Using West Nile virus strain Kunjin virus (WNV\textsubscript{KUN}) as a model system for flavivirus replication, we observed nuclear localization of core protein (C) and of the non-structural protein NS4B (Westaway \textit{et al.}, 1997a) but found no evidence of the replication complex (RC) in the nuclei (Khromykh \textit{et al.}, 2001a, b; Mackenzie & Westaway, 2001; Westaway \textit{et al.}, 2002). In early studies, the replication site of flavivirus species \textit{Japanese encephalitis virus} (JEV) and dengue-2 virus (DEN2V) appeared perinuclear by immunofluorescence (IF) (Cardiff \textit{et al.}, 1973; Edward & Takegami, 1993; Ng & Corner, 1989), and the flavivirus double-stranded RNA (dsRNA) template for WNV\textsubscript{KUN} was shown to be located in the cytoplasm but absent from the nucleus (Ng \textit{et al.}, 1983). Nuclear localization of the RNA-dependent RNA polymerase (RdRp) NS5 was shown by IF in yellow fever virus (YFV)-infected Vero cells (Buckley \textit{et al.}, 1992), DEN2V-infected CV-1 cells (Kapoor \textit{et al.}, 1995) and DEN2V-infected Huh-7 cells (Miller \textit{et al.}, 2006). In JEV-infected PS cells, NS3 and NS5 appeared to co-localize with sites of viral RNA synthesis along the inner periphery of the nucleus by IF and immuno-electron microscopy (IEM) (Uchil \textit{et al.}, 2006).

NS5 is included in the consensus composition of the RC (NS1, NS3, NS5, NS2A and NS4A) defined for WNV\textsubscript{KUN} (Mackenzie \textit{et al.}, 1998; Westaway \textit{et al.}, 1997a, 2002) and is highly conserved (Coia \textit{et al.}, 1988). Recombinant dengue-1 virus (DEN1V) and WNV NS5 species have displayed RdRp activity \textit{in vitro} (Guyatt \textit{et al.}, 2001; Steffens \textit{et al.}, 1999; Tan \textit{et al.}, 1996). Location of replication sites only in the cytoplasm of WNV\textsubscript{KUN}-infected cells was established by RdRp assays of heavy membrane fractions (Chu & Westaway, 1992), by IF using antibodies that co-localized dsRNA with specific non-structural proteins (Mackenzie \textit{et al.}, 1998; Westaway \textit{et al.}, 1997a), by showing that bromo-substituted uridine was incorporated in nascent viral RNA during pulse labelling (Westaway \textit{et al.}, 1999) and by cryo-IEM of thin sections of cells (Mackenzie \textit{et al.}, 1998, 1999; Westaway \textit{et al.}, 1997b). Thus the replication sites containing the RC were located after the latent period in unique induced-membrane structures termed ‘vesicle packets’ (VP), found also only in the cytoplasm of DEN2V-infected cells (Mackenzie \textit{et al.}, 1996a).

Kapoor \textit{et al.} (1995) found that DEN2V NS5 existed as a phosphorylated form that localized primarily to the nucleus, whereas the unphosphorylated form associated with NS3 was found only in the cytoplasm. Interestingly, only one form of YFV phosphorylated NS5 was observed in SW13 cells and it co-migrated with \textsuperscript{35}S\textit{methionine}-labelled NS5 (Reed \textit{et al.}, 1998). In contrast, NS5 of \textit{Tick-borne encephalitis virus} (TBEV) labelled with \textsuperscript{35}S\textit{methionine} migrated as a doublet in gels, but only as the single slower-migrating band after phosphorylation \textit{in vitro} of an infected-cell extract (Morozova \textit{et al.}, 1997).
In view of these inconsistent results described above and the recent report proposing that approximately 20% of the RdRp activity was resident in the nucleus of cells infected with WNV strain E101 (WNV_{E101}), JEV or DEN2V (Uchil et al., 2006), we searched for nuclear localization and phosphorylation of WNV_{KUN} NS5 in Vero cells. We found that WNV_{KUN} NS5 was confined solely to the cytoplasm and was metabolically labelled in medium containing $[^{32}\text{P}]$orthophosphate at serine and threonine sites within the protein.

For direct observation of nuclear localization of flavivirus NS5 (Fig. 1), Vero cells were infected in minimal essential medium containing 0.1% BSA at an m.o.i. of 3 for 24 h (for WNV strains) or 40 h (for DEN2V) corresponding to their peak times of infection. IF assays were conducted using antibodies prepared against WNV_{KUN} NS5 (Khromykh et al., 1996, 1998), guinea pig antibodies to dsRNA to identify sites of WNV_{KUN} replication (Mackenzie et al., 1996b, 1998, 1999; Westaway et al., 1997b) or polyclonal antibodies to DEN2V NS5 known to stain the nuclei of DEN2V-infected cells (Kapoor et al., 1995) for comparative purposes. The bound antibodies were subsequently visualized with FITC- or Texas red-conjugated species-specific IgG (Edward Keller Australia). Differential immunostaining of nuclei and cytoplasm for all cells was achieved by permeabilization of the nuclear membrane with either acetone at –20°C for 20 min (Kapoor et al., 1995) or at 20°C with 4% paraformaldehyde containing 0.1% Triton X-100 (Mackenzie & Westaway, 2001). The results in Fig. 1 show that WNV_{KUN} NS5 localized only to the cytoplasm at 24 h under both conditions of fixation (Fig. 1a, c, d and g), as was NS5 of a closely related WNV strain Sarafend (WNV_{SAR}) (Fig. 1i), and major foci of NS5 were coincident with dsRNA (Fig. 1g, h, i and j). Staining of NS5 appeared to be membrane-associated and was most intense in the perinuclear region, although more diffuse cytoplasmic staining was also present. The relatively small proportion of NS5 associated with the WNV_{KUN} replication foci was not surprising because only minor amounts of NS5 expressed during infections with WNV_{KUN}, JEV and WNV are detectable in biochemical assays of cell fractions with RdRp activity (Chu & Westaway, 1992; Grun & Brinton, 1987; Uchil & Satchidanandam, 2003a, b). In striking contrast to the WNV_{KUN} results, NS5 was strongly immunolabelled with homologous antibodies in both the nucleoplasm and cytoplasm of DEN2V-infected cells at 40 h (Fig. 1b and e), as reported by Kapoor et al. (1995). The DEN2V NS5 antibodies cross-reacted with WNV_{KUN} NS5 but stained only the cytoplasm of WNV_{KUN}-infected cells (Fig. 1c). Like DEN2V NS5, WNV_{KUN} core protein contains a nuclear localization signal (NLS) and was observed previously to translocate to the nucleus of WNV_{KUN}-infected cells (Westaway et al., 1997a); its nuclear IF is confirmed in Fig. 1(f) as a positive control using Triton X-100 as the permeabilizing agent. Similar patterns of IF staining in cytoplasm but no nuclear staining were produced by both anti-NS5 antibodies in WNV_{KUN}-infected cells under fixation conditions that clearly caused nuclear staining of DEN2V NS5 and of WNV_{KUN} core protein. We concluded from the IF assays that WNV_{KUN} NS5 was located in the cytoplasm but not in the nuclei of infected cells and confirmed the nuclear and cytoplasmic locations of DEN2V NS5 (Kapoor et al., 1995).

To investigate the ultrastructural location of WNV_{KUN} NS5, we prepared cryosections of infected cells at 24 h post-infection (p.i.) (Mackenzie et al., 1996a) and labelled them with gold-conjugated antibodies to NS5, core and dsRNA (Fig. 2). Enrichment of NS5 was observed in some
VP (Fig. 2b–f) and within the convoluted membranes (CM; Fig. 2a, d–f). In dual-labelling experiments, co-localization of NS5 and dsRNA was observed only within the VP (Fig. 2c–f). In most cases, only one or two gold particles representing NS5 were observed within the dsRNA-labelled VP, in accordance with the observations noted above that very little NS5 is required for active RNA replication. In the dual-labelling experiments, the DEN2V anti-NS5 antibody appeared to react rather better on the membrane sites than the WNV KUN anti-NS5 antibody, indicating more efficient recognition by the former of the NS5 epitopes under these conditions (compare Fig. 2c with Fig. 2d–f). These results establish the association of WNV KUN NS5 with the sites of RNA synthesis in cytoplasm as a component of the RC late in infection. Cryosections positive for immunogold labelling of WNV KUN NS5 in CM were negative for nuclear staining (Fig. 2h), whereas nuclei were readily labelled with anti-core antibodies (Fig. 2g). Thus no enrichment or foci of labelled WNV KUN NS5 in nucleus was noted, in conformity with the IF results (Fig. 1). These observations indicate that WNV KUN NS5 is confined to the cytoplasm and does not translocate to the nucleus during infection. The immunolabelling results presented are the first visual evidence for the subcellular localization of flavivirus NS5 within two virus-induced membrane structures, including the site of viral RNA synthesis. Some diffuse staining was also observed throughout the cytoplasm, that was also reflected in the IF results (Fig. 1).

Recently, Uchil et al. (2006) reported that approximately 20% of the total RdRp from WNV-, JEV- and DEN2V-infected cells was associated with the nucleus. Additionally, the authors claimed that the JEV NS3 and NS5 proteins were localized within the nuclear membrane by IF (NS3 and NS5) and IEM (NS5). These results appear in conflict with a majority of the published results, as we (Mackenzie et al., 1998, 1999, 2001; Westaway et al., 1997b, 1999) and others (Miller et al., 2006) have shown that dsRNA, bromo-uridine-labelled viral RNA and NS3 are all confined to the cytoplasm. Indeed the recent report by Miller et al. (2006) clearly showed co-localization of DEN2V NS3 and dsRNA in discrete cytoplasmic foci but NS5 was confined to the nucleus that was completely devoid of dsRNA. The assumptions regarding nuclear localization made by Uchil et al. (2006) were based on the loss of normally endoplasmic reticulum- or outer nuclear envelope-membrane resident host proteins associated with the isolated nuclei fractions from infected cells. However, another factor is the possible presence of remnant membrane that has been retained with the purified nuclei, described as ‘fragile and somewhat damaged, often with debris clinging to them’ (Uchil et al., 2006). Additionally the IF images indicate only an association of NS5 and biotin-labelled viral RNA...
with peripheral nuclear membranes and not with the internal nuclear matrix. The supporting IEM indicates an association of the JEV RNA and NS3 mainly on the periphery of the nucleus and a small number of apparently dual-labelled foci just within the nucleoplasm (Uchil et al., 2006).

To investigate whether WNVKUN NS5 was post-translationally phosphorylated like other flavivirus NS5 proteins, Vero cells were infected with WNVKUN at an m.o.i. of 3 and metabolically labelled with 100 μCi (3.7 MBq) [35S]methionine/cysteine (ICN) or with 1 mCi (37 MBq) [32P]orthophosphate (ICN) in appropriate deficient medium (Gibco-BRL), from 16 to 24 h p.i. Cells were subsequently harvested and lysed in SDS lysis buffer (50 mM Tris, pH 7.4, 0.5 % SDS, 1 mM EDTA) containing protease and phosphatase inhibitors (2.5 mM PMSF, 25 μg leupeptin ml−1, 25 mM sodium orthovanadate and 25 mM sodium fluoride). Harvested lysates were diluted by adding 0.5 vols PBS before immuno-isolation with rabbit anti-NS5 antibodies and protein-A Sepharose. Immunoprecipitated proteins were electrophoresed on pre-cast 4–12 % Bis-Tris polyacrylamide gels (Invitrogen) and visualized by autoradiography. A prominent NS5 band was observed after radio-immunoprecipitation of the [35S]methionine/ cysteine-labelled cell extract (Fig. 3a), and a protein corresponding in migration to NS5 was also isolated from the [32P]orthophosphate-labelled WNVKUN-infected cells. To confirm the above observations we infected Vero cells with WNVKUN at an m.o.i. of 3 and at 24 h p.i. the cells were lysed in COP buffer (10 mM Tris 8.2, 150 mM NaCl, 5 mM EDTA and 1 % Nonidet P40 containing protease and phosphatase inhibitors), before immunoprecipitation with mouse anti-NS5 antibodies (5D4.1, kindly provided by Dr Roy Hall, University of Queensland). Immuno-isolated NS5 protein was then separated by PAGE and its phosphorylation status was assessed by Western blotting with antibodies specific to phosphorylated serine, phosphorylated threonine or phosphorylated tyrosine (phospho-protein antibody sampler pack, Zymed Laboratories). The bound antibodies were subsequently visualized with species-specific Alexa Fluor 680 (Molecular Probes) or IRDye 800CW (Rockland Incorporated)-conjugated antibodies and scanned on a LI-COR Odyssey scanner. As can be observed in Fig. 3(b) the IP samples were clearly detected with rabbit polyclonal sera specific for the WNVKUN NS5 protein. Strikingly, the WNVKUN NS5 protein was also recognized by a cocktail of antibodies specific for phosphorylated serine and threonine, but not by antibodies specific for phosphorylated tyrosine. These observations agree with the published results of other flaviviral NS5 proteins, whereby the authors showed that phosphoamino acid analysis of YFV NS5, or HCV and BVDV NS5A, revealed phosphorylation only on serine and threonine residues (Kapoor et al., 1995; Reed et al., 1998). Thus it can be concluded that WNVKUN NS5 is phosphorylated in infected cells but, unlike reports for the NS5 protein expressed during DEN2V, YFV and JEV infections, does not translocate to the nucleus. The phenotype of WNVKUN as a cytoplasmically located phosphoprotein shares homology with the hepatitis C virus RdRp NS5B protein (Hwang et al., 1997) and suggests that phosphorylation of the viral polymerase is an important functional requirement.

In conclusion, it appears that at least for WNVKUN and WNVsAR there is no apparent requirement for nuclear localization of NS5. Nevertheless, it is possible that in a post-replicative event for some flavivirus species, cytoplasmic NS5 becomes hyperphosphorylated, dissociates from NS3 and, via the pathways investigated in vitro for DEN2V NS5 (Brooks et al., 2002; Forwood et al., 1999), is transported to the nucleus where it may influence (other) protein–protein interactions that regulate the expression of cellular genes in response to viral infection (Kapoor et al., 1995; Reed et al., 1998), or induce apoptosis as reported for DEN1V (Marianneau et al., 1997) and JEV (Su et al., 2002). However, our results emphasize that for any uncharacterized flavivirus species, no assumptions should be made in regard to nuclear localization of NS5 (Lindenbach & Rice, 2003). Furthermore, absence from the nucleus of WNVKUN NS5, WNVsAR NS5 and the dsRNA template indicates that nuclear involvement of NS5 is neither a constant nor essential feature of flavivirus replication. Finally we present the first evidence for any flavivirus that NS5 is localized in induced membranes at the site of replication (VP) and within CM.

Fig. 3. (a) Radio-immunoprecipitation and analyses of WNVKUN NS5 in mock-infected or infected Vero cells after metabolic labelling with [35S]methionine/cysteine or [32P]orthophosphate. Radiolabelled NS5 was immunoprecipitated with anti-NS5 antibodies and protein-A Sepharose before PAGE and visualization by autoradiography. (b) Western blot of immunoprecipitated WNVKUN NS5 with antibodies specific for phosphorylated serine and threonine (Rb anti-p-Ser/Thr) and phosphorylated tyrosine (Ms anti-p-Tyr), or for WNVKUN NS5 (Rb anti-NS5). The first lane shows the migration position of stained protein markers.
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