JOURNAL OF VIROLOGY, July 2003, p. 7804–7813 Vol. 77, No. 14
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.14.7804–7813.2003

Molecular and Functional Analyses of Kunjin Virus Infectious cDNA Clones Demonstrate the Essential Roles for NS2A in Virus Assembly and for a Nonconservative Residue in NS3 in RNA Replication†

Wen Jun Liu, Hua Bo Chen, and Alexander A. Khromykh*

Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, and Clinical Medical Virology Centre, University of Queensland, Brisbane, Queensland 4029, Australia

Received 30 December 2002/Accepted 18 April 2003

A number of full-length cDNA clones of Kunjin virus (KUN) were previously prepared; it was shown that two of them, pAKUN and FLSDX, differed in specific infectivities of corresponding in vitro transcribed RNAs by ~100,000-fold (A. A. Khromykh et al., J. Virol. 72:7270-7279, 1998). In this study, we analyzed a possible genetic determinant(s) of the observed differences in infectivity initially by sequencing the entire cDNAs of both clones and comparing them with the published sequence of the parental KUN strain MRM61C. We found six common amino acid residues in both cDNA clones that were different from those in the published MRM61C sequence but were similar to those in the published sequences of other flaviviruses from the same subgroup. pAKUN clone had four additional codon changes, i.e., Ile59 to Asn and Arg175 to Lys in NS2A and Tyr518 to His and Ser557 to Pro in NS3. Three of these substitutions except the previously shown marker mutation, Arg175 to Lys in NS2A, reverted to the wild-type sequence in the virus eventually recovered from pAKUN RNA-transfected BHK cells, demonstrating the functional importance of these residues in viral replication and/or viral assembly. Exchange of corresponding DNA fragments between pAKUN and FLSDX clones and site-directed mutagenesis revealed that the Tyr518-to-His mutation in NS3 was responsible for an ~5-fold decrease in specific infectivity of transcribed RNA, while the Ile59-to-Asn mutation in NS2A completely blocked virus production. Correction of the Asn59 in pAKUN NS2A to the wild-type Ile residue resulted in complete restoration of RNA infectivity. Replication of KUN replicon RNA with an Ile59-to-Asn substitution in NS2A and with a Ser557-to-Pro substitution in NS3 was not affected, while the Tyr518-to-His substitution in NS3 led to severe inhibition of RNA replication. The impaired function of the mutated NS2A in production of infectious virus was complemented in *trans* by the helper wild-type NS2A produced from the KUN replicon RNA. However, replicon RNA with mutated NS2A could not be packaged in *trans* by the KUN structural proteins. The data demonstrated essential roles for the KUN nonstructural protein NS2A in virus assembly and for NS3 in RNA replication and identified specific single-amino-acid residues involved in these functions.

Kunjin virus (KUN) is an Australian flavivirus closely related to other members of the Japanese encephalitis virus subgroup. The KUN genome consists of single-stranded RNA of positive polarity comprising 11,022 nucleotides (10), with one long open reading frame coding 3,433 amino acids in three structural proteins (C, prM, and E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (6). The gene order of KUN genome RNA is 5′-C-(pr)M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′.

Generation of the flavivirus full-length cDNA clones has been hampered by their apparent instability in *Escherichia coli*, leading to extensive mutagenesis of cDNA sequences during preparation of plasmid DNAs (25). These mutations usually resulted in the complete or partial loss of infectivity of RNAs transcribed in vitro from the cDNA templates. Other researchers have applied different approaches to overcome this problem, including splitting the full-length cDNA sequence into two pieces for amplification followed by transcription of RNA from the in vitro-ligated DNA template (24), use of a particular *E. coli* strain, and lowering of the temperature of incubation while growing plasmid DNA in *E. coli* (7, 18); using very low-copy-number vectors (8); avoiding amplification of cDNA in *E. coli* by assembly of full-length cDNA sequence by using long PCR (7); multiple corrections of mutated sequences (23); and inserting introns to separate toxic regions (30).

Over the years a number of stable infectious full-length cDNA clones of KUN that produced RNAs of different specific infectivities have been generated (10, 12). KUN RNA transcribed from our originally prepared full-length cDNA clone pAKUN had very low specific infectivity (~1 PFU per 10 μg of RNA), and the recovered virus differed from the parental KUN by a smaller plaque phenotype and a delayed replication in cells and in mice (10, 12). Later reconstruction of KUN cDNA from viral RNA by using reverse transcription and long PCR amplification with high-fidelity DNA polymerase resulted in generation of cDNA clones FLSD (with a 7-kb fragment in pAKUN replaced) and FLSDX (with a 9.5-kb fragment in pAKUN replaced), which produced RNAs with dramatically improved specific infectivities (2×10^3 and 10^4 PFU/μg of RNA, respectively) (12). Further characterization

* Corresponding author. Mailing address: Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, Brisbane, Queensland 4029, Australia. Phone: 617 36361568. Fax: 617 36361401. E-mail: a.khromykh@mailbox.uq.edu.au.

† This is publication number 161 from the Clinical Medical Virology Centre and the Sir Albert Sakzewski Virus Research Centre.

TABLE 1. Differences between published KUN MRM61C sequences and sequences of infectious KUN cDNA clones[d]

Nucleotide position	Gene	Amino acid position in encoded protein	MRM61C data		FLSDX cDNA data		pAKUN cDNA data		Corresponding residue in related flaviviruses
			Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	
544	PrM	28	C[a]	Pro	**A**	**Thr**	**A**	**Thr**	Thr (WN, JE, MVE)
1500	E	178	G	Leu	T[c]	—	—	—	Leu (WN, JE)/Ala MVE)
1635	E	223	C	Leu	—	—	T	—	Leu (WN, MVE, JE)
1824	E	286	C	Leu	G	—	G	—	Leu (WN, MVE, JE)
2556	NS1	29	T[a]	Ile	**G**	**Met**	**G**	**Met**	Ile (MVE)/Met (WN)/Val (JE)
2924	NS1	152	A[a]	Asn	**G**	**Ser**	**G**	**Ser**	Ser (WN, MVE, JE)
3218	NS1	250	C	Pro	**T**[b]	**Leu**	**T**[b]	**Leu**	Pro (WN, MVE, JE)
3701	NS2A	59	T	Ile	—	—	**A**[b]	**Asn**	Ile (WN, MVE)/Val (JE)
4049	NS2A	175	G	Arg	—	—	**A**[c]	**Lys**	Arg (WN, MVE, JE)
5937	NS3	442	A	Val	**G**	—	**G**	—	Val (WN, MVE, JE)
5996	NS3	462	C[a]	Thr	**T**	**Ile**	**T**	**Ile**	Ile (WN)/Val (JE, MVE)
6017	NS3	469	C[a]	Ala	**T**	**Val**	**T**	**Val**	Val (WN, JE)/Ile (MVE)
6163	NS3	518	T	Tyr	—	—	**C**[b]	**His**	Tyr (WN, MVE)/Phe (JE)
6280	NS3	557	T	Ser	—	—	**C**[b]	**Pro**	Ser (WN)/Gln (JE, MVE)
7112	NS4B	66	C[a]	Thr	**A**	**Asn**	**A**	**Asn**	Asn (WN)/Thr (MVE, JE)
7293	NS4B	126	G	Val	A	—	A	—	Val (WN)/Leu (MVE, JE)
7785	NS5	35	C	Val	T	—	T	—	Val (WN, MVE, JE)
10197	NS5	839	C	Ser	T	—	T	—	Ser (WN)/Thr (JE, MVE)

[a] Apparent errors in the published MRM61C.
[b] Mutations probably introduced during cloning.
[c] Mutation introduced to produce cDNA clones.
[d] Nucleotides in boldface in cDNA clones indicate changes from the original published sequence that lead to changes in corresponding amino acids, also shown in boldface. —, no differences from wild type were found in the indicated nucleotide or in the corresponding encoded amino acid. WN, West Nile virus; JE, Japanese encephalitis virus; MVE, Murray Valley encephalitis virus.

of the virus recovered from FLSD RNAs showed a delayed replication in Vero cells and reduced virulence in mice (9). Selective sequencing of the NS1 gene in the FLSD cDNA clone and in the RNA isolated from virus recovered after transfection of FLSD RNA showed the presence of a proline (Pro)-to-leucine (Leu) substitution of NS1 amino acid codon 250 (Table 1), which was surprisingly stable and was retained in the virus for at least 10 passages (9). Correction of this mutation from Leu back to the Pro codon in the FLSD cDNA clone resulted in the recovery of virus indistinguishable from the wild-type KUN strain MRM61C in the kinetics of viral growth in Vero cells and in virulence in mice (9). However, the effect of this correction in NS1 on specific infectivity of transcribed RNA and on the phenotype of viral plaques, as well as the remaining sequence in different cDNA clones leading to such dramatic differences in their infectivity, was not determined. In this study we utilized sequencing analysis of the entire cDNA of pAKUN and FLSDX clones and site-directed mutagenesis for functional analyses of full-length and replicon RNAs to show that the low efficiency of pAKUN RNA in production of infectious KUN was mainly due to the presence of two amino acid substitutions, one in NS3 that severely inhibited RNA replication and another in NS2A that unexpectedly blocked virus assembly. We also employed here previously described complementation and encapsidation assays (12, 13) with full-length and replicon RNAs to show that only wild-type (and not mutated) NS2A when present either in *cis* or in *trans* can participate in packaging of RNA into secreted virus particles.

MATERIALS AND METHODS

Cells. BHK21 cells were grown in Dulbecco minimal essential medium (DMEM; Invitrogen, San Diego, Calif.) supplemented with 10% fetal bovine serum (FBS) at 37°C in a CO_2 incubator. repBHK cells containing stably replicating KUN replicon RNA (12) were maintained in the same medium supplemented with 0.5 to 1 mg of G418 (Geneticin; Invitrogen) per ml.

Construction of plasmids. Plasmids pAKUN/FLSDX2A, pAKUN/FLSDX3, FLSDX/pAKUN2A, and FLSDX/pAKUN3 were obtained by exchanging the fragments *Sac*II^{1482}-*BssH*II^{5743} and *BssH*II^{5743}-*Age*I^{7897} between previously described KUN full-length cDNA clones pAKUN (10) and FLSDX (12) (Fig. 1). Plasmid FLSDX/pAKUN2A(Ile59) (Fig. 1) was generated by site-directed PCR mutagenesis of the Asn59 codon in NS2A to Ile in FLSDX/pAKUN2A by using high-fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). The mutagenesis was performed on the intermediate plasmid pUC-NS2A containing the *Sph*I^{3628}-*Bam*HI^{4807} fragment from the pAKUN plasmid in the pUC18 vector. After confirmation of the introduced mutation by sequencing analysis, the *Sph*I^{3628}-*Bam*HI^{4807} fragment including the Asn59-to-Ile mutation in NS2A was transferred into FLSDX/pAKUN2A. The mutation in the resulting FLSDX/pAKUN2A (Ile59) plasmid was confirmed by sequencing analysis. KUN replicon plasmids repPACβ-gal/2Amut and repPACβ-gal/3mut were prepared by replacing the *Sph*I^{3628}-*BstB*I^{5148} and *BstB*I^{5148}-*Age*I^{7897} fragments, respectively, in a previously described KUN replicon construct, repPACβ-gal (20), with those from the pAKUN plasmid (Fig. 1). Plasmids repPACβ-gal/3mut(His518) and repPACβ-gal/3mut(Pro557) were constructed by site-directed PCR mutagenesis with high-fidelity *Pfu* DNA polymerase to change Pro557 in NS3 back to the wild-type Ser and His518 in NS3 back to the wild-type Tyr, respectively. The mutagenesis was performed initially on the intermediate plasmid pUC-NS3-5 containing the *Sal*I^{5384}-*Sal*I^{7884} fragment from pAKUN. After confirmation of the introduced mutation by sequencing analysis, the *Sal*I^{5384}-*Sal*I^{7884} fragments including the above mutations were transferred into the repPACβ-gal/3mut plasmid. The plasmids FLSDX/pAKUN3(His518) and FLSDX/pAKUN3(Pro557) (Fig. 1) were generated by replacing *BssH*II^{5743}-*Age*I^{7897} fragments in FLSDX/pAKUN3 with those from repPACβ-gal/3mut(His518) and repPACβ-gal/3mut(Pro557), respectively. All mutations in the resulted replicon and full-length plasmids were confirmed by sequencing.

RNA transcription, transfection, and determination of specific infectivity. All RNA transcripts were prepared with SP6 RNA polymerase from *Xho*I-linearized plasmids DNAs and were purified by using Bio-spin 30 chromatography columns (Bio-Rad, Hercules, Calif.). Purified RNAs were electroporated into BHK21 cells, essentially as described previously (11). Briefly, 1 μg of in vitro-transcribed RNAs was electroporated into 2×10^6 BHK21 (normal BHK) or repBHK cells in 400 μl in a 0.2-cm-electrode-gap cuvette (Bio-Rad) with a Bio-Rad Gene Pulser apparatus. Electroporated cells were then used to prepare serial 10-fold dilutions in DMEM–10% FBS, mixed with 10^6 nontransfected BHK21 cells, and seeded on 60-mm-diameter culture dishes for 8 h to allow cells to attach. Then cells were overlaid with DMEM–2% FBS in 0.75% agarose (FMC, Rockland, Maine). After 4 to 6 days of incubation at 37°C, cells were fixed with 20% formaldehyde and were stained with crystal violet.

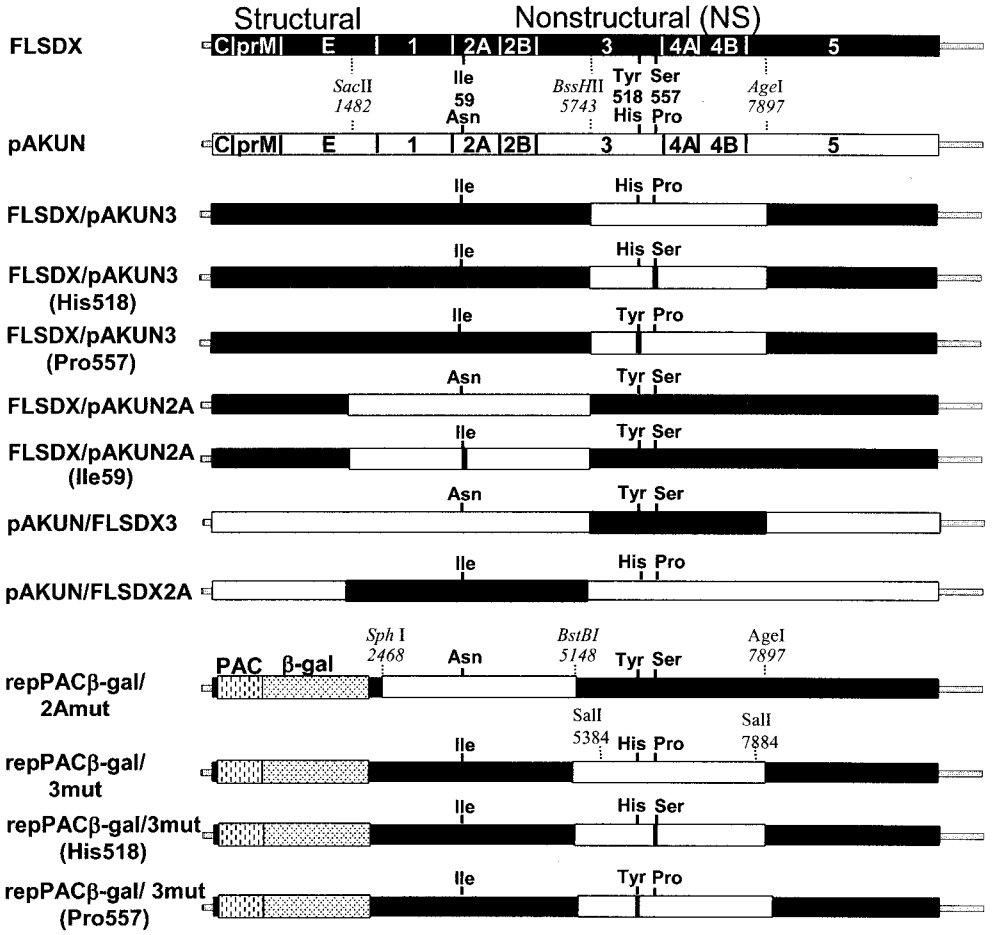

FIG. 1. Schematic representation of the full-length and replicon KUN cDNA constructs with exchanged fragments. The filled box represents the sequence of the FLSDX clone; the open box represents the sequence of the pAKUN clone. *Sac*II, *Sph*I, *BstB*I, *BssH*II, *Age*I, and *Sal*I show restriction sites used in construction of plasmids by fragment exchange; numbers under the restriction sites represent corresponding nucleotide positions in the full-length KUN sequence (6, 10). In the designations of the full-length constructs generated by fragment exchange, first letters represent the name of the clone used as the vector backbone and are separated by a slash from the letters representing the name of the clone used as a source of the cloned fragment. Replicon constructs contain the puromycin acetyltransferase gene (PAC) and β-Gal gene (β-gal) cassette inserted in FLSDX(pro) in place of deleted structural genes (20). Amino acids shown in different recombinant constructs represent corresponding wild-type or mutated residues in NS2A (position 59) and NS3 (positions 518 and 557) genes identified in the original FLSDX and pAKUN constructs. Ile, isoleucine; Asn, asparagine; His, histidine; Pro, proline; Tyr, tyrosine; and Ser, serine.

IF. Replication and expression of mutated full-length RNAs in transfected cells were monitored by immunofluorescence (IF) analysis with mouse monoclonal antibodies to KUN E protein (1) as described elsewhere (12).

X-Gal staining and Northern blotting. Detection of replication and expression of mutated replicon RNAs in BHK21 cells were performed by in situ staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyopyranoside (X-Gal) or by Northern blot with ^{32}P-labeled probes specific for β-galactosidase (β-Gal) and β-actin nucleotide sequences, as described previously (20).

Nucleotide sequence accession number. The pAKUN and FLSDX cDNA sequences have been deposited in GenBank and have been given the accession numbers AY274505 and AY274504, respectively.

RESULTS

Comparison of plaque morphology and specific infectivities of KUN RNAs transcribed from different cDNA clones and of the recovered viruses. As the first step towards identification of the primary determinates of virus replication in different cDNA clones, we compared specific infectivities of transcribed RNAs and plaque morphologies of the recovered viruses. Plaque assays of BHK cells electroporated with RNAs transcribed from AKUN, FLSDX, and FLSDX(Pro) (FLSDX cDNA clone with a proline instead of leucine residue at NS1 amino acid 250) cDNA clones (Fig. 1) and of KUN virion RNA (KUN[wt]) in BHK cells showed specific infectivities of <1, 1.3×10^4, 2×10^4, and 2×10^5 PFU per μg of RNA, respectively (Fig. 2A and Table 2). Incorporation of the antigenomic sequence of hepatitis delta virus ribozyme (HDVr) into the FLSDX(pro) cDNA clone immediately downstream of the last nucleotide of KUN sequence increased specific infectivity of the resulting FLSDX(pro)HDVr RNA by ~2.6-fold (5.3×10^4 PFU/μg [Table 2]) compared to that of FLSDX(pro) RNA. The size of the viral plaques after transfection of FLSDX(pro) and FLSDX(pro)HDVr RNA was similar to that after transfection of KUN(wt) virion RNA, while transfection of FLSDX RNA produced much smaller plaques (Fig. 2A for FLSDX and FLSDX[pro]; results not shown for FLSXD[pro]HDVr). Viruses recovered after transfection of these RNAs into BHK

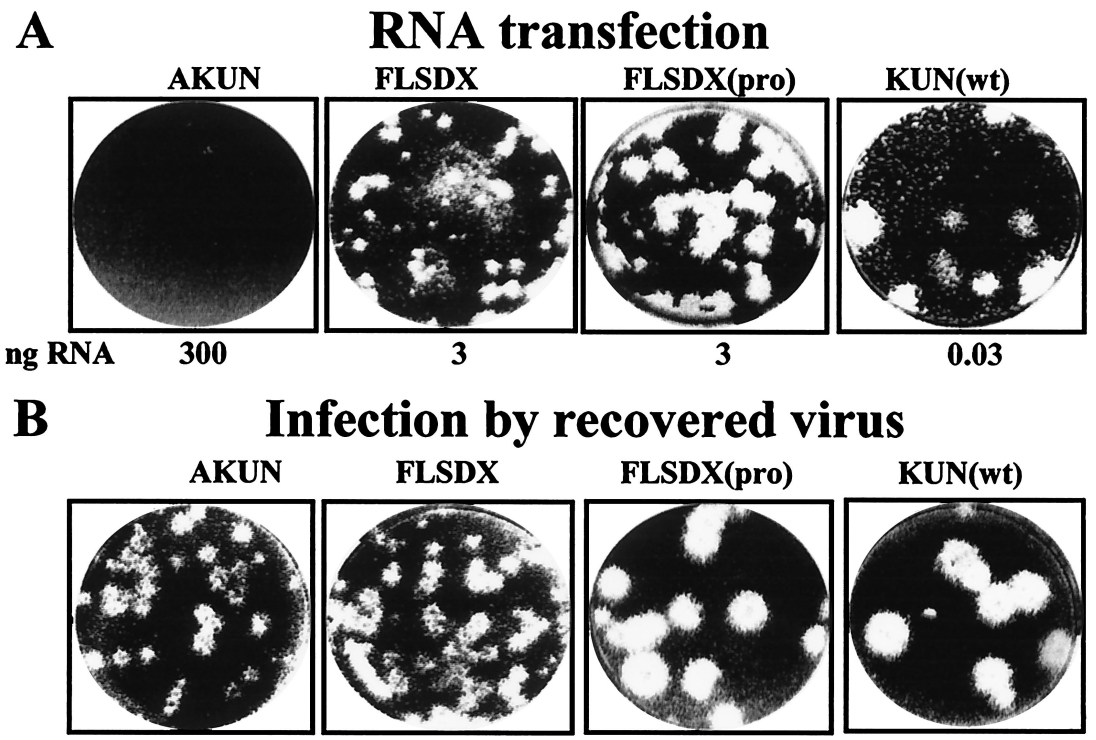

FIG. 2. Viral plaque morphology in BHK cells transfected with the KUN virion RNA (wild type [wt]) and with the indicated engineered RNAs (A) and infected with the corresponding recovered viruses (B). BHK cells were electroporated with indicated RNAs or infected with indicated recovered viruses and were assayed for plaque morphology as described in Materials and Methods. "ng RNA" under the panels in A shows the amount of transfected RNA in nanograms calculated from appropriate dilution of transfected cells in the corresponding dishes. Viral plaques in panel A were visualized at day 4 after RNA electroporation and in panel B at day 5 after infection with corresponding recovered viruses at 10^{-4} dilution.

cells produced plaques similar in size to those formed in BHK cells transfected with corresponding RNAs (Fig. 2B). The increase in size of plaques and specific infectivity of FLSDX(pro) RNA compared to those of FLSDX RNA confirmed previous observations on the importance of amino acid 250 in NS1, shown to be essential for dimerization of this protein, in virus replication in cells and in virulence in mice (9).

Interestingly, in contrast to the extremely low efficiency of plaque formation after transfection of pAKUN RNA, infection by recovered AKUN produced well-defined plaques (Fig. 2B) and it replicated efficiently (10). The retention of the introduced mutation in the NS2A gene (Arg175 to Lys) in the recovered AKUN has previously been confirmed (10); however, the rest of the sequence of pAKUN cDNA and of the recovered AKUN, as well as of FLSDX cDNA (except for the NS1 gene [9]), has not been determined. It was reasonable to assume that the low specific infectivity of pAKUN RNA was due to the presence of mutations inhibiting virus replication and/or assembly and that these mutations were corrected in the recovered AKUN. It was also likely that these inhibiting muta-

TABLE 2. Specific infectivities of KUN RNAs transcribed from different full-length cDNA constructs[a]

RNA	Amino acid in encoded protein					Specific infectivity (PFU/μg of RNA)
	NSI-250	NS2A-59	NS2A-175	NS3-518	NS3-557	
Wild-type virion RNA	Pro	Ile	Arg	Tyr	Ser	2×10^5
FLSDX (pro)	—	—	—	—	—	2.0×10^4
FLSDX (pro)HDVr	—	—	—	—	—	5.3×10^4
FLSDX	Leu	—	—	—	—	1.3×10^4
pAKUN	Leu	Asn	Lys	His	Pro	<1
pAKUN/FLSDX2A	Leu	—	Lys	His	Pro	2×10^3
pAKUN/FLSDX3	Leu	Asn	Lys	—	—	<1
FLSDX/pAKUN3	Leu	—	—	His	Pro	1.8×10^3
FLSDX/pAKUN3 (His518)	Leu	—	—	His	—	2.5×10^3
FLSDX/pAKUN3 (Pro557)	Leu	—	—	—	Pro	1×10^4
FLSDX/pAKUN2A	Leu	Asn	Lys	—	—	<1
FLSDX/pAKUN2A (Ile59)	Leu	—	Lys	—	—	1.4×10^4

[a] —, no differences from the wild type are shown in the indicated encoded amino acid residue.

tions originally present in pAKUN cDNA were corrected in FLSDX cDNA, leading to dramatic improvement in the specific infectivity of FLSDX RNA.

Sequencing analysis of KUN full-length cDNA clones pAKUN and FLSDX. To confirm the above assumption, we decided to determine the entire genomic sequence of pAKUN and FLSDX cDNA clones and compare them with the previously published sequence of the MRM61C strain of KUN (GenBank accession No. D00246) (6). The differences between pAKUN, FLSDX, and the published KUN sequence are shown in Table 1. FLSDX cDNA had 13 nucleotides different from the published KUN sequence, with seven of them leading to amino acid changes; pAKUN cDNA had 17 nucleotides different from the published KUN sequence, with 11 of them leading to amino acid changes. These amino acid mutations were scattered throughout the entire coding region and were located in the prM, E, NS1, NS2A, NS3, and NS4B genes. No mutations were found in NS2B, NS4A, and NS5 or in the 5′ and 3′ untranslated regions. pAKUN and FLSDX cDNA clones had six common amino acid substitutions, mainly conserved, that were different from the original MRM61C published sequence (Table 1). These six common amino acids detected in both FLSDX and pAKUN sequences aligned better with the sequences of other flaviviruses from the same subgroup than with the published MRM61C sequence, suggesting that they represent corrected errors in the originally published MRM61C sequence. The Pro250-to-Leu codon change in the NS1 gene was present in both pAKUN and FLSDX cDNAs and apparently contributed to delayed replication of recovered viruses (9). Thus, with the exclusion of one amino acid difference in NS1 (Pro250 to Leu) that apparently arose during cloning, FLSDX cDNA was identical to the corrected wild-type KUN RNA sequence.

One of two mutations in NS2A in pAKUN clone represented a conserved amino acid change (Arg175 to Lys) that was introduced intentionally as a marker mutation during plasmid construction. It was demonstrated previously that this mutation was retained in the recovered AKUN and did not affect viral replication (10). Thus, it left only 3 amino acid codons in pAKUN cDNA different from those in FLSDX cDNAs and/or to those in corrected MRM61C viral RNA sequences (nucleotide 3701T to A, changing NS2A amino acid Ile59 to Asn; nucleotide 6163T to C, changing NS3 amino acid Tyr518 to His; and nucleotide 6280T to C, changing NS3 amino acid Ser557 to Pro) (Table 1). These changes apparently contributed to the low specific infectivity of transcribed pAKUN RNA. To examine whether these mutations were present in RNA of the recovered AKUN, we performed reverse transcriptase PCR and sequence analysis of the corresponding genomic regions in viral RNA. The results showed that all these three mutations but not the marker mutation (Arg175 to Lys in NS2A) reverted to the wild-type sequence in the recovered viral RNA (data not shown), clearly demonstrating the importance of these amino acid residues in virus replication.

Mutations in NS3 of pAKUN inhibit virus replication. In order to further identify the specific amino acid substitutions that contributed to the low specific infectivity of pAKUN RNA, we prepared a number of cDNA constructs containing these mutations by exchanging corresponding DNA fragments between pAKUN and FLSDX plasmids (Fig. 1). pAKUN/FLSDX3 and pAKUN/FLSDX2A plasmids contained pAKUN backbone and corresponding fragments from FLSDX plasmid, including NS3 and NS2A regions, respectively (Fig. 1). FLSDX/pAKUN3 and FLSDX/pAKUN2A contained FLSDX backbone and fragments from pAKUN plasmids, including the NS3 and NS2A regions, respectively (Fig. 1). We first compared replication efficiencies and specific infectivities of RNAs containing pAKUN-derived NS3 sequence (pAKUN/FLSDX2A and FLSDX/pAKUN3) with that of the parental pAKUN and FLSDX RNAs by using IF analysis with anti-E antibodies and plaque assays. Transfection of pAKUN/FLSDX2A, FLSDX/pAKUN3, and FLSDX RNAs into BHK cells showed an increase in the number of IF-positive cells from 24 to 48 h after transfection, demonstrating the spread of infectious virus (Fig. 3). The detection of IF-positive cells indicated that all RNAs were replicating, since it was previously clearly demonstrated that nonreplicating KUN RNA degraded quickly in cells and that only replicating KUN RNA could express sufficient amount of proteins for IF detection (11). pAKUN/FLSDX2A and FLSDX/pAKUN3 also produced well-defined small plaques (relative to wild-type virus) characteristic for FLSDX RNA (results not shown). As expected from previous experiments, transfection of pAKUN RNA did not lead to the detection of the virus spread within 48 h by IF analysis (Fig. 3) or formation of defined plaques by 4 days in plaque assays (Fig. 2). Specific infectivities of pAKUN/FLSDX2A and FLSDX/pAKUN3 RNAs were similar (2×10^3 and 1.8×10^3 PFU/μg, respectively) and were approximately six- to sevenfold lower than that of FLSDX RNA (Table 2). Site-directed mutagenesis of the His518 and Pro557 mutations to wild-type Tyr and to Ser residues, respectively, showed that the decrease in efficiency of NS3-mutated RNA was largely due to the Tyr518-to-His substitution, while the Ser557-to-Pro substitution had only a marginal effect (Table 2). These data clearly showed that the Tyr518-to-His mutation in NS3 present in pAKUN RNA had an inhibitory effect at least early in virus replication but did not block production and spread of the infectious virus particles.

Mutations in NS2A block production of infectious virus. In contrast to the results with RNAs containing mutations in NS3, transfection of RNAs containing mutations in NS2A, i.e., pAKUN/FLSDX3 and FLSDX/pAKUN2A as well as pAKUN, did not lead to the detection of virus spread from 24 to 48 h by IF analysis (Fig. 3) and did not produce plaques in plaque assays by 4 days (data not shown), demonstrating the inability of these RNAs to produce infectious virus particles at least early in virus replication before the reversion could occur. All these three constructs had only one common amino acid substitution, Ile59 to Asn in NS2A (Table 2), suggesting that residue 59 in NS2A may play a crucial role in ensuring the functioning of NS2A in production of virus particles. To confirm this observation, we prepared another construct, FLSDX/pAKUN2A(Ile59), in which we mutated Asn59 in FLSDX/pAKUN2A back to the wild-type Ile residue (Fig. 1). In contrast to the parental FLSDX/pAKUN2A RNA, transfection of FLSDX/pAKUN2A(Ile59) RNA into BHK cells resulted in production of well-defined viral plaques (results not shown) and the specific infectivity of this RNA was similar to that of the FLSDX RNA (Table 2). Thus, it was clear from these

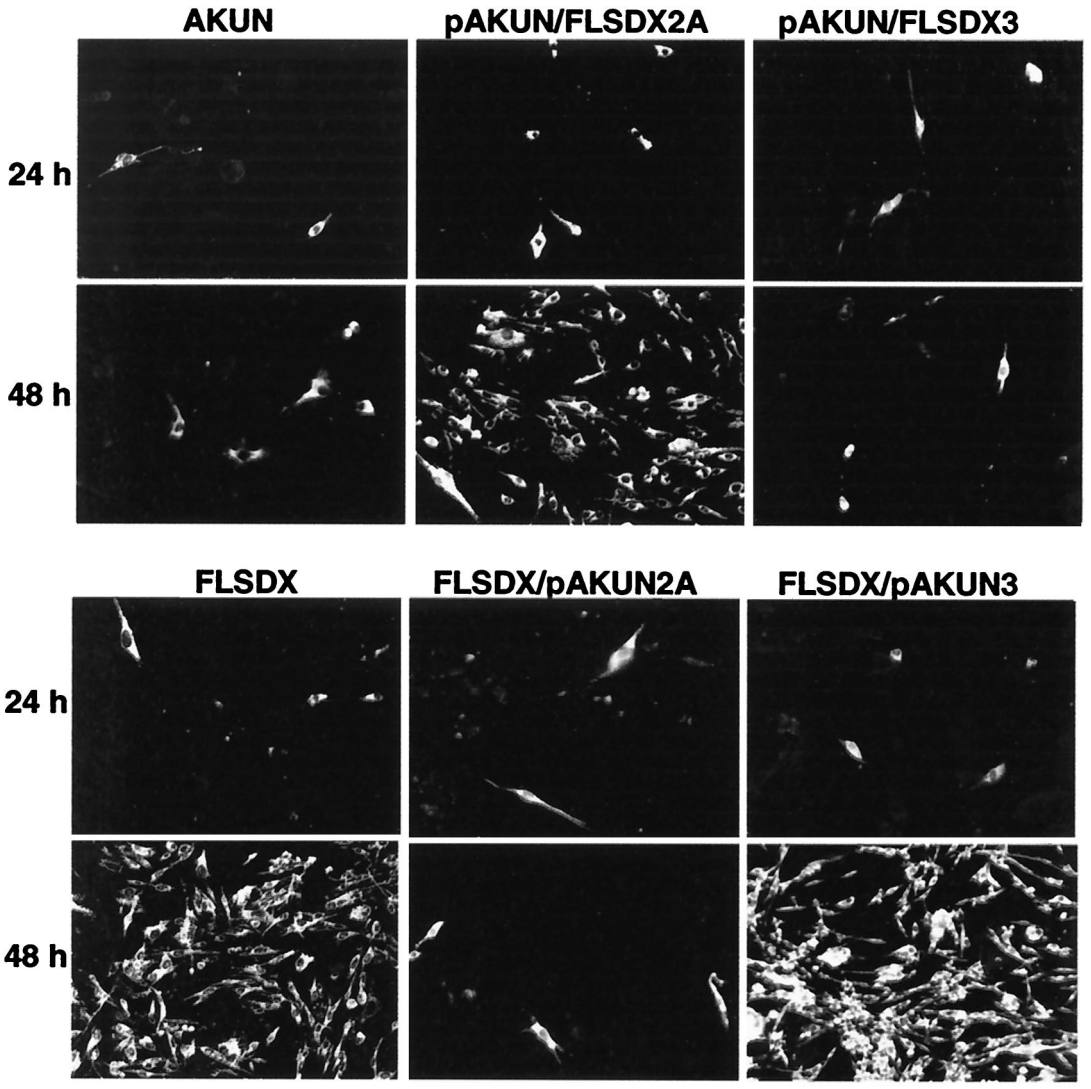

FIG. 3. IF analysis of BHK cells electroporated with mutated RNAs. One microgram of RNAs transcribed from indicated full-length KUN cDNA clones was electroporated into 2×10^6 BHK21 cells, and 2×10^5 cells were seeded on coverslips in 24-well plates. Twenty-four or 48 h later, cells were fixed with acetone and were stained with KUN anti-E antibodies as described in Materials and Methods.

results that a single Ile59-to-Asn substitution in NS2A led to a complete block to the production of infectious virus particles.

Effects of mutations in NS2A and in NS3 on RNA replication. Although in the previous sections we demonstrated the detrimental effect of mutations in NS2A on production of virus particles and the inhibiting effect of mutations in NS3 on virus replication, it was not clear from these results whether these mutations affected RNA replication, virus assembly, or virus release. To examine the effect of the mutations in NS2A and NS3 on RNA replication, we initially prepared two KUN replicon constructs, repPACβ-gal/2Amut and repPACβ-gal/3mut (Fig. 1), by transferring the fragments with NS2A and NS3 mutations, respectively, from pAKUN into the replicon construct repPACβ-gal (20). repPACβ-gal was prepared previously from FLSDX(Pro) clone and therefore represents the wild-type KUN sequence. It encodes β-Gal for easy detection of expression of replicating RNAs. Transfection of BHK cells with repPACβ-gal and repPACβ-gal/2Amut RNAs resulted in the detection of similar number of positive cells after X-Gal staining (~80 to 90%; panels 1 and 2 in Fig. 4A), while a significantly lower number of positive cells was detected by X-Gal staining after a transfection with repPACβ-gal/3mut RNA (~5 to 10%; panel 4 in Fig. 4A). Analysis of β-Gal expression in transfected cell lysates also showed a significantly smaller amount of β-Gal produced from repPACβ-gal/3mut RNA than those produced from repPACβ-gal and repPACβ-gal/2Amut RNAs (compare bar 4 with bars 1 and 2 in Fig. 4B). Northern blot analysis of transfected cells confirmed the results of X-Gal staining and β-Gal assay (Fig. 4C). The amount of housekeeping gene (β-actin) mRNA in cells transfected with each RNA cell line was similar (Fig. 4C). These results demonstrated that mutations in NS3 but not in NS2A were responsible for the inhibition of RNA replication.

To determine which of the two mutations in NS3 inhibited RNA replication, we prepared two more replicon constructs, repPACβ-gal/3mut(His518) and repPACβ-gal/3mut(Pro557),

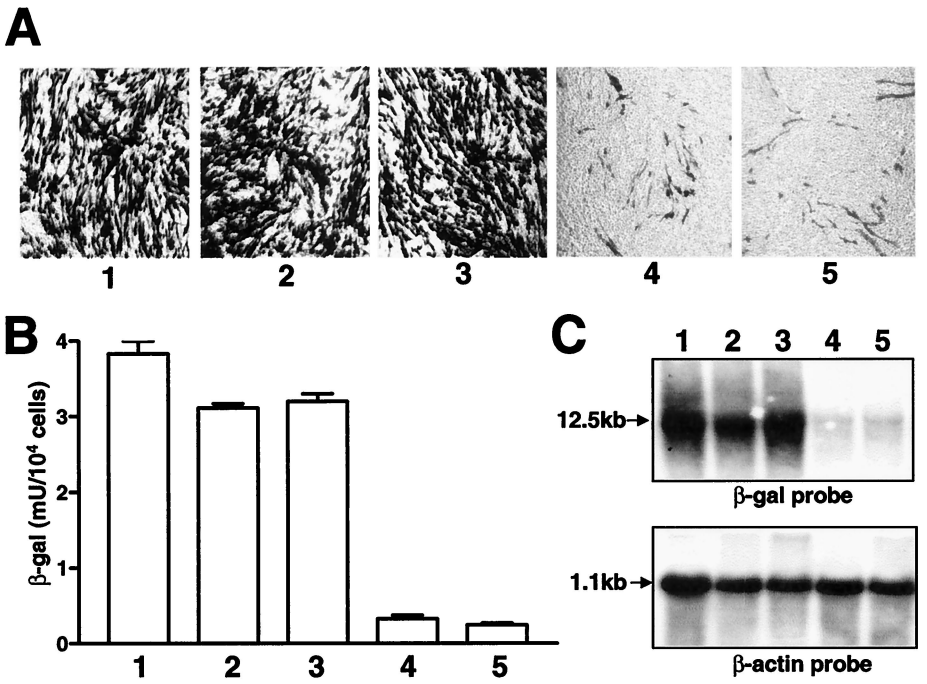

FIG. 4. Comparison of replication efficiencies of KUN replicon RNAs with mutations in NS2A and NS3. BHK21 cells were electroporated with the indicated mutated replicon RNAs. Forty-eight hours after electroporation, cells were either fixed by 4% formaldehyde–phosphate-buffered saline and were stained in situ with X-Gal (A) or lysed for a β-Gal assay (B). Total RNA was also isolated for analysis of accumulation of replicating RNA by Northern blot with ^{32}P-labeled probes specific for β-Gal (C, top row) or for β-actin (C, bottom row) nucleotide sequences. For panel C, ~10 μg of total RNA was used for hybridization; arrows indicate positions in the gel of RNAs of 12.5 kb (C, top row) and 1.1 kb (C, bottom row) determined relative to migration in the same gel of the ethidium bromide-stained 1-kb ladder (Invitrogen). Numbering of corresponding panels (A), bars (B), and lanes (C) corresponds to cells transfected with repPACβ-gal RNA (1), repPACβ-gal/2Amut RNA (containing the Asn59 mutation) (2), repPACβ-gal/3mut(Pro557) RNA (3), repPACβ-gal/3mut RNA (with both His518 and Pro557 mutations) (4), and repPACβ-gal/3mut(His518) RNA (5).

each containing corresponding individual amino acid residues different from the wild-type sequence (Fig. 1). X-Gal staining, β-Gal assay, and Northern blotting clearly showed that replacement of wild-type Tyr518 by His was responsible for severe inhibition of RNA replication, similar to that observed with the double mutant, while another substitution, wild-type Ser557 with Pro, produced only a marginal effect (Fig. 4A to C). These results obtained with the mutated replicon RNAs were in agreement with the results obtained with the mutated full-length RNAs (Table 2).

Complementation of mutated NS2A in *trans*. Recent studies with yellow fever virus (YF) showed that NS2A with a mutation abolishing an internal cleavage site, QK ↓ T, at amino acid 190 and leading to an unexpected block in virus production could be rescued in *trans* by providing helper NS2A protein from another expression vector (17). It was therefore of interest whether KUN NS2A with the Ile59-to-Asn mutation blocking virus production could be complemented in *trans*. To provide helper NS2A for complementation of NS2A-mutated KUN RNA, we employed a complementation system based on the use of repBHK cells stably expressing NS2A as well as other KUN nonstructural proteins from KUN replicon RNA (15). Transfection of repBHK cells with pAKUN/FLSDX3 and FLSDX/pAKUN2A both containing the Ile59-to-Asn mutation in NS2A resulted in detection of viral plaques (Fig. 5), demonstrating successful complementation of their replication by the wild-type NS2A produced from the helper replicon RNA. It has been demonstrated many times previously that recombination does not occur during complementation of defective genomic KUN RNAs in repBHK cells (see, for example, references 12, 14, and 15) and that therefore, viral plaques shown in pAKUN/FLSDX3 and FLSDX/pAKUN2A panels in Fig. 5 represent the result of true complementation.

Replicon RNA with an Ile59-to-Asn substitution in NS2A cannot to be packaged in *trans*. In order to investigate possible mechanisms by which NS2A may be involved in virus assembly, we decided to examine whether replicon RNA with the Ile59-to-Asn mutation in NS2A could be packaged into virus particles by providing structural proteins in *trans*. We used an established *trans* encapsidation system that employs a Semliki Forest virus (SFV) replicon expression vector for production of KUN structural proteins (SFV-MEC105) (13, 26). BHK cells were first electroporated with KUN replicon RNA repPACβ-gal or repPACβ-gal/2Amut containing the NS2A Asn59 mutation. X-Gal staining of transfected cells showed that transfection efficiencies of both RNAs were similar (data not shown). Thirty-two hours after the electroporation with KUN replicon RNAs, cells were electroporated again with SFV-MEC105 RNA. The culture fluids harvested at 48 h after the second electroporation were tested for the presence of virus-like particles containing encapsidated replicon RNAs by infecting Vero cells and staining them with X-Gal 48 h after

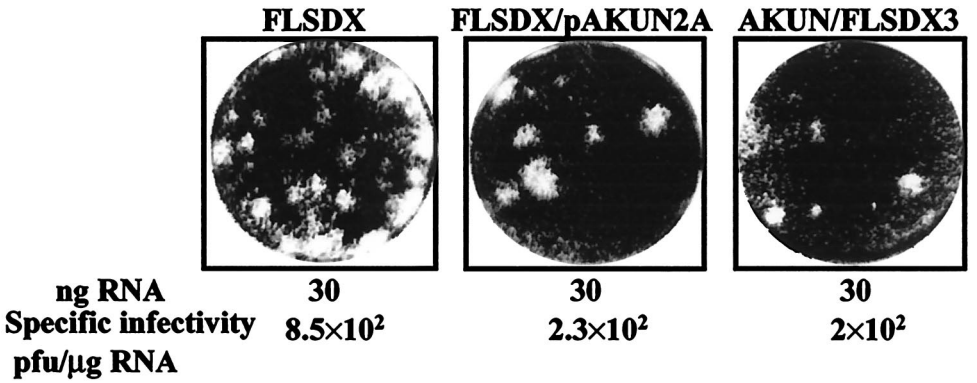

FIG. 5. Complementation of infectivity of KUN RNAs with NS2A mutations in repBHK cells. Electroporation of repBHK cells with RNAs and plaque assays were performed as described in Materials and Methods for normal BHK cells. "ng RNA" under the panels is defined as in the Fig. 1A legend. Viral plaques were stained at day 6 after electroporation.

infection. No β-Gal-positive cells were detected in 100 μl of undiluted culture fluid collected from BHK cells transfected with repPACβ-gal/2Amut and SFV-MEC105 RNAs, while ~160 β-Gal- positive cells were detected in 100 μl of 1:100 dilution of culture fluid collected from BHK cells transfected with repPACβ-gal and SFV-MEC105 RNAs (Fig. 6). This result clearly demonstrated that KUN replicon RNA with the Ile59-to-Asn mutation in NS2A could not be packaged in *trans*.

DISCUSSION

The molecular and functional analyses of two KUN full-length cDNA clones, pAKUN and FLSDX, producing infectious RNAs with substantially different specific infectivities have identified two amino acid codon substitutions responsible for such a difference in their efficiency, i.e., Ile59 to Asn in the NS2A gene and Tyr518 to His in the NS3 gene. The Tyr518-to-His substitution in NS3 led to significant inhibition of RNA replication, while the Ile59-to-Asn substitution in NS2A resulted in selective blockage of virus assembly and/or secretion. Combination of these two substitutions decreased the specific infectivity of less efficient pAKUN RNA by at least 100,000-fold. Sequence analysis of the virus eventually recovered from the pAKUN RNA after its delayed replication showed that both codon substitutions reverted to the more efficient wild-type codons, thus further demonstrating the importance of these amino acids in functions of the corresponding proteins during virus replication.

Further detailed comparisons of entire cDNA sequences of pAKUN and FLSDX cDNAs with the published parent KUN MRM61C sequence revealed six additional, mainly conserved amino acid substitutions present in both pAKUN and FLSDX clones, which aligned better with the sequences of other flaviviruses from the same subgroup than with the published MRM61C sequence (Table 1). It is likely that these mismatches represent errors in the originally published MRM61C sequence determined from the collection of intermediate cDNA clones (6). It is possible that these errors were introduced via mutations during propagation of the intermediate cDNA clones in *E. coli*. Alternatively, it may represent quasispecies of KUN RNA present in the heterogeneous virus pool and selected during reverse transcription and cDNA cloning. In addition to these six mismatches, both FLSDX and pAKUN cDNAs contained a nonconservative Pro250-to-Leu codon change in the NS1 gene. We previously showed that this mutation abolished dimerization of NS1 and contributed to delayed replication of the recovered virus in vitro and in vivo (9). Correction of this mutation led to restoration of NS1 dimer-

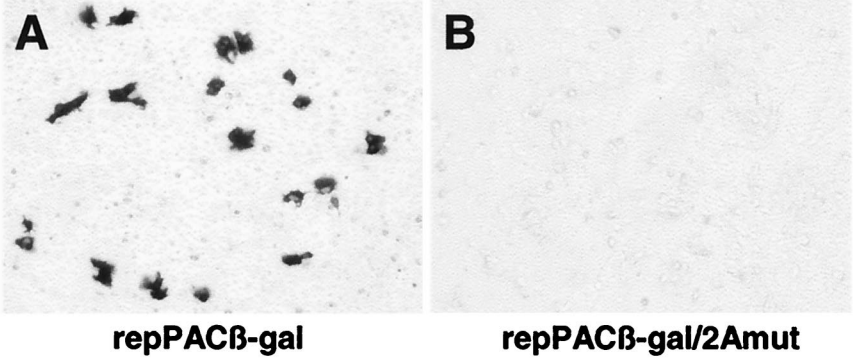

FIG. 6. Detection of packaged KUN replicon RNAs. Panels A and B represent selected fields of Vero cells stained with X-Gal at 48 h after infection with 100 μl of 1:100 dilution (panel A) or undiluted (panel B) culture fluids collected from BHK21 cells sequentially transfected with either repPACβ-gal (A) or repPACβ-gal/2Amut (B) RNAs, respectively, followed by transfection with SFV-MEC105 RNA.

ization and recovery of virus indistinguishable from the wild-type KUN in its growth properties in cells and in mice (9). Here we also show that transfection with RNA containing this corrected mutation (FLSDX[pro]) resulted in production of large viral plaques similar in size to those produced by transfection of purified KUN virion RNA (Fig. 2).

The effect of substitutions in NS3 on infectivity of full-length RNA and on replication of replicon RNA could be expected in view of the demonstrated functions of flavivirus NS3 in helicase and RNA-triphosphatase activities (2, 19, 28, 29). However, both replaced residues (amino acids 518 and 557) were located outside the conserved domains proposed to be involved in these activities. Particularly surprising was the inhibiting effect on RNA replication of a replacement of the nonconserved Tyr518 (intermediate polarity) by His (also intermediate polarity), while a more drastic replacement of highly conserved polar residue Ser557 by nonpolar residue Pro did not have such a dramatic effect on RNA replication and virus assembly. It is not clear why the latter mutation, which did not have a significant effect on virus replication, reverted to the wild-type residue in the virus eventually recovered from pAKUN RNA. pAKUN was recovered as a total virus pool after a prolonged (8 or 9 days) incubation of pAKUN RNA-transfected cells (10). It is possible that this mutation could have been retained in some of the individual virus clones if these were analyzed.

Recent sequence analysis studies of the NS3 gene from different isolates of hepatitis C virus (HCV) revealed that the region between codons 455 and 485 was conserved and was possibly related to HCV RNA helicase activity (27). The amino acid sequence alignment of KUN and HCV NS3 proteins (not shown) suggests that Tyr518 in KUN NS3 corresponds to Gly484 in HCV NS3, which is situated in the conserved helicase region, and that therefore Tyr518 may also be involved in the helicase activity of flavivirus NS3. Further comparative studies of in vitro enzymatic activities of the wild-type KUN NS3 and KUN NS3 with the Tyr518-to-His substitution are required to make any definite conclusion on the role of this residue in the NS3 enzymatic functions in RNA replication.

The complete blockage of KUN production caused by a single amino acid substitution, Ile59 to Asn, in the nonstructural protein NS2A despite efficient RNA replication is intriguing. Recent studies with YF showing that a single amino acid substitution in NS2A at a different position (Lys190) blocked virus production despite efficient RNA replication (17) are completely analogous to our results obtained with the Ile59 substitution in KUN NS2A. The functions of NS2A protein in flavivirus replication are not well understood. NS2A is a highly hydrophobic protein that may span the membrane of endoplasmic reticulum four or five times (6). The mutated residues in NS2A of KUN and of YF are both located in hydrophilic regions. It was shown previously that GST-NS2A bound strongly to 3′ untranslated region RNA and to other components of the RNA replication complex, including NS3 and NS5 in labeled infected-cell lysates by glutathione transferase pull-down assays, and was colocalized with the sites of RNA replication by immunoelectron microscopy of cryosections (22). It was also demonstrated that binding of NS4A to NS2A and RNA was sensitive to RNase digestion, indicating the importance of the complex interactions between NS2A, RNA, and NS4A in formation of the RNA replication complex (22). Based on these results and on earlier data on the composition of the RNA replication complex purified from infected cells (4, 5), members of our group devised the first comprehensive model of formation of the flavivirus replication complex in which NS2A is likely to play an important role by facilitating transport of the partially assembled replicase consisting of RNA, NS2A, NS3, and NS5 to a membrane site of replication via possible hydrophobic interactions with NS4A, which in turn interacts with luminal NS1 (14). Recent immunoelectron microscopy studies with KUN showed that the sites of virus assembly in infected cells are adjacent to the endoplasmic reticulum located outside the periphery of the virus-induced membranes involved in replication (21). These results, combined with other results demonstrating functional coupling between RNA replication and virus assembly (16), indicate that components of the RNA replication complex may be involved in the assembly of progeny RNA into virions possibly via indirect interactions mediated by the viral RNA.

The impaired function of mutated KUN and YF NS2A proteins in virus assembly could be complemented in *trans* by the helper wild-type NS2A, demonstrating that RNAs with these mutations could be packaged into virions by the structural proteins produced in *cis* from the same RNA molecule. This implies that RNA sequences in the vicinity of the mutations in NS2A do not represent a part(s) of the packaging signal. It was therefore intriguing to observe that KUN replicon RNA with the Ile59-to-Asn mutation could not be packaged into virus-like particles when the structural proteins were provided in *trans* from the SFV replicon vector (Fig. 6). These results thus show that the presence of wild-type NS2A produced either in *cis* or in *trans* is absolutely essential for packaging RNA into virions or virus-like particles. Although the YF NS2A mutation blocks an internal cleavage site, this cleavage was not required for production of infectious virus. Moreover, such a cleavage does not occur generally among *Flavivirus* species. Interestingly, a suppressor mutation that restored the infectivity of YF NS2A mutant was located in the helicase domain of YF NS3 (at Asn343) (17), suggesting a possible role for interactions between NS2A and NS3 in virus assembly. Although the YF results remain puzzling, we favor a model in which the NS2A-3 region modulates nucleocapsid assembly or budding.

Another possible scenario of NS2A involvement in RNA packaging that we favor is that wild-type NS2A is required for transport of RNA from the site of RNA replication across the induced membranes to the site of virus assembly, whereas the mutated NS2A cannot perform this function. The RNA binding properties of NS2A and its highly hydrophobic nature may facilitate such a transmembrane RNA transport if it indeed occurs in infected cells. In the *Flavivirus* species, nonpolar amino acids occupy the equivalent site and the flanking sites of the KUN NS2A mutation (Fig. 1 in reference 3); hence, the replacement of Ile by the small polar residues Asn may affect the folding of NS2A such that it is unable to participate in the postulated transport of progeny RNA to the virus assembly site. Further experiments on intracellular colocalization of NS2A, newly synthesized viral RNA, and envelope protein, as well as direct protein-RNA-protein interactions between NS2A, RNA, and envelope protein by using in vitro binding assays and coprecipitation and glutathione transferase pull-

down assays in infected cells, are required to confirm this hypothesis.

In conclusion, by using nucleotide sequencing and functional analyses of KUN full-length and replicon cDNA clones, we have identified two amino acid substitutions that contributed to severely impaired infectivity of our original full-length cDNA clone pAKUN and demonstrated that one of these mutations, Tyr518 to His in the NS3 protein, was responsible for severe inhibition of RNA replication, and that another, Ile59 to Asn in the NS2A protein, was responsible for a blockage in virus assembly. In addition to providing viral cDNA and RNA that is fully characterized, highly efficient, and identical to the wild-type viral RNA, these results have wider implications for the study of flavivirus replication because they demonstrate roles for flavivirus NS3 in RNA replication and for flavivirus NS2A in virus assembly. Previous studies demonstrating an essential role of KUN NS3 in virus assembly (15, 20) and our present results showing the role for KUN NS2A in virus assembly, combined with the recent YF studies demonstrating a role for YF NS2A and NS3 in virus assembly (17), clearly show that the involvement of the nonstructural proteins (i.e., NS2A and NS3) in virus assembly is a common phenomenon for the *Flavivirus* genus. It will be of interest to see whether similar findings can be applied to the members of other *Flaviviridae* genera, the pestiviruses, and the hepaciviruses.

ACKNOWLEDGMENTS

We thank Ed Westaway for critical reading of the manuscript and helpful suggestions.

This work was supported by grants 142983 and 142911 to A.A.K. from the National Health and Medical Research Council of Australia.

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