies and subsequent refolding to neutral pH and detergent-free buffer. Solubilization protocols included extremes of pH, and the alternative use of urea, SDS, deoxycholate, guanidinium isothiocyanate, non-ionic detergents or reducing agents according to published protocols (Marston 1985; Rudolph and Lilie 1996). All procedures, with the exception of the one outlined below, proved unsuccessful in solubilizing the protein (pI:8.3) which

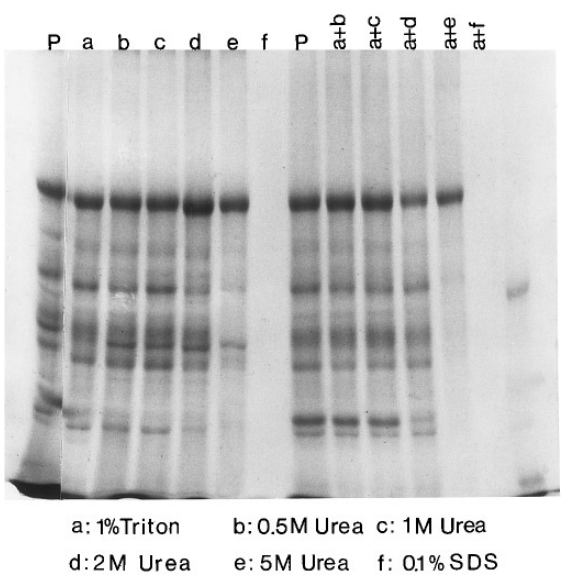


FIG. 3. Purification of E.Coli inclusion bodies containing HCV NS5B protein. P, unwashed pellet; a, 1% Triton; b, 0.5M urea; c, 1M urea; d, 2M urea; e, 5M urea; f, 0.1% SDS. Other lanes (a+b, a+c, etc.) are washes in 1% Triton plus the corresponding other reagent.

invariably formed aggregates during the process of renaturation. The protein was fully dissolved in 8M urea, pH: 10 in the presence of either 100 or 500 mM NaCl but only in high salt (500mM NaCl) at pH:8.0 and similarly when dissolved in 6M urea (not shown). Once the protein was dissolved in 6M urea and 500mM NaCl this enabled us to purify it further using a nickel resin matrix which selectively binds the oligo-histidine domain of the recombinant protein. The final stage, that of protein re-folding, was achieved by step-wise dialysing out the urea to 4M, 2M and urea-free buffer at pH:10.0 and ultimately against pH:8.0 buffer. All four purification stages, i.e. inclusion body purification, protein solubilization, nickel matrix one step purification and finally protein refolding into urea-free pH:8.0 buffer are shown in Fig. 4. Immunoblot was also carried out and showed a clear band with a MW of 68kD (not shown).

Enzymatic activity of NS5B protein. The enzymatic activity of the purified protein was evaluated in the polymerase assay. Table I shows that the purified protein contains a rifampicin resistant poly U polymerizing activity compatible with viral RNA polymerase. This incorporation of ^{3}H UTP is dependent on the presence of polyA (Table II) and is inhibited by 100 uM ribavirin (Table III). The reaction was characterized further by carrying out a time course and by investigating template the effects of temperature and divalent cation concentration (Table III, A,B, and C). The results show a maximum incorporation at 30-45 minutes, optimum Mg $^{++}$ concentration of 12 mM and an optimum reaction temperature of 30°C.

Use of HCV single minus strand and double stranded templates corresponding to the 5′UTR+CORE in unprimed reactions did not produce significant incorporation (not shown)

TABLE I

Characterization of Poly U Polymerase Activity

Enzyme	Template/primer	^{3}H-UTP Incorporation (cpm 10^{-3})
E. Coli RNA polymerase	Poly(A)/Oligo(U)	45.6
	Poly(A)/Oligo(U) + Rifampicin	0.27
HCV NS5B protein	Poly(A)/Oligo(U)	3.29
	Poly(A)/Oligo(U) + Rifampicin	3.18
	Oligo(U) + Rifampicin	0.84

DISCUSSION

In this report we describe the cloning and expression of the product of the NS5B gene in procaryotic cells. The protein is 65KD in molecular weight and possesses RNA polymerase activity.

The gene was first cloned as a fusion protein with GST and a protein of the predicted size of 91kD was expressed albeit at low levels and in an insoluble form associated with inclusion bodies. Several procedures designed to reduce the formation of inclusion bodies and increase the yield of soluble protein were unsuccessful as was further purification using affinity chromatography under denaturing conditions in contrast to the recently reported successful use of a similar strategy and vector for the expression of the Dengue virus 1 RNA polymerase (Tan *et al.* 1996).

We next cloned HCV NS5B into the vector pET 16B where high level expression of the 65kDa protein was achieved. Again the protein was sequestered within inclusion bodies and attempts to reduce inclusion body formation and increase the yield of soluble protein proved unsuccessful. An alternative strategy was adopted which involved the isolation and purification of inclusion bodies followed by protein solubilization, purification by affinity chromatography on a nickel matrix and finally protein re-folding by buffer exchange. Such a protocol produced protein which was 80-90% pure on a stained PAGE.

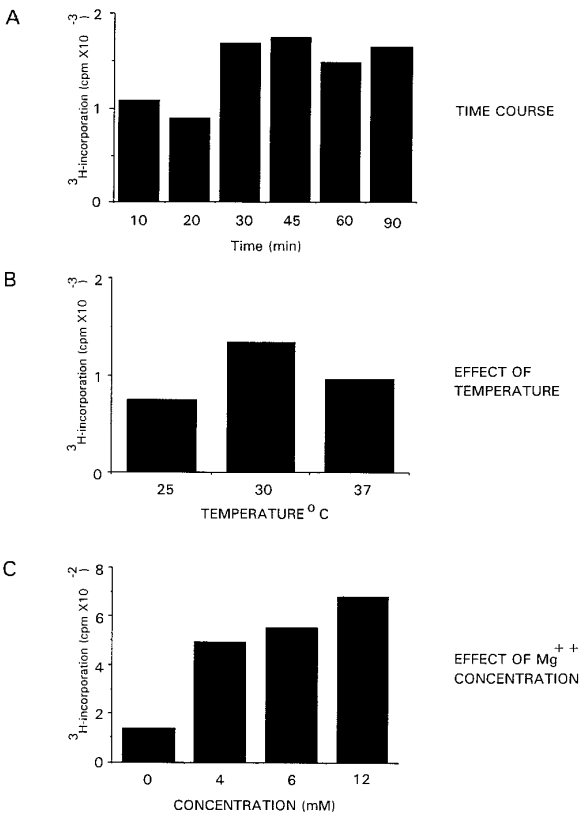
Preliminary characterisation of the purified protein demonstrated RNA polymerase activity by the use of an assay based on the incorporation of ^{3}H UTP using a synthetic polyA/oligoU template. The activity was rifampicin resistant, and therefore not associated with bacterial RNA polymerase contamination, template dependent and required the presence of magnesium. The significantly higher incorporation seen in the presence of polyA/oligoU when compared to oligoU alone demonstrates that the enzyme is not catalyzing the transfer of U residues to the oligoU 3′terminus independently of template, an activity which has been described in a recent study using enzyme expressed in insect cells (Behrens *et al.* 1996). Further analysis is required to demonstrate the processive activity of the polymerisation reaction directed by natural RNA templates. Also as part of this study we studied the effect of ribavirin

TABLE II

Effect of Ribavirin on Poly U Polymerase Activity

Ribavirin (μM)	^{3}H UTP Incorporation (cpm)
100	1380
10	2209
1	2057
0	2671

TABLE III



on the purified RNA polymerase. The observed 50% inhibition of polymerase activity with 100uM ribavirin is in our view unrelated to its observed pharmacological effect since the drug concentration exceeds by more than 30 times the therapeutic blood level.

Low levels of polymerase activity, as seen in this study, have also been reported for RNA polymerases from other flaviviruses when assayed in vitro (Grun and Brinton, 1986; Chu and Wesaway 1987). This finding may either reflect the low stability of the NS5B protein, which has been previously reported (Lin et al. 1994) or the requirement for additional factors, either cellular, viral or both.

The availability of purified HCV polymerase described in this study and information on the complete 3′ Non-coding region of the HCV genome which has recently become available (Tanaka et al. 1995) will now permit the use of the natural template in the *in vitro* polymerase assay in order to elucidate further this essential stage in the virus life cycle and to provide a target for the development of new antiviral drugs.

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REFERENCES

Behrens, S-E., Tomei, L., and De Francesco, R. (1996) *EMBO J.* **15,** 12–22.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., and Rice, C. (1993*a*) *J. Virol.* **67,** 1385–1395.

Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. (1993*b*) *J. Virol.* **67,** 2832–2843.

Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., Kato, N., Tanaka, T., Kimura, K., and Shimotohno, K. (1993) *J. Virol.* **67,** 4665–4675.

Kato, N., Hijikata, M., Outsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., and Shimotohno, K. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9524–9528.

Kumar, U., Thomas, H. C., and Monjardino, J. (1994) Genomic analysis of HCV RNA from a case of chronic sporadic hepatitis. II International Conference on HCV and Related Viruses, San Diego, p. 140.

Kuo, G., Choo, Q-L., Alter, H. J., Gitnick, A., Redeker, A. G., Purcell, R., Miyamura, T., Dienstag, J. L., Alter, M. J., Stevens, C. E., Tegtmeier, G. E., Bonino, F., Colombo, M., Lee, W-S., Kuo, C., Berger, K., Shuster, J. R., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) *Science* **244,** 362–364.

Lin, C., Pagai, B. M., Grakoui, A., *et al.* (1994) *J. Virol.* **68,** 8147–8157.

Marston, F. A. O. (1985) The Purification of Eucaryotic Polypeptides Expressed in *Escherichia Coli* in DNA Cloning (Glover, D., Ed.), pp. 59–88, IRL Press, Oxford.

Rudolph, R., and Lilie, H. (1996) *FASEB J.* **10,** 49–56.

Tan, B-H., Fu, J., Sugrue, R. J., Yap, E-H., Chan, Y-C., and Tan, Y. H. (1996) *Virology* **216,** 317–325.

Tanaka, T., Kato, N., Cho, M-J., and Shimotohno, K. (1995) *Biochem. Biophys. Res. Commun.* **215,** 744–749.

Tomei, L., Failla, C., Santolini, E., De Francesco, R., and La Monica, N. (1993) *J. Virol.* **67,** 4017–4026.