

A stable full-length yellow fever virus cDNA clone and the role of conserved RNA elements in flavivirus replication

Peter J. Bredenbeek,¹ Engbert A. Kooi,^{1†} Brett Lindenbach,^{2‡} Nicolette Huijckman,¹ Charles M. Rice^{2‡} and Willy J. M. Spaan¹

Correspondence

Peter Bredenbeek

p.j.bredenbeek@lumc.nl

¹Department of Medical Microbiology, Center of Infectious Disease, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands

²Department of Molecular Microbiology, Washington University Medical School, St Louis, MO, USA

Received 26 September 2002

Accepted 10 January 2003

Yellow fever virus (YF) is the prototype member of the *Flavivirus* genus. Here, we report the successful construction of a full-length infectious cDNA clone of the vaccine strain YF-17D. YF cDNA was cloned into a low-copy-number plasmid backbone and stably maintained in several *E. coli* strains. Transcribed RNAs had a specific infectivity of 10^5 – 10^6 p.f.u. ($\mu\text{g RNA}$)⁻¹, and the resulting virus exhibited growth kinetics, plaque morphology and proteolytic processing similar to the parental virus in cell culture. This clone was used to analyse the importance of conserved flavivirus RNA sequences and the 3' stem-loop structure in virus replication. The conserved sequences 5'CS and CS1, as well as the 3' stem-loop structure, were found to be essential for virus replication in cell culture, whereas the conserved sequence CS2 and the region containing YF-specific repeated sequences were dispensable. This infectious clone will aid future studies on YF replication and pathogenesis, as well as facilitate the development of YF-17D-based recombinant vaccines.

INTRODUCTION

Yellow fever virus (YF) is the type member of the genus *Flavivirus*, a group of arthropod-borne RNA viruses in the family *Flaviviridae* (Lindenbach & Rice, 2001). The virus is endemic in the central regions of Africa and South America, where it exists primarily as a zoonosis among monkeys. Occasionally, YF is transmitted to human populations, resulting in epidemics with mortality rates of up to 60%. Effective protection against infection is possible by vaccination with the attenuated YF-17D strain (reviewed in Burke & Monath, 2001).

The YF genome is a positive-stranded RNA molecule of 11·8 kb, with a 5' cap structure and a non-polyadenylated 3' terminus (Rice *et al.*, 1985). The RNA encodes a single open reading frame (ORF) flanked by 5' and 3' non-translated regions (NTRs), which are 118 and 565 bases in length, respectively. Translation of YF RNA results in the production of a precursor protein that is cleaved by host and viral proteases to produce the mature viral proteins (see

Lindenbach & Rice, 2001, for a review). The N-terminal one-third of this polyprotein encompasses the structural proteins (C-prM-E). Proteolytic processing of the remainder of the polyprotein yields the viral non-structural (NS) proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5). Replication of the viral genome occurs in the cytoplasm and is associated with cellular membranes (reviewed by Lindenbach & Rice, 2001).

All the mosquito-borne flaviviruses share conserved RNA sequences and structures (Fig. 1A). Sequence comparison and RNA secondary structure predictions of the 3'-NTR have revealed several short, well-conserved sequences and indicated that the 3'-terminal region (approximately 90 bases) can be folded in a conserved stem-loop structure (3'-SS) (Brinton *et al.*, 1986; Hahn *et al.*, 1987; Proutski *et al.*, 1997a; Wengler & Castle, 1986). Apart from the sequence 5'-CACAG-3' in the bulge at the top and A-U and G-C base pairs at the very bottom, this stem-loop structure is not well conserved in primary sequence. A short conserved sequence (CS1; ~26 nucleotides) has been identified upstream of 3'-SS. Complementarity between CS1 and a conserved sequence at the 5' end of the YF ORF (5'-CS) has been proposed to result in a long-range intramolecular RNA interaction (Hahn *et al.*, 1987). Recent experiments suggest that base-pairing between these sequences is essential

[†]Present address: Institute for Animal Science and Health (ID-DLO), 8200AB Lelystad, The Netherlands.

[‡]Present address: Center for the Study of Hepatitis C, Laboratory of Virology and Infectious Disease, The Rockefeller University, New York, USA.

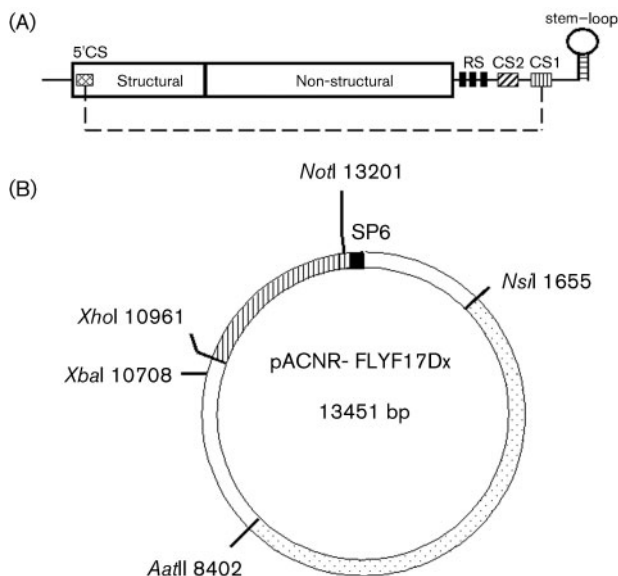


Fig. 1. (A) Schematic representation of the YF genome organization. The large open boxes representing the ORF encoding the viral structural and non-structural proteins is indicated. Cross-hatched box, the conserved sequence (CS) in the capsid gene; black boxes, the repeated sequences; hatched box, CS2; striped box, CS1. The stem-loop structure at the 3' end is depicted as a hairpin. The conserved structure and sequences are enlarged relative to the size of the ORF. (B) Schematic representation of the pACNR/FLYF-17Dx plasmid. The hatched part is the pACNR vector. The DNA fragments taken from pHYF5'3'IV are indicated by the open boxes and the DNA fragment cloned from pYFM5.2 is indicated by the stippled area. The location of the SP6 RNA polymerase promoter and the restriction enzyme sites that were used to construct this full-length YF cDNA are indicated. *XhoI* was used to linearize the plasmid for *in vitro* RNA transcription.

for RNA replication of a Kunjin virus (KUN) replicon (Khromykh *et al.*, 2001).

The 3'-NTR contains another conserved sequence, CS2, which is approximately 24 nucleotides in length. In YF, CS2 is located 22 nucleotides upstream of CS1. CS2 is duplicated in members of the dengue virus (DEN) and Japanese encephalitis virus (JE) subgroups. In addition to the RNA sequences and structures that are conserved among mosquito-borne flaviviruses, YF has an unique domain consisting of three stretches of closely spaced repeated sequences (RS) that are located just downstream of the YF ORF (Hahn *et al.*, 1987).

Based on computer-assisted RNA folding, phylogenetic sequence comparisons and biochemical and biophysical probing, several models for the RNA structure of the 3' end of the genome of the mosquito-borne flaviviruses have been proposed (Blackwell & Brinton, 1997; Brinton *et al.*, 1986; Hahn *et al.*, 1987; Olsthoorn & Bol, 2001; Proutski *et al.*, 1999; Shi *et al.*, 1996). Although these studies do not yield a

consensus model for the flavivirus 3'-NTR structure, it is evident that the folding of the 3'-NTR is complex and involves many stem-loop structures and some potential RNA pseudoknots. DEN mutants with deletions in the 3'-UTR have been described (Men *et al.*, 1996), but their analysis does not favour any particular current RNA structure model.

Attempts to construct a stable, full-length infectious YF cDNA in *E. coli* plasmid and λ phage vectors have been unsuccessful due to problems with the genetic stability of the full-length clone in the prokaryotic host (Rice *et al.*, 1989). This problem was circumvented by using two plasmids and an *in vitro* ligation approach to create a full-length YF cDNA that could be used for the *in vitro* transcription of infectious YF RNA (Rice *et al.*, 1989). Although cumbersome, this approach yielded the first functional flavivirus cDNA for *in vitro* transcription of infectious YF RNA.

In this study, we describe the construction and characterization of full-length YF cDNA in a low-copy-number vector that is stable in several different bacterial strains. The *in vitro*-transcribed RNA from this clone was shown to be highly infectious. The infectious clone was used to analyse the requirement for the conserved flavivirus RNA elements in YF replication.

METHODS

Cell cultures, virus stocks and plaque assays. BHK-21/J (Lindenbach & Rice, 1997) and SW13 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 7.5% foetal calf serum (FCS). Virus stocks were obtained by harvesting the medium of BHK-21/J cells that were transfected with *in vitro*-transcribed RNA of wild-type or mutant YF-17D cDNA. The medium was harvested at 36 h post-transfection, clarified by centrifugation (2500 g for 10 min) and stored at -80°C . Virus stocks were titrated by plaque assays on SW13 and BHK21/J cells as described previously (Rice *et al.*, 1989), except that DMEM instead of Eagle's minimal essential medium (MEM) was used in the overlays.

Plasmid constructions. Standard nucleic acid methodologies were used (Ausubel *et al.*, 2000; Sambrook *et al.*, 1989). The *E. coli* strain MC1061 was used for routine cloning purposes, whereas electro-competent *E. coli* Sure cells (Stratagene) were used as a host for the construction of full-length YF cDNAs. The cDNA fragments for the construction of the full-length YF cDNA were taken from the plasmids pYHF5'3'IV and pYFM5.2. Plasmid pYFM5.2 has been described (Rice *et al.*, 1989). pYHF5'3'IV is a phagemid and contains, in addition to the YF sequences of pYF5'3'IV (Rice *et al.*, 1989), the ϕ 1 origin for filamentous phage replication. Plasmid pACNR1181 was created from the low-copy-number vector pACNR1180, which contains the polylinker cassette of pSL1180 (Pharmacia) (Ruggli *et al.*, 1996). After digestion of pACNR1180 with *AatII* and *NotI*, a short spacer sequence was inserted that resulted in the destruction of the *AatII* site and deleted all the restriction enzyme sites between *NotI* and *Sall* in the polylinker cassette. The resulting pACNR1181 was used as a vector to assemble a full-length YF-17D cDNA. pYHF5'3'IV was digested with *NotI* and *XhoI*. A 5.1 kb DNA fragment that contained the SP6 RNA polymerase promoter directly fused to the YF 5' end, the YF 5' 2271 bp, a small spacer element and the YF 3' 2586 nucleotides was isolated and cloned in *NotI/XhoI*-digested pACNR1181. The resulting plasmid,

pACNR1181YF5'3'IV, was digested with *NsiI* and *AatII* and ligated to the 6747 bp *NsiI*–*AatII* fragment from pYFM5.2 encompassing the middle part of the YF genome (nt 1655–8402). This resulted in the construction of pACNR/FLYF-17Dx (Fig. 1B), which contained a full-length YF-17D cDNA.

Deletion mutagenesis of conserved RNA sequences and structures. All the deletion mutants were initially created in pHYF5'3'IV. The partial deletion of the YF 5'-CS (Table 1) was constructed by fusion PCR (Landt *et al.*, 1990). All the other deletion mutants were created using uridylated single-stranded pHYF5'3'IV DNA as a template to introduce additional restriction enzyme sites (Kunkel, 1985) flanking the conserved RNA sequence and structural elements in the viral 3'-NTR. *HindIII* sites were inserted immediately 5' and 3' of the RS, CS1 and CS2 sequences. The created plasmids were digested with *HindIII* and religated to create the Δ RS, Δ CS1–CS2, Δ CS1 and Δ CS2 mutants (Table 1). Using the same uridylated template and strategy, an additional *XhoI* site was introduced at nt 10776. This plasmid was cut with *XhoI* and religated to yield the Δ SS mutant (Table 1). The relevant parts of these pHYF5'3'IV derivatives were verified by DNA sequencing and then cloned into the full-length YF cDNA.

In vitro transcription. Plasmids containing the full-length YF cDNA were linearized with *XhoI* and purified by phenol/chloroform extraction and ethanol precipitation. Run-off RNA transcripts were synthesized *in vitro* using SP6 RNA polymerase (Rice *et al.*, 1987). Trace amounts of [³H]UTP were included in the reaction to quantify the yield. Transcripts were used for transfection without any additional purification.

RNA transfections. A transfection plaque assay (Grakoui *et al.*, 1989) on SW13 cells was used to determine the infectivity of YF-17D transcripts in p.f.u. (μ g RNA)⁻¹. In short, almost confluent monolayers of SW13 cells in 35 mm diameter dishes were washed twice with PBS lacking Ca²⁺ and Mg²⁺. A mixture of 0.1–1.0 ng wt or mutant YF-17D RNA transcripts and 4 μ g lipofectin (Life Technologies) in 200 μ l PBS was added to the cells. After 15 min, the transfection mixture was removed, the cells were washed once with PBS and a DMEM/agarose overlay was applied as described for the plaque assays. Plaques were identified by crystal-violet staining after incubation for 4 days at 37 °C (Rice *et al.*, 1989).

For direct analysis of experiments in which viral RNA synthesis and protein expression was analysed directly in the transfected cells, BHK-21J cells were electroporated with 5 μ g of *in vitro*-transcribed YF RNA

as described previously (Lindenbach & Rice, 1997). Aliquots were taken from the medium of the transfected cells to quantify the virus yields.

Immunofluorescence. Transfected cells were grown on coverslips. At 24 h post-transfection or infection, the cells were fixed with 3% paraformaldehyde in PBS (pH 7.4) for at least 30 min and washed with PBS containing 10 mM glycine. Following permeabilization with 0.1% Triton X-100 in PBS, the cells were incubated in PBS containing 2% horse serum for 1 h to minimize non-specific immunofluorescence. Indirect immunofluorescence was carried out with a 1:1000 dilution of mAb 1A5 (provided by J.J. Schlesinger) in PBS, which is specific for the YF NS1 protein (Schlesinger *et al.*, 1983) and visualized with a secondary Cy3-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 1:1000.

Labelling and analysis of viral RNAs. In general, 3 ml of the electroporated BHK-21J cell suspension (approximately 1.5×10^6 cells) was placed in 35 mm plates in DMEM containing 2% FCS. At the indicated times post-electroporation (p.e.), the medium was replaced with 750 μ l medium containing 2 μ g actinomycin D ml⁻¹ and 50 μ Ci [³H]uridine ml⁻¹. At 24 h p.e., RNA was isolated with Trizol (Life Technologies) and resuspended in 21 μ l H₂O. One-third of the RNA was denatured with glyoxal and DMSO and analysed by electrophoresis in 0.8% MOPS/agarose gels (Sambrook *et al.*, 1989). Gels were prepared for fluorography as described previously (Bredenbeek *et al.*, 1993).

RESULTS

Construction and characterization of a stable, full-length yellow fever cDNA clone

The low-copy-number vector pACNR1181, derived from pACNR1180 (Ruggli *et al.*, 1996), was used as a vector for the construction of a full-length YF-17D cDNA (see Methods; Fig. 1B). Transformation of *E. coli* Sure cells with the ligation reaction that was expected to yield the full-length YF cDNA construct resulted in the production of small and large bacterial colonies after incubation of the plates for 24 h at 37 °C. Both types of colonies were grown in liquid medium and plasmids were isolated. Based on restriction enzyme digestion patterns all the small colonies ($n=14$) carried the plasmid containing the full-length YF cDNA (pACNR/FLYF-17Dx) whereas the bacteria of the faster-growing

Table 1. Characteristics of YF mutants containing mutations in conserved nucleotide sequences and/or conserved secondary RNA structures

Construct	Position of deleted nucleotides in YF	No. of deleted nucleotides	RNA synthesis*	Plaque formation†
YF-17D Δ 5'-CS	155–160	6	–	No
YF-17D Δ RS	10333–10520	188 (+ aagcuu)‡	++	Yes
YF-17D Δ CS1–CS2	10705–10772	68 (+ aagcuu)‡	–	No
YF-17D Δ CS2	10705–10729	25 (+ aagcuu)‡	+	Yes
YF-17D Δ CS1	10748–10772	25 (+ aagcuu)‡	–	No
YF-17D Δ SS	10776–10861	86	–	No
YF-17Dx	–	–	++	Yes

*As determined by [³H]uridine labelling of electroporated BHK-21J cells.

†As determined by lipofection of SW13 cells.

‡Due to engineering an extra *HindIII* site, six extra nucleotides were inserted.

colonies ($n=4$) carried only the parental vector pACNR1181YF5'3'IV (data not shown).

*Xho*I-linearized pACNR/FLYF-17Dx template was used for *in vitro* RNA transcription and the resulting full-length YF transcripts were electroporated into BHK-21J cells. The transfected cells were fixed at 24 h p.e. and analysed for the expression of YF NS1 by immunofluorescence microscopy. Many of the electroporated cells showed a perinuclear, punctated signal when stained with an NS1-specific antibody (Fig. 2A). As a control, cells were transfected with a truncated YF-17Dx transcript lacking the 3' terminal 155 nucleotides of the YF genome (*Xba*I site in Fig. 1B) and therefore unlikely to be replication-competent. The cells transfected with the truncated YF transcripts failed to express any detectable NS1 protein (Fig. 2B). These results demonstrated that YF RNA transcribed from pACNR/FLYF-17Dx was replication-competent and that the NS1 signal was not merely the result of translation of the input RNA.

Stability of the infectious YF cDNA in *E. coli*

The initial *in vitro* ligation approach to obtain infectious YF-17D RNA was developed because of severe stability problems with the full-length YF-17D cDNA in *E. coli*. Therefore it was of great importance to examine the stability of pACNR/FLYF-17Dx in *E. coli*. The recombination-deficient *E. coli* Sure and DH5 α strains and recombination-competent *E. coli* MC1061 strain were transformed with pACNR/FLYF-17Dx. Two colonies from each transformation were propagated for ten cycles consisting of alternating growth in liquid medium and plating on a selective medium. No significant change in the growth characteristics or colony morphology of the bacteria was observed during these passages. Restriction enzyme digestion of plasmid DNA purified after 5 and 10 cycles generated the expected DNA pattern (data not shown). YF-17Dx RNA transcribed from these purified plasmids was used to determine the specific infectivity of the YF-17D transcripts. As shown in Table 2, the specific infectivity of the RNA transcripts was comparable [$3.5\text{--}4.5 \times 10^5$ plaques ($\mu\text{g RNA})^{-1}$], irrespective of the *E. coli* host that had been used to grow the plasmid. More importantly, no significant changes

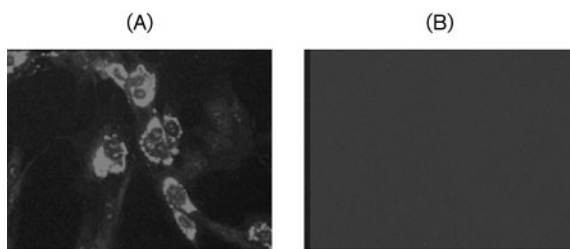


Fig. 2. Immunofluorescence staining of BHK-21 cells with the YF NS1-specific antibody, 1A5, at 24 h p.e. (A) Cells transfected with full-length YF-17Dx transcripts. (B) Cells transfected with YF transcripts lacking the 3' 155 nucleotides of the YF sequence.

Table 2. Specific infectivity of plasmid-derived YF-17Dx RNA after repeated passaging of pACNR-FLYF-17Dx in *E. coli*

<i>E. coli</i> strain	Specific infectivity [YF plaques ($\mu\text{g RNA})^{-1}$]*		
	Passage 1	Passage 5	Passage 10
Sure	2.8×10^5	3.8×10^5	3.5×10^5
DH5 α	ND	3.7×10^5	3.5×10^5
MC1061	ND	4.3×10^5	4.5×10^5

*For each *E. coli* strain, the specific infectivity is the average of the transfection of *in vitro*-transcribed RNA from two individually passaged bacteria cultures.

ND, Not determined.

in the number of plaques ($\mu\text{g RNA})^{-1}$ were detected after repeated passages of the plasmid in any of the studied *E. coli* strains.

Kinetics of viral RNA synthesis and virus production

To study the kinetics of YF RNA synthesis and virus production directly in transfected cells, BHK-21J were electroporated with YF-17Dx transcripts and labelled with [^3H]uridine in the presence of actinomycin D for 6 h at 6 h intervals. RNA replication was first detected after labelling the cells from 12 to 18 h p.e. and synthesis peaked between 18 and 30 h (Fig. 3A). After 30 h, viral RNA synthesis decreased, which correlated with the increasing YF-induced cytopathic effect on the transfected cells. Virus could be detected in the medium of the electroporated cells at 12 h p.e. (Fig. 3B). There was a significant rise in virus titre between 12 and 18 h p.e., which levelled off between 24 and 30 h (Fig. 3B) and remained at this level for the remaining period of the experiment.

Next, the growth properties of the parental YF-17D virus and the YF-17Dx virus released into the medium of the transfected cells were compared. BHK-21J cells were infected at an m.o.i. of 10 with a laboratory strain of YF-17D and YF-17Dx virus. Virus was harvested at 12 h intervals and the yield was quantified as described previously (Rice *et al.*, 1989). Similar results were obtained for both virus strains, with virus titres in the medium peaking at 36 h and being slightly lower at later time points (Fig. 4). In addition, the expression of YF proteins was analysed by radio-immunoprecipitation of YF proteins in lysates of [^{35}S] methionine-labelled BHK-21J cells infected with either YF-17D or YF-17Dx virus. No apparent difference in migration or relative levels of the prM, E, NS1, NS2B, NS3, NS4B or NS5 proteins was observed (data not shown).

Conserved flavivirus RNA elements that are essential for virus replication

Several mutant YF-17D cDNAs were constructed by deleting either the conserved flavivirus sequence elements ($\Delta 5'$ -CS,

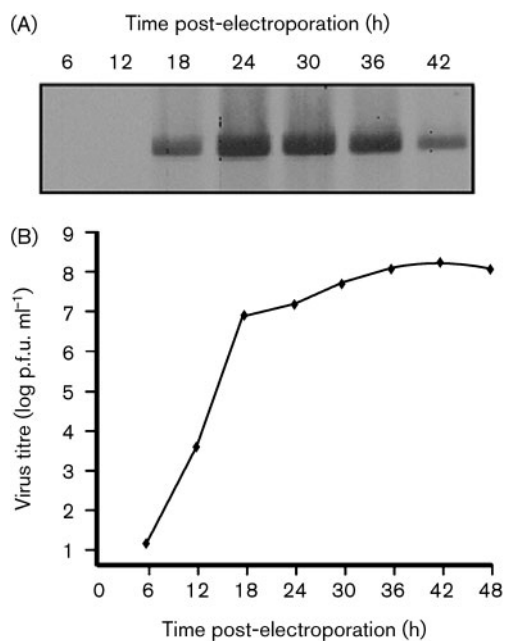


Fig. 3. Kinetics of YF RNA synthesis and virus production in BHK-21J cells electroporated with YF-17Dx transcripts. (A) Kinetics of YF RNA synthesis. The transfected cells were labelled for 6 h with 50 μCi [³H]uridine ml^{-1} in the presence of actinomycin D and lysed at the indicated times p.e. (B) Production of YF in electroporated BHK-21J cells. Samples ($n=2$ per time point) of the medium of the transfected cells were taken at the indicated times p.e. and used for a plaque assay on BHK-21J cells to determine the YF titre.

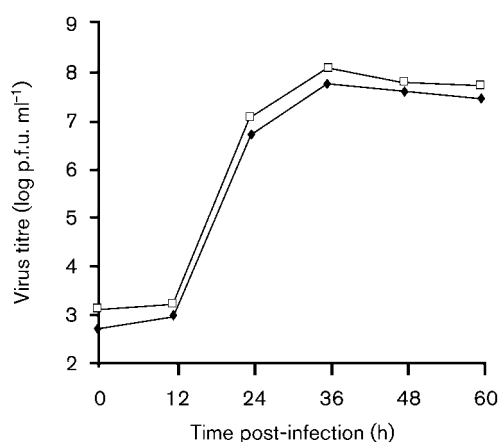


Fig. 4. Comparison of the growth characteristics of YF-17Dx virus derived from the infectious clone (◆) with YF-17D virus stocks derived from vaccine lots (□). BHK-21J cells were infected at an m.o.i. of 10. The medium of the infected cells was sampled at the indicated times post-infection. Plaque assays on BHK-21J cells were used to determine the virus titre.

ΔCS1 , ΔCS2 and $\Delta\text{CS1-CS2}$), RNA structures (ΔSS), or the subgroup-specific repeated sequences (ΔRS) (Fig. 1A). *In vitro* RNA transcripts were transfected into SW13 and BHK cells by lipofection and electroporation, respectively. Only the ΔRS and ΔCS2 mutants were able to form plaques after lipofection into SW13 cells at either 31 or 37 °C. Plaques were not observed for the $\Delta 5'$ -CS, $\Delta\text{CS1-CS2}$, ΔCS1 and ΔSS mutants. The plaques that were formed by the ΔRS mutant were somewhat smaller than the plaques obtained with YF-17Dx (Fig. 5A). The plaque morphology of the ΔCS2 mutant varied between experiments. Turbid plaques as well as more clear plaques were observed. The size of the ΔCS2 mutant plaques was significantly smaller than observed for YF-17Dx plaques (Fig. 5A).

First cycle RNA analysis was performed to exclude the possibility that the lack of plaque formation for $5'$ -CS, $\Delta\text{CS1-CS2}$, ΔCS1 and ΔSS mutants was due to the deletion of viral sequences that are involved in virion assembly, e.g. the encapsidation signal. BHK-21J cells were electroporated with *in vitro* transcripts of either wt YF-17Dx or the deletion mutants and labelled with [³H]uridine from 18 to 24 h p.e. in the presence of actinomycin D. Total RNA was isolated from the transfected cells and analysed on a denaturing agarose gel. As shown in Fig. 5(B), synthesis of YF RNA could only be detected in the cells transfected with the ΔRS and ΔCS2 mutants. No viral RNA synthesis was detected for the $\Delta 5'$ -CS, $\Delta\text{CS1-CS2}$, ΔCS1 and ΔSS mutants.

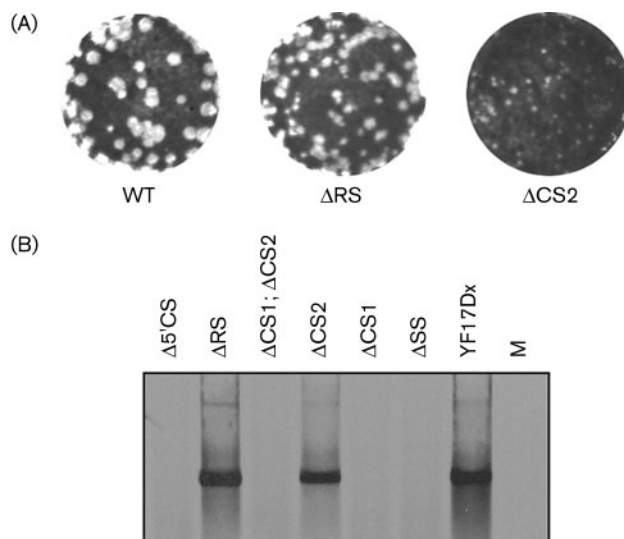


Fig. 5. (A) Plaque morphology of YF-17Dx, YF-17D ΔRS and YF-17D ΔCS2 after transfection plaque assays on SW13 cells. The transfected cells were incubated at 37 °C, 5% CO₂ for 96 h, fixed with 8% formaldehyde and stained with crystal violet. (B) Analysis of YF RNA synthesis in BHK-21J cells electroporated with *in vitro*-transcribed RNA of the indicated YF mutants. Transfected cells were labelled with [³H]uridine in the presence of actinomycin D between 18 and 24 h p.e.

To compare the growth characteristics of the mutants and the parental viruses BHK-21J cells were infected at an m.o.i. of 10 with either the Δ RS or Δ CS2 mutants or with wt YF-17Dx virus. Virus was harvested at 8 h intervals and the yield was determined by plaque assay (Fig. 6). All three viruses showed a rapid increase in titre between 8 and 24 h, with virus production peaking at around 32 h and then levelling off. The kinetics of Δ RS and Δ CS2 virus production appeared a little slower than wt virus and the maximum titre was also somewhat lower. Both the Δ RS and Δ CS2 mutants showed a clear cytopathic effect.

DISCUSSION

Recombinant cDNA clones from which full-length infectious RNA can be transcribed are a valuable tool for studying the molecular biology of positive-strand RNA viruses (Boyer & Haenni, 1994). The approach relies on the infectious nature of the genome RNA of these viruses when transfected into permissive host cells. However, the construction of such functional cDNAs for flaviviruses has proven difficult (Ruggli & Rice, 1999, for a review). Often the plasmids containing a full-length cDNA of these viruses are unstable in *E. coli*. For YF, this problem was initially circumvented by using two plasmids and an *in vitro* ligation approach (Rice *et al.*, 1989). This strategy yielded the first functional flavivirus cDNA for *in vitro* transcription of infectious viral RNA. In this report, the construction and characterization of a stable, full-length YF cDNA in an *E. coli* plasmid vector has been described. The YF cDNA fragments that were used to assemble this clone were taken from the plasmids pYF5'3'IV and pYFM5.2, which were previously used to create the full-length cDNA template for the production of infectious YF RNA by *in vitro* ligation (Rice *et al.*, 1989). The

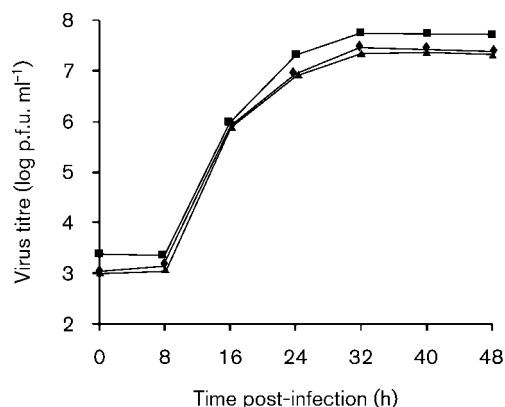


Fig. 6. Growth characteristics YF-17D Δ RS (◆) and YF-17D Δ CS2 (▲) virus compared with YF-17Dx (■). BHK-21J cells were infected with YF-17Dx, YF-17D Δ RS and YF-17D Δ CS2 virus at an m.o.i. of 10. Samples of the media were collected at the indicated times post infection. The virus titre was determined by plaque assay on BHK-21J cells. The growth curves are based on the average virus titre in two samples per time point per mutant.

low-copy-number plasmid pACNR1180 was used as a vector for the construction of the full-length YF cDNA. This vector is derived from pACYC177 and has previously been used to construct stable, infectious pestivirus cDNAs (Mendez *et al.*, 1998; Ruggli *et al.*, 1996). The RNA transcribed from pACNR/FLYF-17Dx had a specific infectivity in the order of 10^5 – 10^6 p.f.u. (μ g RNA)⁻¹ as determined by lipofection. This was significantly higher than reported for the YF RNA obtained from *in vitro*-ligated templates, even when these values are corrected for the percentage of full-length YF RNA in the RNA transcribed from the ligated template (Rice *et al.*, 1989). Similar specific infectivities have only been reported for a few other flavivirus clones, such as tick-borne encephalitis virus (Mandl *et al.*, 1997) and Murray Valley encephalitis virus (Hurrelbrink *et al.*, 1999). The progeny virus obtained from the pACNR/FLYF-17Dx clone showed similar kinetics in proteolytic processing, viral RNA synthesis and growth as the parental virus in cell culture.

The stability of pACNR/FLYF-17Dx was extensively tested in the *E. coli* strains Sure, DH5 α and MC1061. No changes in *E. coli* colony morphology or growth characteristics were observed during the passaging of pACNR/FLYF-17Dx in these bacteria. More importantly, RNA transcribed from plasmid DNA isolated at passages 5 and 10 showed a similar specific infectivity as RNA derived from the originally isolated plasmids. These results demonstrate the successful construction of a stable full-length YF cDNA in a plasmid vector that can be used for *in vitro* transcription of highly infectious viral RNA. Apart from the mutagenesis studies that are described in this report, the pACNR/FLYF-17Dx clone has already been used successfully in other studies (Amberg & Rice, 1999; Kummerer & Rice, 2002; Lindenbach & Rice, 1999).

As for all RNA viruses, the 5'- and 3'-NTRs of the YF genome are believed to play a crucial role in the initiation of viral RNA synthesis. Both the YF 5'- and 3'-NTRs contain sequence motifs and/or stem-loop structures (Fig. 1) that are well conserved among flaviviruses. It has been suggested that these domains are essential for viral RNA synthesis. In this study, the role of the conserved flavivirus sequences (5'-CS, RS, CS2 and CS1) in virus replication was analysed, as well as the stem-loop structure (SS) at the 3' end of the viral genome.

Deleting the RS domain was well tolerated by YF and resulted in a virus with similar biological properties to the YF-17Dx virus. A domain comparable with the YF RS sequences has also been found in the JE virus serogroup, but there is no sequence similarity in the RS domains of YF and JE-like viruses. For KUN, a deletion of 352 nucleotides in the 5'-proximal half of the 3'-NTR resulted in a five- to tenfold reduction in replication (Khromykh & Westaway, 1997). This deletion was upstream of CS2 and included the RS sequences. RS is lacking in DEN. Deletions in this region of DEN type 4 yielded viruses with delayed growth properties and a smaller plaque size (Men *et al.*, 1996). These deletions

always included the DEN CS2B element and are therefore difficult to compare with the YF-17DΔRS mutant, which retained CS2.

RNA secondary structure analysis of flaviviruses predicts that the YF CS2 sequence forms an independent stem-loop structure within the 3'-NTR (Olsthoorn & Bol, 2001; Proutski *et al.*, 1997b). The fact that this stem-loop structure is well conserved in both pathogenic and vaccine strains of YF suggests that this sequence is essential for virus replication. However, analysis of YF-17DΔCS2 showed that this sequence can be deleted with relatively minor effects on virus replication. Compared with YF-17Dx, the rate of RNA synthesis by YF-17DΔCS2 was somewhat decreased. However, the kinetics of virus production of YFΔCS2 was similar to YF-17Dx. The reason for the observed variability in YF-17DΔCS2 plaque size and morphology is unclear. Small and turbid plaques have also been reported for DEN type 4 deletion mutants involving CS2 (Men *et al.*, 1996). RT-PCR and sequence analysis showed that the deletion was still present in YF-17DΔCS2 progeny virus; however, second-site revertants cannot be excluded.

All the mosquito-borne flaviviruses contain a 5' conserved sequence that is located a few nucleotides downstream of the translation initiation codon. This 5'-CS is actually part of the flavivirus coding sequence. It has been suggested that the flavivirus 5'-CS sequence base pairs with CS1 via a long-range RNA interaction (Hahn *et al.*, 1987; Khromykh *et al.*, 2001). This interaction is predicted to result in a 'pan-handle'-like structure that is hypothesized to be required for virus replication by modulating virus translation (Khromykh *et al.*, 2001). Recently it was shown, using a KUN replicon and a DEN NS5-based *in vitro* polymerase assay, that complementarity between 5'-CS and CS1 is a prerequisite for viral RNA synthesis (Khromykh *et al.*, 2001; You *et al.*, 2001). The observation that the partial deletion of YF CS1 is lethal for viral RNA synthesis, as reported in this study, is in agreement with a model that requires 'circularization' at some stage of the flavivirus replication cycle. The involvement of CS1 in the 'cyclization' of the viral genomic RNA does not exclude the possibility that the CS1 sequence might also take part in an alternative structure involving base-pairing to other domains within the 3'-NTR. (Proutski *et al.*, 1997a, b; Rauscher *et al.*, 1997; Shi *et al.*, 1996). It can be hypothesized that these different RNA structures involving CS1 are metastable and in equilibrium with each other. This equilibrium may be influenced by factors like RNA-protein interactions and the cellular environment, thereby regulating negative-strand RNA synthesis versus positive-strand RNA synthesis or availability of the RNA for translation.

Another important element of the 3'-NTR RNA is formed by the 3'-terminal 86 nucleotides that are deleted in the YF-17DΔSS mutant. In all the models describing the flavivirus 3'-NTR RNA, these nucleotides are involved in the formation of a hairpin structure (Hahn *et al.*, 1987; Mackenzie *et al.*, 2001; Proutski *et al.*, 1997b; Shi *et al.*, 1996). Deletion

of the 3' 86 nucleotides was lethal for YF RNA synthesis as shown by the results with the YF-17DΔSS mutant.

Finally, as one of the safest and most effective human vaccines, YF-17D recombinants are being vigorously explored as candidate vaccines for other flavivirus diseases, such as JE (Monath, 2002), DEN (Der Most *et al.*, 2000) and West Nile virus (Monath, 2001), as well as for cancer vaccines (McAllister *et al.*, 2000). The availability of full-length stable YF cDNA clones should help in these efforts.

ACKNOWLEDGEMENTS

We thank Linda Lutfiya for initiating the mutagenesis experiments to create the YF deletion mutants. E. K. was supported by Grant 901-02-173 from the Dutch Organization for Sciences (NWO-MW). This work was supported in part by a grant from the Public Health Service (AI31501). We thank Richard Molenkamp for helpful discussions and critical reading of the manuscript.

REFERENCES

- Amberg, S. M. & Rice, C. M. (1999). Mutagenesis of the NS2B-NS3-mediated cleavage site in the flavivirus capsid protein demonstrates a requirement for coordinated processing. *J Virol* **73**, 8083-8094.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (editors) (2000). *Current Protocols in Molecular Biology*. New York: John Wiley.
- Blackwell, J. L. & Brinton, M. A. (1997). Translation elongation factor-1 alpha interacts with the 3' stem-loop region of West Nile virus genomic RNA. *J Virol* **71**, 6433-6444.
- Boyer, J. C. & Haenni, A. L. (1994). Infectious transcripts and cDNA clones of RNA viruses. *Virology* **198**, 415-426.
- Bredenbeek, P. J., Frolov, I., Rice, C. M. & Schlesinger, S. (1993). Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. *J Virol* **67**, 6439-6446.
- Brinton, M. A., Fernandez, A. V. & Disposito, J. H. (1986). The 3'-nucleotides of flavivirus genomic RNA form a conserved secondary structure. *Virology* **153**, 113-121.
- Burke, D. S. & Monath, T. P. (2001). Flaviviruses. In *Fields Virology*, 4th edn, pp. 1043-1125. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.
- Der Most, R. G., Murali-Krishna, K., Ahmed, R. & Strauss, J. H. (2000). Chimeric yellow fever/dengue virus as a candidate dengue vaccine: quantitation of the dengue virus-specific CD8 T-cell response. *J Virol* **74**, 8094-8101.
- Grakoui, A., Levis, R., Raju, R., Huang, H. V. & Rice, C. M. (1989). A cis-acting mutation in the Sindbis virus junction region which affects subgenomic RNA synthesis. *J Virol* **63**, 5216-5227.
- Hahn, C. S., Hahn, Y. S., Rice, C. M., Lee, E., Dalgarno, L., Strauss, E. G. & Strauss, J. H. (1987). Conserved elements in the 3' untranslated region of flavivirus RNAs and potential cyclization sequences. *J Mol Biol* **198**, 33-41.
- Hurrelbrink, R. J., Nestorowicz, A. & McMinn, P. C. (1999). Characterization of infectious Murray Valley encephalitis virus derived from a stably cloned genome-length cDNA. *J Gen Virol* **80**, 3115-3125.
- Khromykh, A. A. & Westaway, E. G. (1997). Subgenomic replicons of the flavivirus Kunjin: construction and applications. *J Virol* **71**, 1497-1505.

- Khromykh, A. A., Meka, H., Guyatt, K. J. & Westaway, E. G. (2001).** Essential role of cyclization sequences in flavivirus RNA replication. *J Virol* **75**, 6719–6728.
- Kummerer, B. M. & Rice, C. M. (2002).** Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. *J Virol* **76**, 4773–4784.
- Kunkel, T. A. (1985).** Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc Natl Acad Sci U S A* **82**, 488–492.
- Landt, O., Grunert, H. P. & Hahn, U. (1990).** A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene* **96**, 125–128.
- Lindenbach, B. D. & Rice, C. M. (1997).** Trans-complementation of yellow fever virus NS1 reveals a role in early RNA replication. *J Virol* **71**, 9608–9617.
- Lindenbach, B. D. & Rice, C. M. (1999).** Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. *J Virol* **73**, 4611–4621.
- Lindenbach, B. D. & Rice, C. M. (2001).** *Flaviviridae*: The viruses and their replication. In *Fields Virology*, 4th edn, pp. 991–1041. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.
- McAllister, A., Arbetman, A. E., Mandl, S., Pena-Rossi, C. & Andino, R. (2000).** Recombinant yellow fever viruses are effective therapeutic vaccines for treatment of murine experimental solid tumors and pulmonary metastases. *J Virol* **74**, 9197–9205.
- Mackenzie, J. M., Khromykh, A. A. & Westaway, E. G. (2001).** Stable expression of noncytopathic Kunjin replicons simulates both ultrastructural and biochemical characteristics observed during replication of Kunjin virus. *Virology* **279**, 161–172.
- Mandl, C. W., Ecker, M., Holzmann, H., Kunz, C. & Heinz, F. X. (1997).** Infectious cDNA clones of tick-borne encephalitis virus European subtype prototypic strain Neudoerfl and high virulence strain Hypr. *J Gen Virol* **78**, 1049–1057.
- Men, R., Bray, M., Clark, D., Chanock, R. M. & Lai, C. J. (1996).** Dengue type 4 virus mutants containing deletions in the 3' non-coding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. *J Virol* **70**, 3930–3937.
- Mendez, E., Ruggli, N., Collett, M. S. & Rice, C. M. (1998).** Infectious bovine viral diarrhoea virus (strain NADL) RNA from stable cDNA clones: a cellular insert determines NS3 production and viral cytopathogenicity. *J Virol* **72**, 4737–4745.
- Monath, T. P. (2001).** Prospects for development of a vaccine against the West Nile virus. *Ann N Y Acad Sci* **951**, 1–12.
- Monath, T. P. (2002).** Japanese encephalitis vaccines: current vaccines and future prospects. *Curr Top Microbiol Immunol* **267**, 105–138.
- Olsthoorn, R. C. & Bol, J. F. (2001).** Sequence comparison and secondary structure analysis of the 3' noncoding region of flavivirus genomes reveals multiple pseudoknots. *RNA* **7**, 1370–1377.
- Proutski, V., Gould, E. A. & Holmes, E. C. (1997a).** Secondary structure of the 3' untranslated region of flaviviruses: similarities and differences. *Nucleic Acids Res* **25**, 1194–1202.
- Proutski, V., Gaunt, M. W., Gould, E. A. & Holmes, E. C. (1997b).** Secondary structure of the 3'-untranslated region of yellow fever virus: implications for virulence, attenuation and vaccine development. *J Gen Virol* **78**, 1543–1549.
- Proutski, V., Gritsun, T. S., Gould, E. A. & Holmes, E. C. (1999).** Biological consequences of deletions within the 3'-untranslated region of flaviviruses may be due to rearrangements of RNA secondary structure. *Virus Res* **64**, 107–123.
- Rauscher, S., Flamm, C., Mandl, C. W., Heinz, F. X. & Stadler, P. F. (1997).** Secondary structure of the 3'-noncoding region of flavivirus genomes: comparative analysis of base pairing probabilities. *RNA* **3**, 779–791.
- Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L. & Strauss, J. H. (1985).** Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* **229**, 726–733.
- Rice, C. M., Levis, R., Strauss, J. H. & Huang, H. V. (1987).** Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *J Virol* **61**, 3809–3819.
- Rice, C. M., Grakoui, A., Galler, R. & Chambers, T. J. (1989).** Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. *Nat New Biol* **1**, 285–296.
- Ruggli, N. & Rice, C. M. (1999).** Functional cDNA clones of the Flaviviridae: strategies and applications. *Adv Virus Res* **53**, 183–207.
- Ruggli, N., Tratschin, J. D., Mittelholzer, C. & Hofmann, M. A. (1996).** Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. *J Virol* **70**, 3478–3487.
- Sambrook, J., Fritsch, T. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schlesinger, J. J., Brandriss, M. W. & Monath, T. P. (1983).** Monoclonal antibodies distinguish between wild and vaccine strains of yellow fever virus by neutralization, hemagglutination inhibition, and immune precipitation of the virus envelope protein. *Virology* **125**, 8–17.
- Shi, P. Y., Brinton, M. A., Veal, J. M., Zhong, Y. Y. & Wilson, W. D. (1996).** Evidence for the existence of a pseudoknot structure at the 3' terminus of the flavivirus genomic RNA. *Biochemistry* **35**, 4222–4230.
- Wengler, G. & Castle, E. (1986).** Analysis of structural properties which possibly are characteristic for the 3'-terminal sequence of the genome RNA of flaviviruses. *J Gen Virol* **67**, 1183–1188.
- You, S., Falgout, B., Markoff, L. & Padmanabhan, R. (2001).** In vitro RNA synthesis from exogenous dengue viral RNA templates requires long range interactions between 5'- and 3'-terminal regions that influence RNA structure. *J Biol Chem* **276**, 15581–15591.