Journal of General Virology (2001), **82**, 735–745. Printed in Great Britain

A small region of the dengue virus-encoded RNA-dependent RNA polymerase, NS5, confers interaction with both the nuclear transport receptor importin-β and the viral helicase, NS3

Magnus Johansson,[1] Andrew J. Brooks,[1] David A. Jans[2] and Subhash G. Vasudevan[1]

[1] Department of Biochemistry and Molecular Biology, James Cook University, Townsville, Queensland 4811, Australia

[2] Nuclear Signalling Laboratory, Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra, Australia

The dengue virus RNA-dependent RNA polymerase, NS5, and the protease/helicase, NS3, are multidomain proteins that have been shown to interact both *in vivo* and *in vitro*. A hyperphosphorylated form of NS5 that does not interact with NS3 has been detected in the nuclei of virus-infected cells, presumably as the result of the action of a functional nuclear localization sequence within the interdomain region of NS5 (residues 369–405). In this study, it is shown by using the yeast two-hybrid system that the C-terminal region of NS3 (residues 303–618) interacts with the N-terminal region of NS5 (residues 320–368). Further, it is shown that this same region of NS5 is also recognized by the cellular nuclear import receptor importin-β. The interaction between NS5 and importin-β and competition by NS3 with the latter for the same binding site on NS5 were confirmed by pull-down assays. The direct interaction of importin-β with NS5 has implications for the mechanism by which this normally cytoplasmic protein may be targetted to the nucleus.

Introduction

Dengue fever is a mosquito-borne flavivirus disease that affects approximately 50 million people annually in tropical and subtropical areas (Barrett, 1997). The family *Flaviviridae* contains small, enveloped, positive-sense single-stranded RNA viruses and in addition to the dengue viruses has members associated with human diseases such as yellow fever (YF) and Japanese encephalitis (JE) and also hepatitis C virus (HCV). There are four serologically and phylogenetically distinguishable dengue viruses (Barrett, 1997). Dengue virus infections with a single subtype produce a classical febrile illness, whereas consecutive infections by different serotypes can lead to the more serious illness called dengue haemorrhagic fever, of which there are about 1 million cases each year with a mortality rate of around 5% (Barrett, 1997; Gubler & Clark, 1995). There is no cure for dengue fever, although a multivalent vaccine against all four serotypes is in development (Barrett, 1997).

Author for correspondence: Subhash G. Vasudevan.
Fax +61 7 47251394. e-mail Subhash.Vasudevan@jcu.edu.au

The ~ 11 kb single-stranded dengue virus RNA genome is capped but not polyadenylylated and encodes a single polyprotein including three structural and at least seven nonstructural (NS) proteins in the order C–prM–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5. The maturation of the polyprotein is achieved by the viral NS2B–NS3 proteinase complex as well as host signal peptidases (Chambers *et al.*, 1990). Replication of dengue virus occurs at the membrane-associated replicase complex (RC). RC has been characterized extensively in other flaviviruses (e.g. Kunjin and YF) and includes NS1, NS2A, NS3, NS4A and NS5 (Mackenzie *et al.*, 1998; Westaway *et al.*, 1997*a*).

The NS3 protein (69 kDa) has protease activity localized within 167 N-terminal residues (Li *et al.*, 1999). The immediate C-terminal region of the NS3 protease domain contains conserved motifs found in nucleoside triphosphate (NTP)-binding proteins and the DEXH family of RNA helicases. It was demonstrated recently that amino acid residues 160–187 of NS3 are essential for its NTPase activity (Li *et al.*, 1999).

The NS5 protein (104 kDa) is predicted to contain at least two distinct domains: the N-terminal region is predicted to be an *S*-adenosyl-methionine (SAM) transferase domain, on the

0001-7388

basis of similarity to several groups of methyltransferases from a wide variety of species (Koonin, 1993), whilst the C-terminal domain of NS5, from residue 455 onwards, contains eight highly conserved sequence motifs that have been recognized in many RNA-dependent RNA polymerases (RdRps; POL domain) (Koonin, 1991). RdRp activity has been demonstrated for *Escherichia coli*-expressed dengue virus 1 NS5 protein (Tan *et al*., 1996).

In dengue virus-infected cells, three RNA species have been observed: (i) the RNase-sensitive single-stranded genomic RNA, (ii) the double-stranded RNA replicative form (RF) and (iii) the partially RNase-sensitive replicative intermediate (RI) (Cleaves *et al*., 1981). The polymerase activity of NS5 probably requires the helicase and the NTPase activity of NS3 to replicate the genome from RF at RC. Also, the capping of the newly synthesized genome may require the combination of NS3 and NS5 activities, since capping is dependent on 5′-RNA triphosphatase activity (previously detected for NS3 from West Nile virus) (Wengler & Wengler, 1993) as well as the putative SAM transferase activity of NS5. This is concordant with the demonstrated cytoplasmic form of NS5 interacting with NS3 both *in vivo* as well as *in vitro* (Kapoor *et al*., 1995). However, the detailed molecular nature and function of this interaction in virus RNA replication has not been determined.

Although all suggested functions of NS5 are generally thought to occur in the cytosol, a hyperphosphorylated form of NS5 has been located in the cell nucleus (Kapoor *et al*., 1995). The NS5 of YF virus can also be detected in the nucleus (Buckley *et al*., 1992) and recombinant NS5/NS5A of members of all three genera of the *Flaviviridae* have been shown to be phosphorylated by serine/threonine kinases (Morozova *et al*., 1997). Other flavivirus proteins detected in the nucleus include the C (capsid) protein of dengue virus 2 (Bulich & Aaskov, 1992) and the C and NS4B proteins of Kunjin virus (Westaway *et al*., 1997*b*). However, hyperphosphorylation and nuclear localization of NS5 have not been demonstrated for other species of the genus *Flavivirus*. Proteins larger than 45 kDa in size generally require specific targetting signals called nuclear localization sequences (NLSs) in order to enter the nucleus (Jans, 1995; Jans *et al*., 1991) by using an intricate nuclear import machinery that involves recognition by the cellular NLS receptor, the importin-α/importin-β heterodimer, and other cellular factors, including the guanine nucleotide-binding protein Ran (Jans *et al*., 1998). NLSs generally have no specific consensus sequence, consisting of hydrophilic residues that can be located at any position within the polypeptide that carries them as long as the NLS is accessible in the overall protein. The NLS of simian virus 40 (SV40) large tumour antigen (T-ag) is a typical example that consists of a single cluster of positively charged amino acids (PKKKRKV) (Jans *et al*., 1998). A second type of NLS, called a bipartite NLS, has been identified in various nuclear-targetted proteins such as the steroid hormone receptors for glucocorticoid and progesterone. These consist of two clusters of positively charged residues that are separated by a 10–12 amino acid residue spacer (Jans *et al*., 1998). Importin-α binds the NLS region of nuclear-targetted proteins and importin-β then binds importin-α to enhance the former interaction. The complex consisting of the import proteins and the nuclear-targetted protein is transported through the nuclear pore and released in the nucleoplasm in a Ran–GTP-dependent process (Jans *et al*., 1998; Ribbeck *et al*., 1999).

We have demonstrated previously both *in vivo* and *in vitro* that a 37 amino acid domain of NS5 (aa 369–405) can function as an NLS capable of targetting β-galactosidase to the nucleus. This interdomain linker region of NS5 is recognized by the importin-α/β complex and contains a functional protein kinase CK2 phosphorylation site (threonine-395) that appears to inhibit nuclear targetting (Forwood *et al*., 1999). The functional significance of nuclear transport and phosphorylation of NS5 has not been established and needs to be addressed. On the basis of our previous study, it was hypothesized that the NS5 NLS may be masked by interaction with NS3 in the early stages of replication. This site may become exposed at a late stage, either through conformational changes in NS5 resulting from hyperphosphorylation at serine residues or through autoproteolysis of NS3 that may provide access to the NLS for importins.

The picture that is emerging is of the RC as a protein 'machine', where many critical protein–protein contacts and also RNA–protein interactions may underpin its replication function. The fine molecular mapping of these interactions is important both as a means to characterize them to reveal their significance in the replication mechanism and also to investigate the suitability of these interaction sites as specific targets for new antiviral compounds. The yeast two-hybrid (Y2H) system detects protein–protein interactions *in vivo* in yeast by taking advantage of the modular nature of the transcription factor GAL4 (Ma & Ptashne, 1987). Briefly, a protein of interest is fused to a DNA-binding domain (BD), while a second protein is fused to a transcription-activation domain (AD). If the two proteins interact, the chimeric complex couples the BD domain bound at an upstream sequence element to the AD domain, which activates transcription of a reporter gene(s), e.g. *lacZ* or *HIS3* (Chien *et al*., 1991). The Y2H system has also previously been shown to be suitable for mapping interactions between the polymerase and helicase of the positive-stranded brome mosaic virus (O'Reilly *et al*., 1997).

In this paper, we report the characterization of the interaction between NS3 and NS5 from dengue virus type 2 strain TSV01 by mapping the binding sites between these proteins using the Y2H system. Furthermore, we investigate the Y2H interaction for NS5 with the host-encoded nuclear import proteins, demonstrating for the first time that NS5 interacts with importin-β. This interaction is verified and shown to occur independently of importin-α in pull-down assays. The function of the observed interactions, specifically a competitive regulatory role, is discussed.

Methods

■ **Cell culturing and extraction of RNA from dengue 2 virus TSV01.** Cells of the C6/36 line derived from *Aedes albopictus* (Igarashi, 1978) were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, as described previously (McBride *et al.*, 1995). Extraction of RNA at 6 days post-infection from C6/36 cell culture supernatant infected with dengue 2 virus TSV01 (McBride & Vasudevan, 1995) was performed by using the QIAamp Viral RNA Mini kit (Qiagen) as recommended by the manufacturer. The RNA was eluted with diethyl pyrocarbonate-treated water at 80 °C and stored at −70 °C.

■ **Isolation of NS3 and NS5 cDNAs.** Aliquots of the dengue 2 virus TSV01 RNA template (5 µg) were annealed with 2 pmol of the reverse gene-specific primers for NS3 or NS5 (Table 1). cDNA synthesis was performed with SuperScript II RNase H–reverse transcriptase according to manufacturer's instructions (Life Technologies). The NS3 and NS5 gene-specific primers (Table 1) were used to amplify the NS3 and NS5 genes separately from the TSV01 cDNA, using the EXPAND high-fidelity PCR enzyme mixture (Boehringer Mannheim). The amplified genes were subsequently inserted into the pGEM-T vector (Promega) to produce plasmids pMJNS3 AND pGEM-TNS5. The nucleotide sequences of the inserts in pMJNS3 and pGEM-TNS5 were identical to the nucleotide sequence obtained directly from RT–PCR products of TSV01. The plasmid pMJNS5 was generated from pGEM-TNS5, by digestion with the restriction endonuclease *Sac*II in order to remove an 8 bp non-coding region between the *Sac*II site in the NS5 forward primer (Table 1) and the same site within the pGEM-T vector, followed by ligation to reclose the plasmid. The two plasmids pMJNS3 and pMJNS5 were used in the construction of the various Y2H plasmids as well as the expression plasmids for the production of HISNS3 and HISNS5 (see Fig. 1) in this study.

■ **Construction of plasmids for the Y2H system.** All recombinant DNA techniques and cloning procedures were carried out by standard methods. The cDNAs encoding NS3 and NS5 as well as suitable truncations of the proteins were cloned into the GAL4 Y2H system 2 plasmids pAS2-1 (BD) or pACT2 (AD) (Clontech) in order to produce proteins/truncated polypeptides that were fused in-frame with the BD or AD domains of GAL4. The correctness of the clones was verified by nucleotide sequencing with the GAL4 Y2H BD or AD sequencing primers (Clontech). The plasmid constructs used to express the

Table 1. Primers used for generation of NS3 and NS5 full-length and truncation constructs and bacterial and yeast strains used

Primer names indicate the gene targetted, the orientation (in parentheses) and the restriction site added. Underlined nucleotides within primers indicate introduced restriction sites. Nucleotides in capitals represent the authentic dengue virus sequence and those in lower case are introduced sequences.

(*a*) Primer	Sequence (5′ → 3′)	Gene region (nt)
NS3 (forward) *Nco*I	gaaacccatgGCTGGAGTATTGTGGCATGTCC	1–22 (+sense)
NS3 (reverse) *Sac*I	ctggagctcttaCTTTCTTCCAGCTGCAAACT	1854–1835 (−sense)
NS5 (forward) *Sac*II	ggtaccgcggatccaatgGGAACTGGCAACATAGGAGAGACGCTTGG	1–29 (+sense)
NS5 (reverse) *Xba*I	cctctagattaCCACAGGACTCCTGCCTC	2700–2683 (−sense)
Truncated NS5 (forward) *Sma*I	ggcccccgGGAACTGGCAACATAGGAG	1–19 (+sense)
NS5 aa 1–368 (reverse) *Xho*I	agtttctcgagtcaTTCTTTCGGTTCTTG	1104–1090 (−sense)
NS5 aa 1–405 (reverse) *Xho*I	ctaactcgagctaGCTTCTCACCTTTCTT	1215–1200 (−sense)
Truncated NS5 (forward) *Bam*HI	aaggggatccgcAATGCAGCCTTAGGTGC	1216–1232 (+sense)
NS5 aa 406–900 (reverse) *Xho*I	ccccctcgagctaCCACAGGACTCCTGCCTC	2700–2683 (−sense)

(*b*) Strain	Genotype	Reference
E. coli		
DH5α	*deoR*, *endA1*, *gyrA96*, *hsdR17*(r_k^- m_k^+), *recA1*, *relA2*, *supE44*, *thi-1*, Δ(*lacZYA–argFV169*), ϕ80*lacZ*ΔM15, F^-	Hanahan (1983)
AD494 (DE3) (RIL)	Δ*ara–leu7697*, Δ*lacX74*, Δ*phoAPvuII*, *phoR* Δ*malF3*, F′[*lac*$^+$(*lacI*^{q})*pro*], *trxB*::kan (DE3), (RIL)	Derman *et al.* (1993); M. Johansson (unpublished)
S. cerevisiae		
Y187	Mat-α, *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3*, *112*, *gal4*Δ, *met*$^-$, *gal80*Δ, *URA3*::*GAL1*$_{UAS}$–*GAL1*$_{TATA}$–*lacZ*	Harper *et al.* (1993)
Y190	Mat-α, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *112*, *gal4*Δ, *gal80*Δ, *cyh*^{r}*2*, *LYS2*::*GAL1*$_{UAS}$–*HIS3*$_{TATA}$–*HIS3.URA3*::*GAL1*$_{UAS}$–*GAL1*$_{TATA}$–*lacZ*	Harper *et al.* (1993); Flick & Johnston (1990)

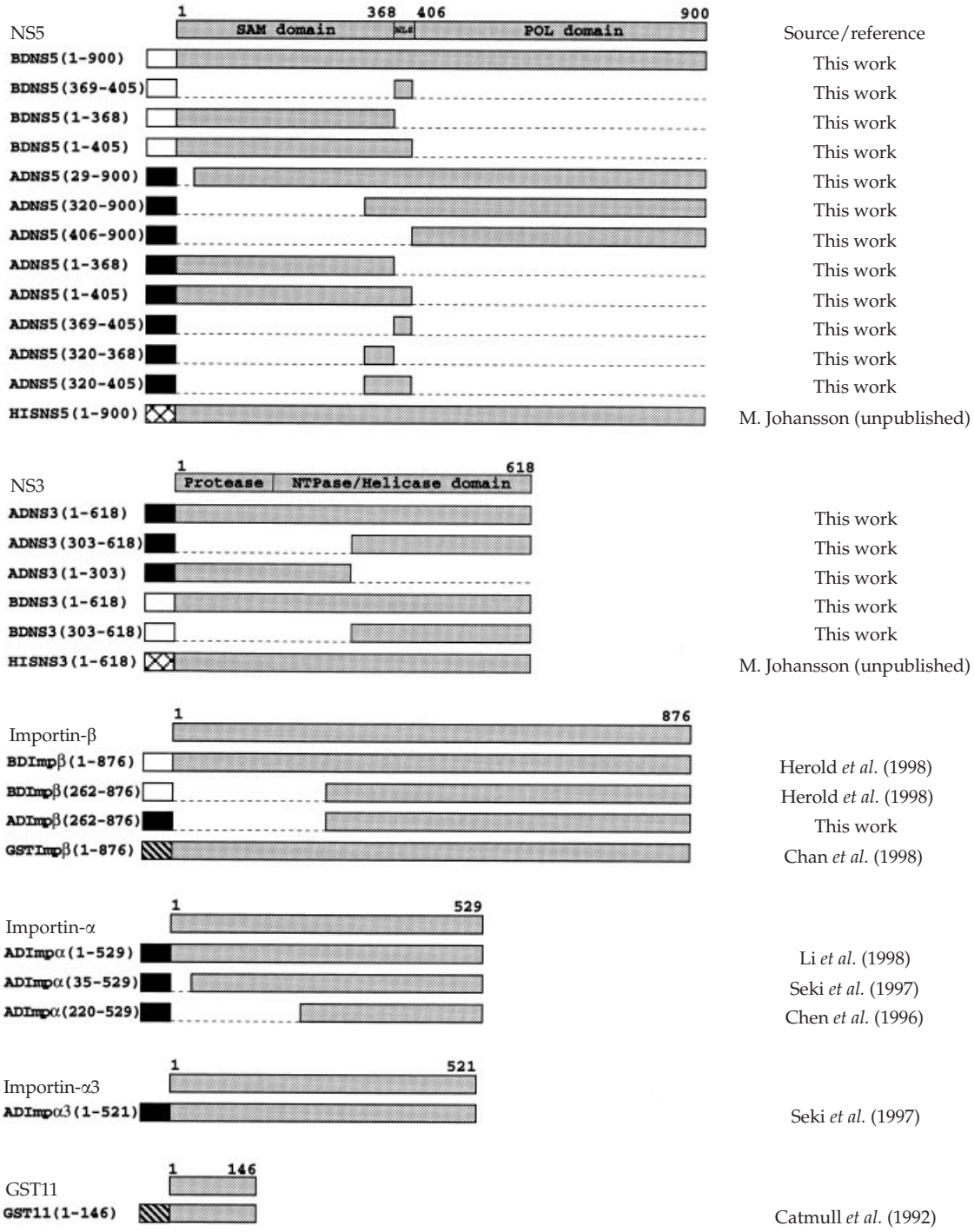

Fig. 1. Schematic representation of fused and/or truncated proteins used either in the Y2H assay or in the GST pull-down assay. The domains of NS5, with the previously characterized NLS region, and the domains of NS3 are indicated. The GAL4 BD (open boxes) and GAL4 AD (filled boxes), the HIS tag (cross-hatched boxes) and the GST tag (hatched boxes) are indicated. The regions of the proteins not included in the various fusion constructs are indicated by broken lines.

appropriate fusion proteins for Y2H interaction tests and *in vitro* pull-down assays in this work are presented in Fig. 1.

■ **The Y2H assay for reporter gene activity.** Y2H interactions were assayed with the *in vitro* plate filter lift assay (Clontech). Interactions were assayed on at least five independent transformants in both of *Saccharomyces cerevisiae* strains Y190 and Y187 (Table 1) to give conclusive results, but the data obtained with strain Y190 are shown exclusively in the figures since the interaction was stronger in that strain. Yeast colonies were patched onto SD minimal media lacking leucine or tryptophan in order to select for plasmids originating from pACT2 or

pAS2-1, respectively. The plates were incubated at 30 °C for 48 h and the cells were transferred to nitrocellulose membrane and assayed according to the *Yeast Protocol Manual* (Clontech) with the modification of three freeze–thaw cycles. The development of the blue colour resulting from β-galactosidase activity started within 1 h for strong interactions, but required up to 8 h for the weaker interactions. The negative controls did not develop any colour for at least 24 h.

■ **Detection of proteins expressed in yeast.** Yeast cells transformed with plasmids encoding the hybrid proteins were grown overnight at 30 °C in a suitable SD selection liquid medium. The cultures were inoculated 1:10 into YPD medium and grown to an OD_{600} of 0·6. The cells were collected on ice, frozen in liquid nitrogen and stored at −80 °C until required for protein extraction. Proteins were prepared by the urea–SDS extraction method (Printen & Sprague, 1994), separated on 10% SDS–PAGE (Laemmli, 1970), transferred to nitrocellulose membrane and detected with monoclonal AD or BD antibodies (Clontech) visualized by enhanced chemiluminescence (ECL) (Pharmacia–Amersham Biotech).

■ **Glutathione *S*-transferase (GST) pull-down assays of NS5 with GST-tagged importin-β.** Histidine-tagged NS5 and NS3 (HISNS5, HISNS3) were expressed in *E. coli* strain AD494 (DE3) (RIL) (Table 1) and purified by nickel-chelating chromatography (M. Johansson, A. J. Brooks and S. G. Vasudevan, unpublished), whereas GST–importin-β (GSTImpβ) was expressed and purified as described previously (Chan *et al.*, 1998; Forwood *et al.*, 1999). GSTImpβ or the control protein GST11 (Catmull *et al.*, 1992) at 2 μg/μl (final concentration) in buffer A (20 mM Tris–HCl, pH 7·9, 140 mM NaCl, 20 mM $MgCl_2$ and 0·1% Triton X-100) was supplemented with NS5 at 120 ng/μl (final concentration). Glutathione–Sepharose 4B (Pharmacia–Amersham Biotech) was added to the proteins, which were then incubated with gentle agitation at 4 °C for 14 h. The beads were washed five times with buffer A and proteins were eluted with 40 mM reduced glutathione in buffer A. The eluted fractions were analysed on 10% SDS–PAGE, transferred to nitrocellulose membrane and detected with mouse polyclonal NS5 antibody (M. Johansson and S. G. Vasudevan, unpublished) visualized by ECL. The protein concentration used in the pull-down assays was dependent on the amount of pure NS5 available; however, the specificity of the interaction was confirmed by the competition studies (see below). Furthermore, many pull-down experiments reported in the literature (e.g. Chung *et al.*, 2000) have used *in vitro* transcription–translation products that were also in the same range of protein concentration. Competition assays were set up essentially as described above, but HISNS3 (equimolar or 10-fold excess compared with NS5) or BSA (equimolar with NS5) was added with HISNS5 in the presence of GSTImpβ and glutathione–Sepharose 4B.

Results

The C-terminal domain of NS3 confers interaction with NS5

The interaction between NS3 and NS5 has been shown both *in vivo* and *in vitro* by using dengue 2 virus New Guinea C strain (Kapoor *et al.*, 1995). The NS3–NS5 interaction has also been demonstrated in JE virus-infected extracts by UV cross-linking, Western blot and immunoprecipitation studies (Chen *et al.*, 1996). However, the interaction has not previously been studied *in vivo* in yeast for any member of the genus *Flavivirus*. The NS3 and NS5 sequences from the dengue 2 virus strain TSV01 are 98·4 and 98·8% identical, respectively, to those of the New Guinea C strain (M. Johansson, A. J. Brooks and S. G. Vasudevan, unpublished results). In order to ensure that the TSV01-derived NS5 and NS3 behaved similarly, a pull-down assay was carried out to confirm that histidine-tagged NS5 (TSV01) could co-purify full-length NS3 (TSV01) from an *E. coli* extract (data not shown). In order to demonstrate the interaction between NS3 and NS5 in the Y2H system, the genes encoding the full-length proteins were fused in-frame with genes encoding BD and AD, respectively (Fig. 1). Surprisingly, no reporter activity was detected when these constructs were expressed in yeast (see Fig. 3*a*). However, there are similar examples in the literature where proteins that have been shown to interact by *in vitro* methods fail to provide a positive Y2H interaction (e.g. Herold *et al.*, 1998). Possible explanations for this include misfolding of the fusion protein, blocking of the interaction site by cellular factors and/or incorrect localization through exclusion of the proteins from the nucleus.

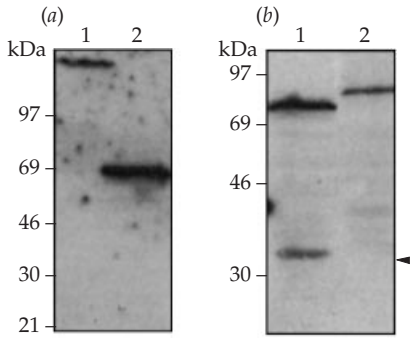

Fig. 2. Expression levels of fusion proteins expressed in yeast. (*a*) Proteins were extracted as described in Methods, separated by 10% SDS–PAGE and visualized by immunoblotting with anti-BD monoclonal antibody. Lanes: 1, BDNS5; 2, control protein BDp53 (Iwabuchi *et al.*, 1993). (*b*) Proteins extracted as outlined in (*a*) were separated on 12·5% SDS–PAGE and visualized by immunoblotting with anti-AD monoclonal antibody. Lanes: 1, ADNS3; 2, AD–SV40 T-ag. The arrowhead indicates the proteolytic fragment referred to in the text.

In order to examine this further, the level of expression of the chimeric proteins in yeast was assessed by separating the extracts by SDS–PAGE followed by Western blot detection using AD- or BD-specific monoclonal antibodies. Lane 1 of Fig. 2(*a*) shows that BDNS5(1–900) was expressed at low levels, especially in comparison with the control protein, BDp53 (Fig. 2*a*; lane 2), whereas ADNS3(1–618) (Fig. 2*b*; lane 1) was expressed reasonably well compared to the corresponding control protein, AD–SV40 T-ag (Fig. 2*b*; lane 2). Intriguingly, a ∼ 34 kDa proteolytic fragment was also detected with the AD antibody, implying cleavage of a peptide bond around amino acid positions 140–150 of NS3 (Fig. 2*b*; lane 1) and raising the possibility that a stable C-terminal domain fragment may be released from the fusion protein that competes with ADNS3(1–618) for the binding site on BDNS5(1–900) that is expressed at a lower level. Interestingly, a potential protease-sensitive site, $KKGK^{145}$, can be identified in NS3 at around the point predicted to yield a proteolytic fragment of the size observed. It was shown previously that internal proteolysis of

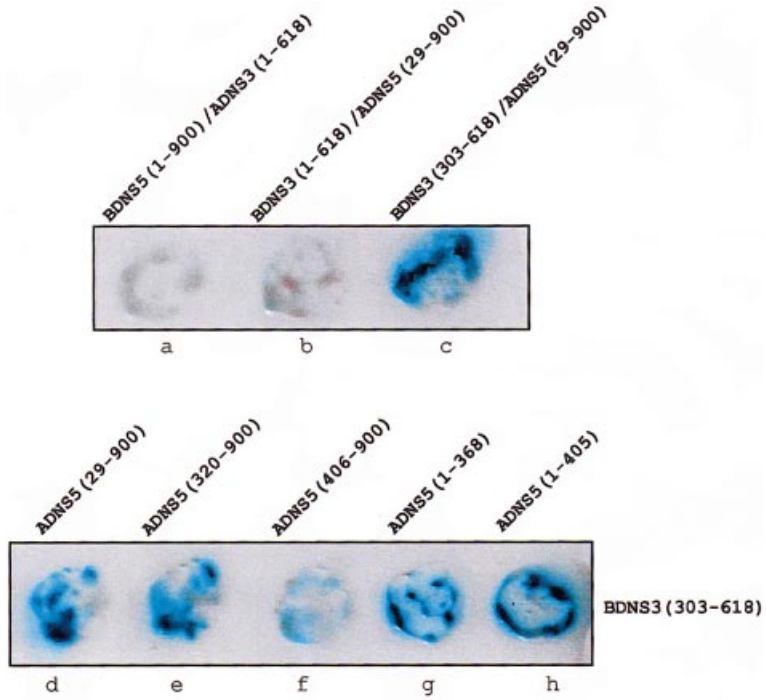

Fig. 3. Y2H assay to examine the interaction between NS5 and NS3 proteins from dengue virus type 2. Filter lift assays are presented showing the β-galactosidase activities of the yeast transformants; the strength of interaction is indicated by increased blue colour. The constructs being tested are indicated above each panel. Panels (*d*)–(*h*) show colony-lift assays for clones expressing almost full-length NS5 or various truncations with BDNS3(303–618).

the NS3 protein of dengue 2 virus occurred at the site RRGR^{460} within the RNA helicase motif (Teo & Wright, 1997), and the same site has been implicated in internal processing of the HCV NS3 protein (Shoji *et al.*, 1999). However, in the present study, ADNS3 is not expected to behave in a similar way, since NS2B is not present in the yeast cells. Intriguingly, an 18 kDa fragment was noted in the previous study by Teo & Wright (1997) that may be due to proteolysis by cellular proteases at the KKGK^{145} site within the protease domain of NS3. Reciprocal constructs of NS3 and NS5 fused to BD and AD, respectively, did not give rise to any reporter activity (Fig. 3*b*).

In order to examine further the interaction between NS3 and NS5 in yeast, plasmids were constructed to direct the synthesis of various truncated forms of the proteins (Fig. 1). The levels of the recombinant polypeptides were assessed by immunoblotting. BDNS3(303–618) was expressed well in yeast (data not shown) and did show an interaction with ADNS5 (Fig. 3*c*, *d*), as indicated by expression of the reporter β-galactosidase. This was consistent with the idea outlined above that the C-terminal portion of NS3, released by proteolysis around amino acid 150, may interact with NS5. Upon interaction with BDNS3(303–618), ADNS5(320–900) showed reporter activity (Fig. 3*e*) comparable to that of ADNS5. This suggested that the NS5 region C-terminal of position 319 is important for interaction with NS3. The truncated constructs ADNS5(1–368) and ADNS5(1–405) (Fig. 3*g*, *h*) showed reporter gene expression levels higher than those observed for ADNS5(406–900) (Fig. 3*f*). The reduced reporter activity in the latter indicated that the region between residues 320 and 405 of NS5 is probably important for interaction with NS3. However, the low reporter activity in ADNS5(406–900) may also suggest weak interactions with the POL domain of NS5 (i.e. the region C-terminal of residue 405). Interestingly, the results with the truncated NS5 proteins map the NS3 interaction site close to the interdomain linker containing the NLS that was characterized previously (Forwood *et al.*, 1999).

ADNS3(1–303) (Fig. 1), which contains the protease domain as well as the protease-sensitive site mentioned above, was expressed in yeast (data not shown), but showed no detectable reporter activity when tested with the BDNS5 constructs, suggesting that the NS5 interaction site is indeed within the C-terminal region of NS3.

NS5 interacts with the nuclear import receptor importin-β

We have demonstrated previously that the interdomain linker region (residues 369–405) of NS5 functions as an NLS both *in vivo* and *in vitro* (Forwood *et al.*, 1999) and is recognized by the importin-α/importin-β heterodimeric complex. A number of recent studies have shown that importin-β can interact directly with nuclear-targetted proteins independently of importin-α to mediate their nuclear import (Chan *et al.*, 1998; Henderson & Percipalle, 1997; Tiganis *et al.*, 1997). We decided to use the Y2H assay to examine the interaction of NS5 with importins, with particular interest in mapping the potential sites of interaction in relation to the NS5–NS3 interaction site.

Full-length importin-β and an N-terminally truncated importin-β [Impβ(262–876)], both fused to BD, were analysed

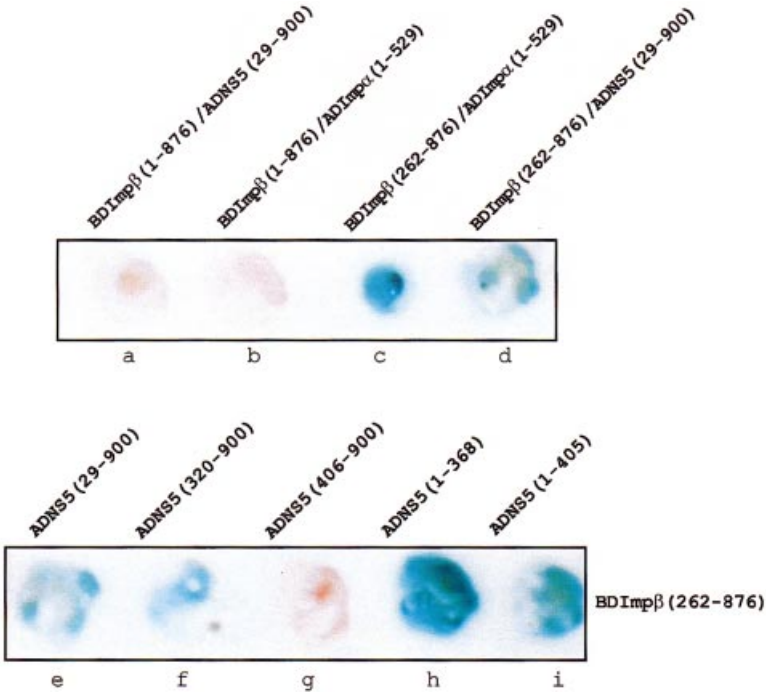

Fig. 4. The interaction of dengue virus 2 NS5 protein with human importin-β examined by Y2H assay. Filter-lift assays are presented showing the β-galactosidase activities of the yeast transformants; increased interaction is indicated by increased blue colour. (*a*)–(*d*) Yeast strains bearing plasmids that expressed the polypeptides indicated. (*e*)–(*i*) Yeast strains carrying the various NS5 constructs fused to AD indicated together with a plasmid that encodes BDImpβ(262–876).

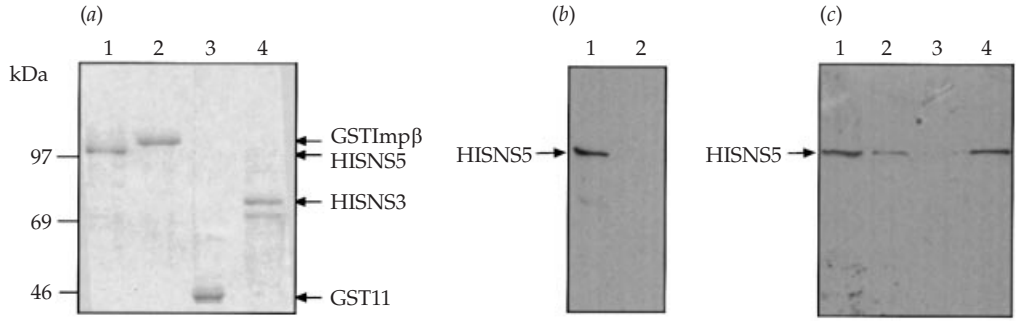

Fig. 5. NS5 pull-down assay with GSTImpβ. (*a*) Partially purified HISNS5 (lane 1), mouse GSTImpβ (2), the unrelated negative control GST11 (3) and partially purified HISNS3 (4) were separated on 10% SDS–PAGE and visualized by Coomassie blue staining. (*b*) HISNS5 was mixed with GSTImpβ or GST11 and subjected to a pull-down test as described in Methods. The pulled-down fractions were separated by 10% SDS–PAGE and visualized by immunoblotting with anti-NS5 polyclonal antibody. Lanes: 1, the fraction pulled down with GSTImpβ; 2, the fraction pulled down with GST11. (*c*) NS3 competition for the GSTImpβ–NS5 interaction. The pulled-down fractions were separated by 10% SDS–PAGE and visualized by immunoblotting with anti-NS5 polyclonal antibody. Lanes: 1, GSTImpβ pull down of NS5; 2, GSTImpβ pull down with addition of equal amounts of NS5 and NS3; 3, GSTImpβ pull down of NS5 with 10-fold excess of NS3 added; 4, control with addition of equal amounts of NS5 and BSA.

against ADNS5. No reporter activity was detected using full-length BD–importin-β [BDImpβ(1–876)] with ADNS5 (Fig. 4*a*). It has been shown previously that BDImpβ(1–876) and AD–importin-α (ADImpα) do not interact in the Y2H system (Fig. 4*b*), due to the fact that the N-terminal domain of importin-β can bind Ran–GTP, which prevents the importin-α/importin-β interaction (Herold *et al.*, 1998). In contrast, BDImpβ(262–876), which lacks the ability to bind Ran, shows strong interaction with importin-α (Fig. 4*c*). Interestingly, BDImpβ(262–876) also exhibited an interaction with ADNS5 (Fig. 4*d*, *e*), implying that nuclear import of NS5 might be mediated directly by importin-β. ADNS5(320–900) showed similar reporter activity to the full-length NS5 (Fig. 4*f*), whereas the truncation ADNS5(406–900) showed no detectable activity (Fig. 4*g*). To test whether the previously demonstrated NLS sequence, NS5(369–405), may be the region responsible for interaction with importin-β, ADNS5(1–368) and ADNS5(1–405) were analysed, both of which were expressed at similar levels in yeast (data not shown). Unexpectedly, ADNS5(1–368) (Fig. 4*h*), which does not include the previously characterized NS5 NLS, showed reporter activity upon interaction with BDImpβ(262–876) that was much greater than that observed with ADNS5(1–405) (Fig. 4*i*) or ADNS5 (Fig. 4*d*, *e*). These data indicate that the

region immediately N-terminal of the previously characterized NLS is critical for binding of importin-β to NS5. Human importin-α and -α3 were also analysed for their ability to interact with NS5 in the Y2H system, but neither showed reporter gene activity indicative of interaction with either ADNS5 or the truncated constructs of NS5 (data not shown).

Interaction of NS5 with importin-β, independent of importin-α, in pull-down assays

The NS5–importin-β interaction has not been reported previously, so we decided to confirm it biochemically. Purified HISNS5 (Fig. 5*a*; lane 1) was used in a GST pull-down assay with full-length GSTImpβ protein (Fig. 5*a*; lane 2), together with a GST-fused, unrelated protein (GST11) as a negative control (Fig. 5*a*; lane 3). The result of the pull-down assay was analysed directly by SDS–PAGE (data not shown) followed by immunoblotting (Fig. 5*b*), showing that GSTImpβ can pull down NS5 (Fig. 5*b*; lane 1). No detectable NS5 protein was pulled down by a molar excess of GST11 (Fig. 5*b*; lane 2). This shows that the interaction between full-length importin-β and NS5 is direct.

In order to show that the importin-β-binding site on NS5 is similar or related to the NS3-binding site, HISNS3 was used in a competition experiment (Fig. 5*a*; lane 4). SDS–PAGE analysis followed by immunoblotting with anti-NS5 antibodies showed that equimolar NS3/NS5 incubated with GSTImpβ bound to glutathione beads resulted in a reduction in pull-down of NS5 (Fig. 5*c*; lanes 1 and 2). A 10-fold excess of NS3 blocked NS5 binding to GSTImpβ (Fig. 5*c*; lane 3), while equimolar BSA did not affect the interaction between NS5 and GSTImpβ (Fig. 5*c*; lane 4). This shows that the binding sites for NS3 and importin-β on NS5 either overlap or are closely related. A duplicate SDS–PAGE analysis of the samples in Fig. 5(*c*) was transferred to a nitrocellulose membrane and probed with anti-NS3 antibodies to ensure that the observed competition was not due to direct binding of NS3 and importin-β. After extended exposure, ECL detection showed a weak band corresponding to NS3 in the lane containing a 10-fold excess of NS3 (data not shown). This indicates that the reduction in NS5 pulled down by GSTImpβ shown in lane 2 of Fig. 5(*c*) and the complete absence of NS5 in lane 3 of Fig. 5(*c*) are due to competition by NS3 for the same or a related site.

Mapping of the NS5 interaction regions by Y2H analysis

Analyses of the full-length and domain constructs of NS5 indicated that the region 320–368/(405) is important for the NS5–NS3 and NS5–importin-β interactions. Plasmids expressing the smaller constructs ADNS5(369–405), ADNS5(320–368) and ADNS5(320–405) were constructed to determine more precisely the site of protein interactions within this region. ADNS5(369–405) was expressed well (data not shown) but was found to interact only weakly with importin-β (Fig. 6*a*). Although the reporter activity was low, it could be detected reproducibly, and a similar result was obtained for plasmids expressing the reciprocal peptides (data not shown). No reporter gene activity was detected for BDNS3(303–618) interacting with ADNS5(369–405) (Fig. 6*d*) or the reciprocal peptides (data not shown). ADNS5(320–368) was expressed at lower levels than ADNS5(369–405) but did show interactions with both BDImpβ(262–876) and BDNS3(303–618), indicating that the region including residues 320–368 is important for interactions of NS3 as well as importin-β with NS5 (Fig. 6*b*, *e*). The longer peptide ADNS5(320–405) was expressed poorly (data not shown) and showed no reporter activity with BDImpβ(262–876) (Fig. 6*c*). On the other hand, some interaction could be detected with BDNS3(303–618) (Fig. 6*f*). The truncation constructs demonstrate quite clearly that both NS3 and importin-β interact with NS5 within ~ 50 amino acids.

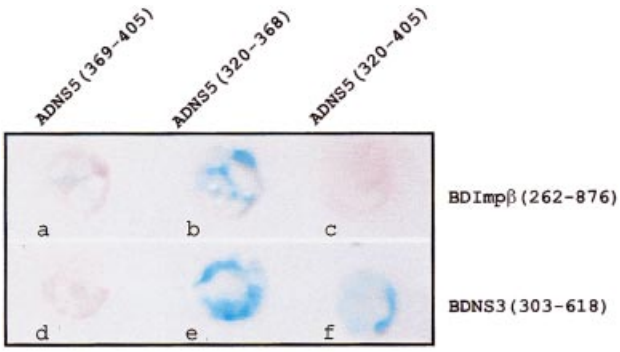

Fig. 6. Y2H interaction assay-based mapping to show convergence of NS3 and importin-β interactions to a small region within NS5. Two separate filter assays were carried for the three AD–NS5 constructs indicated with either BDImpβ(262–876) (*a*–*c*) or BDNS3(303–618) (*d*–*f*), but the results are comparable because the plates were grown, assayed and processed at the same time. The strength of interaction between the tested peptides is reflected by the β-galactosidase activities of the yeast transformants, where the strength of interaction activity is indicated by increased blue colour.

Discussion

The results of this study using the Y2H system show for the first time that dengue virus NS5 is able to interact with the nuclear import receptor importin-β. Intriguingly, the region of NS5 responsible is not the previously characterized NLS of NS5 (residues 369–405) recognized by the importin-α/β heterodimer (Forwood *et al.*, 1999) but, rather, the N-terminally flanking region (residues 320–368) that also appears to be the site of interaction with NS3. Taking together with the previously demonstrated binding of the NS5 NLS by the importin-α/β heterodimer complex, the present results indicate that at least three different proteins are able to bind a short, 85 amino acid sequence of NS5 (residues 320–405). In the context of dengue virus infection, this is quite intriguing, raising the possibility of complex co-operative and/or competitive interactions. Characterizing these interactions in the Y2H system

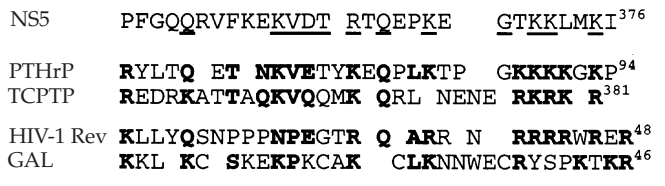

Fig. 7. The single-letter amino acid code is used to compare NS5 residues 348–376 to previously reported minimal importin-β-binding regions (Lam *et al.*, 1999). The underlined NS5 residues are similar to conserved amino acids within the consensus alignment (indicated in bold) of PTHrP, TCPTP, HIV-1 Rev and GAL4 (Lam *et al.*, 1999 and references therein). Sequences in NS5 that are similar to PTHrP are also underlined.

may lead to a test that can be used to screen for highly specific small molecules that prevent the protein–protein interaction.

In terms of specific results, we made the interesting observation that, in contrast to truncated versions, full-length NS5 and full-length NS3 do not interact in the Y2H assay. This can probably be attributed to proteolytic cleavage of NS3 (around residue 150), liberating the NS3 C terminus from the GAL4 BD to compete with the full-length protein for NS5, which is consistent with the fact that NS3 (residues 303–618) interacts with full-length NS5 as well as with various truncations of NS5. In fact, the results obtained from the reporter assays indicate that the interaction of the NS3 C-terminal domain is stronger in the presence than in the absence of the N-terminal domain of NS5, since the truncation construct ADNS5(406–900) showed the weakest interaction with BDNS3(303–618). The data clearly indicate that the region N-terminal of the NS5 NLS and POL domain region is responsible for NS3 binding. Similarly, Y2H studies indicate that interaction of brome mosaic virus helicase-like protein 1a and the 2a polymerase is mediated by a region N-terminal of the polymerase homology region (O'Reilly *et al.*, 1997).

We found that an importin-β construct with a deleted N terminus interacted strongly with NS5, although previous *in vitro* studies had indicated that the NS5 NLS is recognized with reasonably high affinity by the importin-α/β complex (Forwood *et al.*, 1999). Several recent reports indicate that there are a number of different pathways by which nuclear-targetted proteins can be transported to the nucleus in addition to the well-characterized importin-α/β heterodimer-mediated pathway (Jans *et al.*, 1998). The ability of importin-β to interact directly with NS5 independently of importin-α suggests that the latter may be transported to the nucleus by the importin-β-mediated pathway. This direct interaction of importin-β with NS5 independent of importin-α was confirmed in pull-down assays. Interestingly, it is possible to find some significant similarity within the NS5 region that interacts with importin-β to the minimal importin-β-binding regions of parathyroid hormone-related protein (PTHrP) and T-cell protein tyrosine phosphatase (TCPTP) (Lam *et al.*, 1999). This region of NS5 is aligned against a comparison of the PTHrP region with TCPTP as well as human immunodeficiency virus type 1 (HIV-1) Rev and GAL4 in Fig. 7.

Detailed study of the interactions of NS3 and importin-β with NS5 mapped the region of interaction down to amino acids 320–368. This region of NS5 does not contain any serine residues or any other readily recognizable putative phosphorylation sites, however, so it would seem that this alone does not shed light on the observation that hyperphosphorylated NS5 does not appear to interact with NS3 (Kapoor *et al.*, 1995). It seems possible that the NS3–NS5 interaction may be regulated by conformational changes within NS5, triggered by phosphorylation events that occur within other domains of the protein.

A recent report showed that NS5A from HCV, a member of the family *Flaviviridae*, interacts with the importin-β homologue karyopherin-β3 (44·4% similarity, 17·6% identity) (Chung *et al.*, 2000). This interaction has been characterized by using the Y2H system and shown to occur between the N-terminal region of NS5A and the C-terminal part of karyopherin-β3 (Chung *et al.*, 2000). HCV NS5A is not detected in the nuclei of infected cells, although a region resembling an NLS has been fused N-terminally to β-galactosidase and shown to target it to the nucleus (Ide *et al.*, 1996). A putative 'hijacking' role for NS5A was suggested, whereby the viral protein functions by sequestering nuclear karyopherin-β3 (RanBP5/importin 5) so that it is not available for other cellular functions (Chung *et al.*, 2000). There is no apparent similarity between the interacting sequence of NS5A and the dengue virus NS5 sequence to suggest a similar role, not even within NS5 residues 320–405.

There is no known role for nuclear localization of dengue virus NS5, but the facts that nuclear localization does occur *in vivo* in dengue virus-infected cells (Kapoor *et al.*, 1995) and that the protein contains a functional NLS (Forwood *et al.*, 1999) clearly imply that it is significant. Detailed investigation using the approaches described here should help to unravel the mechanism and events that trigger the nuclear import of NS5 and may provide a new target for the development of antiviral agents. This process necessarily involves investigation of protein interaction sites that may be amenable to high throughput assays in order to find suitable inhibitors. The Y2H system clearly represents an attractive genetic screen to do exactly this, and hence will be highly useful in future, as soon as the functional consequences of the different competing NS3–NS5–importin interactions are fully understood through complementary studies involving reverse genetics with infectious clones.

In conclusion, the NS3 protein must work in concert with NS5 for replication of the viral genome, since the RF is a double-stranded RNA. This process occurs in the perinuclear membrane of infected cells. Since NS3 also interacts with the importin-β-binding site defined in this work, it is possible that an additional role for NS3 may be to ensure that NS5 remains in the cytoplasmic environment, where it is necessary for virus replication and capping. Detailed characterization of this region by specific mutagenesis as well as nuclear localization studies

with recombinant NS5 are currently in progress in this laboratory.

Dr M. Johansson was supported by a post-doctoral fellowship from the Wenner-Gren foundations, also contributed by the Swedish Medical Research council, the Swedish Institute and Bengt Lundqvist Memorial Fund. The research was carried out with funds provided by the Australian Research Council Small Grant Scheme and support from the Clive and Vera Ramaciotti Foundation.

References

Barrett, A. D. (1997). Japanese encephalitis and dengue vaccines. *Biologicals* **25**, 27–34.

Buckley, A., Gaidamovich, S., Turchinskaya, A. & Gould, E. A. (1992). Monoclonal antibodies identify the NS5 yellow fever virus non-structural protein in the nuclei of infected cells. *Journal of General Virology* **73**, 1125–1130.

Bulich, R. & Aaskov, J. G. (1992). Nuclear localization of dengue 2 virus core protein detected with monoclonal antibodies. *Journal of General Virology* **73**, 2999–3003.

Catmull, J., Dan, Z. & Miller, D. J. (1992). Nucleotide sequence of a cDNA from *Onchocerca gibsoni* encoding a novel repetitive antigen. *International Journal of Parasitology* **22**, 685–687.

Chambers, T. J., Hahn, C. S., Galler, R. & Rice, C. M. (1990). Flavivirus genome organization, expression, and replication. *Annual Review of Microbiology* **44**, 649–688.

Chan, C. K., Hubner, S., Hu, W. & Jans, D. A. (1998). Mutual exclusivity of DNA binding and nuclear localization signal recognition by the yeast transcription factor GAL4: implications for nonviral DNA delivery. *Gene Therapy* **5**, 1204–1212.

Chen, C. F., Li, S., Chen, Y., Chen, P. L., Sharp, Z. D. & Lee, W. H. (1996). The nuclear localization sequences of the BRCA1 protein interact with the importin-α subunit of the nuclear transport signal receptor. *Journal of Biological Chemistry* **271**, 32863–32868.

Chien, C. T., Bartel, P. L., Sternglanz, R. & Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proceedings of the National Academy of Sciences, USA* **88**, 9578–9582.

Chung, K. M., Lee, J., Kim, J. E., Song, O. K., Cho, S., Lim, J., Seedorf, M., Hahm, B. & Jang, S. K. (2000). Nonstructural protein 5A of hepatitis C virus inhibits the function of karyopherin β3. *Journal of Virology* **74**, 5233–5241.

Cleaves, G. R., Ryan, T. E. & Schlesinger, R. W. (1981). Identification and characterization of type 2 dengue virus replicative intermediate and replicative form RNAs. *Virology* **111**, 73–83.

Derman, A. I., Prinz, W. A., Belin, D. & Beckwith, J. (1993). Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. *Science* **262**, 1744–1747.

Flick, J. S. & Johnston, M. (1990). Two systems of glucose repression of the GAL1 promoter in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **10**, 4757–4769.

Forwood, J. K., Brooks, A., Briggs, L. J., Xiao, C. Y., Jans, D. A. & Vasudevan, S. G. (1999). The 37-amino-acid interdomain of dengue virus NS5 protein contains a functional NLS and inhibitory CK2 site. *Biochemical and Biophysical Research Communications* **257**, 731–737.

Gubler, D. J. & Clark, G. G. (1995). Dengue/dengue hemorrhagic fever: the emergence of a global health problem. *Emerging Infectious Diseases* **1**, 55–57.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**, 557–580.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**, 805–816.

Henderson, B. R. & Percipalle, P. (1997). Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta. *Journal of Molecular Biology* **274**, 693–707.

Herold, A., Truant, R., Wiegand, H. & Cullen, B. R. (1998). Determination of the functional domain organization of the importin alpha nuclear import factor. *Journal of Cell Biology* **143**, 309–318.

Ide, Y., Zhang, L., Chen, M., Inchauspé, G., Bahl, C., Sasaguri, Y. & Padmanabhan, R. (1996). Characterization of the nuclear localization signal and subcellular distribution of hepatitis C virus nonstructural protein NS5A. *Gene* **182**, 203–211.

Igarashi, A. (1978). Isolation of a Singh's *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. *Journal of General Virology* **40**, 531–544.

Iwabuchi, K., Li, B., Bartel, P. & Fields, S. (1993). Use of the two-hybrid system to identify the domain of p53 involved in oligomerization. *Oncogene* **8**, 1693–1696.

Jans, D. A. (1995). The regulation of protein transport to the nucleus by phosphorylation. *Biochemical Journal* **311**, 705–716.

Jans, D. A., Ackermann, M. J., Bischoff, J. R., Beach, D. H. & Peters, R. (1991). p34cdc2-mediated phosphorylation at T124 inhibits nuclear import of SV-40 T antigen proteins. *Journal of Cell Biology* **115**, 1203–1212.

Jans, D. A., Chan, C. K. & Huebner, S. (1998). Signals mediating nuclear targeting and their regulation: application in drug delivery. *Medical Research Review* **18**, 189–223.

Kapoor, M., Zhang, L., Ramachandra, M., Kusukawa, J., Ebner, K. E. & Padmanabhan, R. (1995). Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. *Journal of Biological Chemistry* **270**, 19100–19106.

Koonin, E. V. (1991). The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *Journal of General Virology* **72**, 2197–2206.

Koonin, E. V. (1993). Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and λ2 protein of reovirus. *Journal of General Virology* **74**, 733–740.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lam, M. H., Briggs, L. J., Hu, W., Martin, T. J., Gillespie, M. T. & Jans, D. A. (1999). Importin β recognizes parathyroid hormone-related protein with high affinity and mediates its nuclear import in the absence of importin α. *Journal of Biological Chemistry* **274**, 7391–7398.

Li, S., Ku, C. Y., Farmer, A. A., Cong, Y. S., Chen, C. F. & Lee, W. H. (1998). Identification of a novel cytoplasmic protein that specifically binds to nuclear localization signal motifs. *Journal of Biological Chemistry* **273**, 6183–6189.

Li, H., Clum, S., You, S., Ebner, K. E. & Padmanabhan, R. (1999). The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. *Journal of Virology* **73**, 3108–3116.

Ma, J. & Ptashne, M. (1987). Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell* **48**, 847–853.

McBride, W. J. H. & Vasudevan, S. G. (1995). Relationship of a dengue

2 isolate from Townsville, 1993, to international isolates. *Communicable Diseases Intelligence* **19**, 522–523.

McBride, W. J. H., Breinl, R. A. & Vasudevan, S. G. (1995). Rapid diagnosis of dengue by nucleic acid hybridization. *Southeast Asian Journal of Tropical Medicine and Public Health* **26**, 488–494.

Mackenzie, J. M., Khromykh, A. A., Jones, M. K. & Westaway, E. G. (1998). Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. *Virology* **245**, 203–215.

Morozova, O. V., Tsekhanovskaya, N. A., Maksimova, T. G., Bachvalova, V. N., Matveeva, V. A. & Kit, Y. Y. (1997). Phosphorylation of tick-borne encephalitis virus NS5 protein. *Virus Research* **49**, 9–15.

O'Reilly, E. K., Paul, J. D. & Kao, C. C. (1997). Analysis of the interaction of viral RNA replication proteins by using the yeast two-hybrid assay. *Journal of Virology* **71**, 7526–7532.

Printen, J. A. & Sprague, G. F., Jr (1994). Protein–protein interactions in the yeast pheromone response pathway: Ste5p interacts with all members of the MAP kinase cascade. *Genetics* **138**, 609–619.

Ribbeck, K., Kutay, U., Paraskeva, E. & Gorlich, D. (1999). The translocation of transportin-cargo complexes through nuclear pores is independent of both Ran and energy. *Current Biology* **9**, 47–50.

Seki, T., Tada, S., Katada, T. & Enomoto, T. (1997). Cloning of a cDNA encoding a novel importin-alpha homologue, Qip1: discrimination of Qip1 and Rch1 from hSrp1 by their ability to interact with DNA helicase Q1/RecQL. *Biochemical and Biophysical Research Communications* **234**, 48–53.

Shoji, I., Suzuki, T., Sato, M., Aizaki, H., Chiba, T., Matsuura, Y. & Miyamura, T. (1999). Internal processing of hepatitis C virus NS3 protein. *Virology* **254**, 315–323.

Tan, B. H., Fu, J., Sugrue, R. J., Yap, E. H., Chan, Y. C. & Tan, Y. H. (1996). Recombinant dengue type 1 virus NS5 protein expressed in *Escherichia coli* exhibits RNA-dependent RNA polymerase activity. *Virology* **216**, 317–325.

Teo, K. F. & Wright, P. J. (1997). Internal proteolysis of the NS3 protein specified by dengue virus 2. *Journal of General Virology* **78**, 337–341.

Tiganis, T., Flint, A. J., Adam, S. A. & Tonks, N. K. (1997). Association of the T-cell protein tyrosine phosphatase with nuclear import factor p97. *Journal of Biological Chemistry* **272**, 21548–21557.

Wengler, G. & Wengler, G. (1993). The NS 3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. *Virology* **197**, 265–273.

Westaway, E. G., Mackenzie, J. M., Kenney, M. T., Jones, M. K. & Khromykh, A. A. (1997*a*). Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. *Journal of Virology* **71**, 6650–6661.

Westaway, E. G., Khromykh, A. A., Kenney, M. T., Mackenzie, J. M. & Jones, M. K. (1997*b*). Proteins C and NS4B of the flavivirus Kunjin translocate independently into the nucleus. *Virology* **234**, 31–41.

Received 4 September 2000; Accepted 20 December 2000