Infectious cDNA clones of tick-borne encephalitis virus European subtype prototypic strain Neudoerfl and high virulence strain Hypr

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Infectious cDNA clones of two strains of tick-borne encephalitis (TBE) virus, i.e. European subtype prototypic strain Neudoerfl and the closely related but more virulent strain Hypr, were constructed. The recombinant constructs consisted of cDNAs stably inserted into the bacterial plasmid pBR322 under the control of T7 promoter elements. The genome of TBE virus strain Neudoerfl was successfully cloned, both as a full-length cDNA and as two partial cDNAs. In the case of strain Hypr, the genome is represented by two cDNA clones corresponding to the 5' - and 3' - terminal halves of the genome. Highly infectious RNAs can be produced from the full-length cDNA clone or from the partial clones ligated in vitro to form full-length cDNA templates prior to T7 transcription. The biological properties of the recombinant progeny viruses, including virulence characteristics, were indistinguishable from the corresponding parent virus strains. Thus, the described infectious cDNA clones represent a useful and reliable experimental system for the specific mutagenesis of TBE virus.

Introduction

Tick-borne encephalitis (TBE) virus is a human pathogenic member of the genus flavivirus (family Flaviviridae), which also includes other medically important pathogens, such as yellow fever (YF) virus, Japanese encephalitis (JE) virus and the four serotypes of dengue (DEN 1–4) virus (Wengler et al., 1995). The structural and functional properties of TBE virus have been studied intensively during the past years (for review see Heinz & Mandl, 1993). Molecular analyses led to the recent elucidation of the three-dimensional structure of the major surface protein E by X-ray crystallography (Rey et al., 1995), establishing the TBE virus serocomplex as a study model for flaviviruses in general. Site-specific mutagenesis of TBE virus genomes is a desirable and powerful technique with which to study their molecular biology, but so far this has been hampered by the lack of an appropriate experimental system.

The flavivirus genome consists of an unsegmented positive-stranded RNA genome of approximately 11 kb in length (for review see Chambers et al., 1990). It encodes three structural proteins (the capsid protein C, the small membrane protein M, which is formed by furin-mediated cleavage from its precursor, prM, and the large envelope protein E) and seven non-structural proteins (the glycoprotein NS1, NS2A, the protease component NS2B, the protease/helicase protein NS3, NS4A, NS4B and the polymerase NS5). All of the viral proteins are encoded within a single long open reading frame, which is flanked by short noncoding regions (NCRs). Viral genome RNA is infectious, i.e. it gives rise to virus progeny when introduced into susceptible cells.

Directed mutagenesis of flaviviruses can be achieved by using so-called infectious clones, i.e. cDNA clones from which full-length and infectious RNA can be transcribed in vitro. In many cases, the generation of stable and efficient flavivirus infectious clones has been found to be quite tedious because of instability problems observed with bacterial vectors containing long flavivirus-specific inserts. This problem was first overcome by Rice et al. (1989), who successfully generated infectious YF virus RNA from a pair of cDNA clones ligated in vitro prior to RNA transcription. Since then, full-length infectious clones have become available for a number of other mosquito-borne flaviviruses, namely JE virus (Sumiyoshi et al., 1992), DEN 2 (Kapoor et al., 1995; Pryor et al., 1996), DEN 4 (Lai et al., 1991) and Kunjin virus (Khromykh & Westaway, 1994). The generation of infectious RNA from cloned cDNA of tick-borne flaviviruses has so far not been reported. Pletnev et al. (1992, 1993) replaced sequences from a DEN 4 infectious cDNA clone...
with the corresponding TBE viral sequences and were able to isolate viable chimeric viruses from these constructs. This approach enabled them to study the biological effects of specific mutations within TBE viral sequences in a DEN 4 genetic backbone. More recently, a novel alternative approach to generate infectious TBE viral RNA from PCR-amplified, but uncloned, cDNAs was presented by Gritsun & Gould (1995).

In this report we describe the generation of a stable full-length cDNA clone of a tick-borne flavivirus, TBE virus European subtype prototypic strain Neudoerfl. RNA derived from this clone in vitro exhibits a specific infectivity approximately equal to that of genomic RNA purified from virions. Infectious RNA was also derived from two partial strain-Neudoerfl-specific clones after in vitro ligation. In addition, we report on the generation of two clones representing the genome of a more virulent strain of TBE virus (strain Hypr), which are suitable for generating infectious RNA of this strain. Viruses derived from these infectious clones were found to be indistinguishable from the corresponding parent virus strains with respect to antigenic structure, growth properties and virulence in mice.

Methods

Strains Neudoerfl and Hypr are two well-characterized representatives of European subtype TBE virus, and their complete genomic sequences were reported previously (Neudoerfl: Mandl et al., 1988, 1989; GenBank accession no. U27495; Hypr: Wallner et al., 1996; GenBank accession no. U39292).

As starting materials for cDNA synthesis and cloning, stock preparations of these two virus strains were prepared from primary chick embryo cells infected with virus in suckling mouse brain suspensions, and purified from the supernatant medium by sucrosegradient centrifugation as described previously (Heinz & Kunz, 1981).

For the comparative characterization of parent and recombinant viruses, stock solutions were prepared as 20% (w/v) suspensions of infected suckling mouse brain in medium 199 supplemented with Hank’s salts, buffered with 15 mM HEPES, 15 mM HEPPS [N-(2-hydroxyethyl)piperazine-N′-(3-propane sulfonic acid), pH 7.6], containing 1% neomycin and 0.1% BSA. Recombinant viruses were harvested from the supernatants of transfected BHK-21 cells (see below) 3 days after transfection and passaged twice in suckling mouse brains.

Reagents and general procedures.

RNA was extracted from purified virus stocks by Proteinase K digestion, phenol–chloroform extraction and subsequent ethanol precipitation as described previously (Mandl et al., 1988). Approximately 1 µg of RNA was used in each cDNA synthesis reaction (AMV reverse transcriptase cDNA synthesis kit; Boehringer Mannheim) and either random hexanucleotides or TBE virus sequence-specific 20-mer oligonucleotides were used as primers. As outlined in detail below, some genomic clones were obtained directly from these cDNA preparations and, in other cases, specific fragments were first amplified by PCR and subsequently cloned. In all cases, pBR322 was used as plasmid vector and all clones were propagated in E. coli strain HB101. Transfection of bacteria with plasmids was performed by electroporation using electrocompetent cells and the GenePulser apparatus from Bio-Rad (settings: 1.8 kV, 25 µF, 200 Ω). Plasmids were purified using the Wizard Miniprep system (Promega) or, for the preparation of larger amounts, QIAfilter Plasmid Midi or Maxi kits (Qiagen). For DNA ligations, T4 DNA ligase (Stratagene) or the Ligation Express kit (Clontech) were used. Restriction enzymes were purchased from New England Biolabs or Boehringer Mannheim. The virus-specific sequence of each intermediate cloning product was checked by sequence analysis before it was used in a subsequent cloning step. The viral sequences of the five final plasmids were verified again by sequence analysis of both strands after propagation in E. coli HB101 and large-scale plasmid preparation. Sequence analysis was performed in an automated DNA sequencer (ABI-Perkin Elmer) using virus-specific 20-mer primers and fluorescent deoxyribonucleotides.

Specific cloning strategies (Fig. 1). pTNd/5′. A fragment spanning the genome of TBE virus strain Neudoerfl from the 5′ terminus to the unique Clal (nt 3155) site was amplified by PCR with the addition of an artificial 5′-terminal SalI site, and cloned between the Clal and SalI sites of pBR322. Sequence errors caused by PCR were corrected by replacing the BamHI–Clal (nt 1988–3155) fragment with unamplified cDNA. For the removal of the unique Nhel (nt 3142) site, the short Nhel–Clal (nt 3142–3155) fragment was replaced by two complementary synthetic oligonucleotides containing the appropriate silent point mutation. Finally, a T7 promoter sequence was added at the 5′ terminus by replacing the SalI–MluI (5′ terminus to nt 208) fragment with a new PCR fragment synthesized with a primer containing the desired 5′-terminal sequences.

pTNd/c. The Clal–AattII (nt 3155 to the 3′ terminus) fragment from pTNd/5′ was introduced between the corresponding sites of pBR322 to yield a full-length genomic insert of strain Neudoerfl.

pTHy/5′. This clone was assembled from two directly cloned Hypr cDNA fragments, SpIli–Clal (nt 277–3155) and Clal–SalI (nt 3155–5123), and a PCR fragment ranging from the 5′ terminus to the SpIli (nt 277) site. An AattII site, a Nhel site and the T7 promoter were added in front of the viral 5′ terminus using an appropriate primer for the 5′-terminal PCR fragment.

pTHy/3′. As with pTHy/5′, the 3′ half of the strain Hypr genome was assembled from two directly cloned cDNA fragments, SalI–BamHI (nt 5123–8949) and BamHI–AattII (nt 8949–10103), and a PCR fragment spanning the 3′ NCR from the AattII (nt 10103) site to the 3′ terminus situated within an artificial Nhel site.

RNA transcription and transfection. Plasmid pTNd/c (500 ng) was digested with Nhel. After phenol–chloroform extraction and ethanol precipitation the 5′ overhang was partially filled-in using Klenow polymerase (New England Biolabs) in the presence of 12.5 µM dCTP and dTTP (30 min/25 °C). Alternatively, 1 µg of pTNd/5′ was digested with Nhel, treated with Klenow polymerase as described above, digested with Clal and subsequently ligated with 1 µg of pTNd/5′ DNA which had also been digested previously with Clal. Similarly, 1 µg of pTNd/3′ was digested with Nhel, reacted with Klenow, then digested with SalI and ligated with 1 µg of pTHy/5′ previously cut with SalI. The in vitro-ligated DNAs or the linearized full-length DNA (pTNd/c) were purified...
Fig. 1. Schematic drawing of plasmids containing cDNA derived from TBE virus strain Neudoerfl (a) and strain Hypr (b). Viral cDNA sequences are represented by wide, shaded boxes (light for noncoding, darker for coding regions). Gene designations and relevant sites (numbering corresponds to the viral genome sequence) are indicated. Functional elements of the pBR322 vector sequences are shown as thinner, filled boxes. Amp, ampicillin-resistance gene; Tet, tetracycline-resistance gene; ORI, origin of replication. Truncated, non-functional resistance genes are in parentheses.
by phenol–chloroform extraction, precipitated with ethanol and washed with 70% ethanol. The pellet was then resuspended in 40 µl RNase-free double-distilled water and put into the T7 transcription reaction using the reagents and protocols supplied within the MEGAscript T7 kit (Ambion). The transcription reaction contained 7.5 mM each of ATP, CTP and UTP, 1.5 mM GTP and 1 mM cap analogue (m7G(5')ppp(5')G; Ambion). The reaction mixture (20 µl) was incubated for 3 h at 37°C. The amount of full-length RNA synthesized was estimated by comparing band intensities of various sample dilutions with standard RNA preparations of known concentrations after fractionation on denaturing glyoxal–agarose gels and staining with acridine orange.

For RNA transfection, subconfluent BHK-21 cells were collected with trypsin, washed once in serum-free MEM–Earle’s medium and once in ice-cold PBS buffer. Aliquots of approximately 5 x 10⁶ cells were then resuspended in 800 µl ice-cold PBS and mixed in a 0.4 cm gene pulser cuvette with 100 µl PBS containing various amounts of synthetic RNA molecules (between 10 fg and 1 µg). The cells were electroporated by two successive pulses (settings: 1.5 kV, 25 µF, 500 Ω) usually resulting in a time constant of 0.8 milliseconds. Then, 1 ml MEM–Earle’s medium containing 5% foetal calf serum, 1% neomycin and 1% glutamine were added and the cells were resuspended in 800 µl PBS containing various amounts of synthetic RNA molecules (between 10 fg and 1 µg). After 3 h incubation on ice, the RNA-transfected BHK-21 cells were homogenized with a syringe in a 1 ml cuvette with 100 µl PBS containing various amounts of RNA (between 10 fg and 1 µg). The amount of remaining virus inoculum or previously generated virus), cells were washed twice every time the medium was replaced.

**Virulence assay.** Virulence of parent and recombinant viruses was compared by infecting groups of ten Swiss-albino mice-GP (5 weeks old, body weight approximately 20 g) subcutaneously with 10000 p.f.u. of virus, and recording survival of mice for a period of 28 days.

### Results

#### Construction of plasmids

The infectious cDNA clones of TBE virus described in this paper are represented by five plasmids, termed pTNd/c, pTNd/5', pTNd/3', pTHy/5' and pTHy/3'. The structures of these plasmids are shown as schematic drawings in Fig. 1. The specific experimental procedures and strategies involved in the generation, cloning, sub-cloning and sequence analyses of cDNAs leading to the generation of these plasmids are described in detail in Methods. In general, each cloning step was evaluated by complete sequence analysis of the cDNA inserts, and all five plasmids shown in Fig. 1 were found to be stable during transfections into E. coli and large-scale plasmid productions.

All five plasmids are simple derivatives of pBR322. They contain cDNAs derived from two strains of TBE virus, prototypic strain Neudoerfl and strain Hypr. Both strains are closely related representatives of European subtype TBE virus, but they differ significantly with respect to their virulence in mice (Wallner et al., 1996). The complete genomic sequences of both strains have been published previously (Mandl et al., 1988, 1989; Wallner et al., 1996), revealing a high sequence identity (97.2%) between the two strains, except for a major size difference within the 3' NCR, which is approximately 300 nucleotides longer in strain Neudoerfl than in strain Hypr (Wallner et al., 1995).

For TBE virus strain Neudoerfl we obtained a cDNA clone corresponding to the complete genome (pTNd/c), as well as two partial clones containing approximately the 5'-terminal third (pTNd/5') and the 3'-terminal two-thirds (pTNd/3') of the genomic sequences (Fig. 1 a). In the case of TBE virus strain Hypr, two plasmids, termed pTHy/5' and pTHy/3', were constructed containing approximately the 5'- and 3'-terminal halves of the genomic sequences, respectively (Fig. 1 b).

The sequence design surrounding the termini of the genomic sequences is depicted in Fig. 2. T7 promoter sequence elements were placed in front of the cDNA inserts of plasmids pTNd/c, pTNd/5' and pTHy/5'. Between the T7 promoter sequences and the authentic viral 5' termini, an additional G residue was introduced to facilitate efficient transcription (Fig. 2). The 3' termini (plasmids pTNd/c, pTNd/3' and pTHy/3') were constructed to form part of an Nhel restriction enzyme recognition sequence, which was utilized to generate RNA run-off transcripts with authentic 3' termini, as described in Methods.

The 3' NCR of TBE virus strain Neudoerfl contains an internal poly(A) region of apparently variable length (Wallner et al., 1995). In our constructs (plasmids pTNd/c and pTNd/3') this was arbitrarily chosen to be 49 residues long.
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Fig. 2. Nucleotide sequences surrounding the termini of the viral cDNA inserts of the five plasmids shown in Fig. 1. (a) 5’ terminus of strain Neudoerfl as present in plasmids pTNd/5’ and pTNd/c. (b) 3’ terminus of strain Neudoerfl as present in plasmids pTNd/3’ and pTNd/c. (c) 5’ terminus of strain Hypr as present in pTHy/5’. (d) 3’ terminus of strain Hypr as present in pTHy/3’. Virus cDNA sequences are shown in italics. The additional G residue at the 5’ termini is depicted in bold face. Artificially introduced restriction sites are underlined; the T7 promoter sequence is depicted by bold underlining.

Table 1. Differences between the sequence of the infectious cDNA clone and the published genomic sequence of TBE virus strain Neudoerfl

<table>
<thead>
<tr>
<th>Nucleotide number</th>
<th>Neudoerfl genome*</th>
<th>cDNA clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>930</td>
<td>T</td>
<td>C†</td>
</tr>
<tr>
<td>3147</td>
<td>C</td>
<td>T‡</td>
</tr>
<tr>
<td>4860</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>7434</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>7464</td>
<td>G</td>
<td>T</td>
</tr>
<tr>
<td>7617</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>7638</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>8493</td>
<td>A</td>
<td>G</td>
</tr>
</tbody>
</table>

* Numbering and sequence according to GenBank accession number U27495.
† Most likely due to sequence heterogeneity of viral RNA.
‡ Introduced by specific mutagenesis to abolish NheI (nt 3142) site.

The nucleotide sequences of the viral inserts contained in all five plasmids were thoroughly checked by complete sequence analysis on both strands and found to be identical to the previously published sequences (strain Neudoerfl, GenBank accession number U27495; strain Hypr, U39292) except for a few differences present in strain Neudoerfl cDNAs as listed in Table 1. Most of these mutations were caused by the PCR steps during the construction of these clones. At least one difference (nt 930) seems to have been caused by sequence heterogeneity at this position in the original viral RNA pool. Finally, the mutation at nucleotide 3147 was specifically introduced to abolish the unique NheI recognition site within the strain Neudoerfl sequence. The removal of this site was necessary in order to be able to use an NheI-cleavage for the formation of correct 3’ termini (compare Fig. 2). The experimental strategy for this site-specific mutation is described in Methods. All of the nucleotide differences listed in Table 1 are silent mutations, i.e. they do not cause mutations at the amino acid level.

Generation of TBE virus from cloned cDNA

RNA transcripts corresponding to the complete genome of TBE virus strain Neudoerfl were obtained either from the NheI-linearized plasmid pTNd/c, or after ClaI digestion and subsequent in vitro ligation of the two partial-genome-containing plasmids pTNd/5’ and pTNd/3’. In the case of strain Hypr (for which a full-length cDNA clone was not constructed), infectious RNA was obtained by the latter procedure, in this case taking advantage of the unique SalI site within the sequence of this strain. RNAs generated by either of these approaches were found to be highly infectious when introduced into BHK-21 cells by electroporation. Infectious virus could be harvested from the supernatants of transfected BHK-21 cells within 2 or 3 days. At this time, a cytopathic effect indistinguishable from parent TBE virus controls was observed in these cell cultures.

In other experiments, RNA transcripts were injected into the brains of suckling mice. These mice died within 3–4 days (as did baby mice injected with parent TBE virus), showing typical symptoms of TBE virus encephalitis. TBE virus could also be harvested from these mouse brains, but further characterization was not carried out.

Specific infectivity of RNA synthesized in vitro

In order to evaluate the efficiency and reliability of our system, the specific infectivity of RNA transcribed from the full-length clone pTNd/c was determined and compared to genomic RNA extracted from purified virus suspensions. As summarized in Table 2, RNA purified from TBE virus was found to exhibit a specific infectivity of 10^6 infectious units (i.u.) per µg of RNA under the experimental conditions used (i.e. electroporation into BHK-21 cells). In comparison,
Table 2. Specific infectivities of TBE strain Neudoerfl
RNAs isolated from virus or generated in vitro

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>Infectious units/µg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus†</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Infectious cDNA clone (pTNd/c)</td>
<td></td>
</tr>
<tr>
<td>Standard transcription protocol</td>
<td>$10^3$–$10^6$</td>
</tr>
<tr>
<td>No cap analogue</td>
<td>$10^3$</td>
</tr>
<tr>
<td>3′ end by AatII digest</td>
<td>$10^4$</td>
</tr>
<tr>
<td>DNase after transcription</td>
<td>$10^3$–$10^8$</td>
</tr>
</tbody>
</table>

* Determined by electroporation of a log₁₀ end-point dilution series of viral or engineered RNA into BHK-21 cells.
† Genomic RNA extracted from purified virus.

specific infectivity of in vitro-generated RNA varied among individual experiments between $10^6$ and $10^8$ i.u./µg. The specific infectivities of RNAs transcribed from ligated cDNAs could not be determined exactly due to variable ligation efficiencies and the formation of incorrect ligation products, but was estimated to be approximately 10–100-fold lower than that of RNA obtained from the full-length cDNA clone (data not shown).

We also analysed the influence of several modifications of the standard RNA transcription procedure on the specific infectivities of in vitro-generated RNAs: omission of the cap analogue from the T7 transcription resulted in a 1000-fold decrease. RNA transcribed from plasmids linearized with AatII instead of Nhel, which generates transcripts that are slightly too long at their 3′ ends (compare Fig. 2), exhibited a 100-fold lower specific infectivity (Table 2). In contrast, DNase treatment of the reaction mixture after T7 transcription did not have a measurable influence (Table 2) and was consequently omitted from the standard transcription protocol.

Characterization of recombinant viruses

Stock preparations of recombinant TBE virus strains Neudoerfl (R-Neudoerfl) and Hypr (R-Hypr) were produced by passaging the virus harvested from the supernatant medium of RNA-transfected BHK-21 cells twice in suckling mouse brains, as outlined in more detail in Methods. Virus titres in these stock solutions were determined, by plaque tests on PS cells and end-point dilution experiments on BHK-21 and primary chick embryo cells, to be $3 \times 10^8$ and $3 \times 10^9$ i.u./ml for R-Neudoerfl and R-Hypr, respectively. Likewise, stock solutions prepared with parent virus strains Neudoerfl and Hypr contained $3 \times 10^8$ and $1 \times 10^9$ i.u./ml, respectively. The analyses described in this section were all performed using these four virus stocks.

Partial sequence analysis using RT–PCR confirmed the presence of the mutations listed in Table 1 in recombinant virus R-Neudoerfl, including the intentionally introduced silent mutation within the Nhel site (nt 3142) at position 3147 (data not shown). Otherwise, the partial sequence data derived from both recombinant viruses matched the corresponding parent genome sequences exactly.

The antigenic profiles of these viruses were compared to the parent viruses using a well-established set of 18 monoclonal antibodies directed against the envelope protein E (Guirakhoo et al., 1989; Holzmann et al., 1995). As reported previously, TBE virus strain Hypr can be clearly distinguished from strain Neudoerfl by these profiles (Wallner et al., 1996). As expected, the antigenic profiles of the recombinant viruses R-Neudoerfl and R-Hypr were identical to those obtained with the corresponding parent virus strains (data not shown).

The recombinant and parent viruses were further compared using plaque morphology and temperature-sensitivity assays. Neither the parent nor the recombinant viruses were found to be temperature sensitive and no differences in plaque size or appearance were detectable at 37 °C or at 40 °C (data not shown).

The growth properties were further analysed by monitoring the release of virus after infection of primary chick embryo cell cultures. The resulting growth curves shown in Fig. 3 indicate no significant differences between the recombinant viruses and their corresponding parent virus strains. The most efficient virus release occurred between 15 and 18 h post-infection and amounted to $10^9$ f.u./ml/h in the cases of TBE virus strain Neudoerfl and its recombinant derivative R-Neudoerfl. In comparison, the maximum titres achieved by strains Hypr and R-Hypr were found to be between 10- and 100-fold higher.

Finally, we compared the virulence of recombinant and parent viruses in the mouse model. Adult mice were injected...
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subcutaneously with 10,000 p.f.u. of each virus and survival was recorded for 28 days. Results of this experiment are shown in Fig. 4. TBE virus strain Hypr is clearly more virulent, killing all infected mice within 10–12 days, whereas the mean survival time after inoculation with strain Neudoerfl is longer and survival of up to 30% of the mice is usually observed even when doses as high as 10,000 p.f.u. are used for infection. The data presented in Fig. 4 demonstrate that the virulence characteristics of the recombinant viruses R-Neudoerfl and R-Hypr closely match those of the corresponding parent virus strains.

Discussion

Site-specific mutagenesis is an invaluable technique for studying the biology and pathogenesis of viruses. Together with several other flaviviruses (Rice et al., 1989; Lai et al., 1991; Sumiyoshi et al., 1992; Khromyk & Westaway, 1994; Kapoor et al., 1995; Pryor et al., 1996), infectious cDNA clones are now also available for two strains of TBE virus, thus making it possible to specifically mutate the genome of this positive-stranded RNA virus. The actual usefulness of an infectious cDNA clone for the generation of defined virus mutants depends on at least two basic requirements.

(i) The cDNA must be stably inserted into a cloning vector. With several other flavivirus genomes, cloning large cDNAs into bacterial vector systems was very difficult due to instability of such constructs during propagation in the bacterial host. The inability to obtain complete genomic cDNA clones originally hampered the construction of flavivirus infectious clones. This problem was first overcome by Rice et al. (1989), who generated infectious YF virus RNA from two partial cDNA clones after in vitro ligation. With TBE virus prototype strain Neudoerfl, we are now able to obtain a full-length genomic clone, which appears to be perfectly stable. This clone is a simple derivative of pBR322 and is propagated in E. coli HB101. Besides the full-length clone, partial clones of both Neudoerfl and Hypr strains were also obtained, which can be used to generate infectious RNA by the in vitro ligation procedure of Rice et al. (1989). These clones may in fact prove to be more convenient working systems for the generation of site-specific mutations due to the larger number of unique restriction sites in these smaller cDNA inserts.

(ii) The cloned DNA must be completely sequenced and should not carry biologically relevant mutations compared to the parent sequence. Sequence mutations may arise during the cloning procedure and may influence the biology of the recombinant virus, as observed, for example, in the case of the JE virus infectious cDNA clone (Sumiyoshi et al., 1995). This makes it difficult to evaluate the biological effect of a subsequently introduced mutation. In the case of our TBE virus system, the cloned sequences of both TBE virus strains were checked completely and a few silent mutations were found only in the strain Neudoerfl clones. Since these mutations do not alter the amino acid sequence we consider them most likely to be irrelevant, although a biological effect caused solely at the nucleotide level cannot be completely excluded.

If RNA is generated, using an efficient and reliable transcription system, from an infectious cDNA clone that meets both requirements discussed above, this RNA is expected to exhibit a high specific infectivity and the recombinant progeny virus should be phenotypically indistinguishable from its parent virus. Both these characteristics were met by our TBE infectious clone system, indicating that it is, in fact, a reliable experimental tool for the specific mutagenesis of this virus.

Up to now there have been two approaches to generate viruses with specifically mutated TBE viral sequences. In the chimera approach reported by Pletnev et al. (1992, 1993), structural proteins of DEN 4 virus were replaced by the corresponding TBE proteins. Mutations introduced into the TBE virus coding sequences were shown to have significant effects on the biology of the chimeric virus progeny. Due to the combination of TBE and dengue virus genetic information in these constructs it remained difficult to determine to what extent these effects were influenced by the interaction of the heterologous nucleotide and protein sequences present in these chimeras.

Recently, a fast and straightforward approach to generate infectious TBE viral RNA from uncloned cDNA amplified by PCR was introduced by Gritsun & Gould (1995). This attractive alternative, however, seems to be restricted by the fact that the uncloned PCR-derived cDNAs will give rise to heterogeneous RNA populations. As a consequence, the presence of random mutations in the recombinant virus progeny derived from such an experimental system cannot be excluded.

A remarkable feature of the TBE viral genome is its unusual size heterogeneity of the 3’ NCR, even among closely related strains (Mandl et al., 1991; Wallner et al., 1995; Dobrikova &
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References


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