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TERMINAL SEQUENCES OF THE REPLICATIVE FORM OF RNA OF THE JAPANESE ENCEPHALITIS VIRUS

MASAAKI NAGAMATSU, *YO KIKUCHI, AKIRA IGARASHI

Department of Virology, Institute of Tropical Medicine, Nagasaki University, 12-4 Sakamoto-machi, Nagasaki City 852, Japan, and *Mitsubishi-kasei Institute of Life Sciences, 11 Minami-ooya, Machida City, Tokyo 194, Japan

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Summary. — The 5′ and 3′ terminal sequences of the replicative form (RF) of RNA of a flavivirus, the Japanese encephalitis (JE) virus, strain Ja0ArS982, have been determined by in vitro labelling and mobility shift analysis. The plus strand sequence was 5′AGAAGUUUAUCUGUGUGAA...UCU_{OH}3′, while the minus strand sequence was 5′AGAUCCUGUGUUCUUCCUCA... UCU_{OH}3′. These sequences were similar to those of West Nile (WN) virus being identical in 12 nucleotides at the 5′terminal of the minus strand, and in the 5′terminal dinucleotides, 5′AG3′. Somewhat more internal hexanucleotides 5′CUGUGU3′ are conserved among 3 flaviviruses, the JE, WN, and yellow fever viruses.

Key words: RNA sequence; RNA replicative form; Japanese encephalitis virus; two dimensional mobility shift analysis

Introduction

We have determined the complete nucleotide sequence of the single--stranded genomic RNA of Japanese encephalitis (JE) virus, a member of family *Flaviviridae*, by cDNA cloning and sequencing. In order to confirm the terminal sequences of genomic RNA, it was necessary to identify the terminal sequences of the replicative form (RF) of viral RNA extracted from the infected cells. Here, we report the data obtained by direct sequencing using 2-dimensional mobility shift analysis.

Materials and Methods

Cells. Aedes albopictus, clone C6/36, cells (Igarashi, 1978) were grown as suspension culture in spinner bottles at 28°C with Eagle's minimal essential medium supplemented with 0.2 mmol/l each of nonessential amino acids and 10 % heat-inactivated foetal calf serum. When the cell density reached around 10^6 cells/ml, microcarrier (Cytodex 1, Pharmacia, Sweden) was added to final concentration of 0.2 mg/ml at 6—18 hr before virus inoculation in order to attach the cells onto the beads.

Purification of the virus and preparation of viral RNA. The procedures were similar as described by Hori *et al.* (1986). Growth medium was removed from C6/36 cell culture in suspension with

microcarrier by decantation and a wild strain of JE virus, Ja0ArS982, was inoculated at high multiplicity of infection about 10 PFU/cell. After 2 hr of adsorption, the cells were fed with the maintenance medium (the cell growth medium in which serum concentration was reduced to 2 %) and were incubated again at 28 °C in suspension. Infected culture fluid was harvested every day and the cells were replenished with fresh medium until 7 days post infection. The harvested medium was clarified by filtration and high speed centrifugation (10,000xg, 15 min). The wirus concentrated from the supernatant by polyethylene glycol precipitazion, resuspended in STE buffer (0.1 mol/l NaCl, 0.01 mol/l Tris-HCl, 0.001 mol/l EDTA, pH 7.4), clarified again and pelleted by ultracentrifugation through 15 % sucrose in STE using a Beckman SW 41 rotor at 37,000 rev/min for 120 min. Virion RNA was phenol extracted from the pellet and ethanol precipitated. The purity of the RNA was examined by agarose gel electrophoresis with ethidium bromide staining.

Preparation and analysis of RF RNA. Total cellular RNA was phenol-extracted from infected C6/36 cells in the presence of 0.1 % SDS, ethanol precipitated and fractionated by LiCl. The RNA soluble in 2 mol/l LiCl was ethanol precipitated, phenol extracted and subjected to 0.7 % agarose gel electrophoresis. The RF RNA at approximately 11 kbps position was electroeluted, phenol extracted, and ethanol precipitated. The 3′ ends of the RF RNA were labelled with (5′—^{32}P)pCp and RNA ligase (England and Uhlenbeck, 1978). After ethanol precipitation, the labelled RNA was purified by 6 % polyacrylamide gel electrophoresis (PAGE). The RF RNA was dephosphorylated at its 5′-ends by calf intestine alkaline phosphatase and labelled with γ-^{32}P-ATP and polynucleotide kinase, followed by the purification with Sephadex G-50 gel filtration (Keene *et al.* 1978) and 0.7 % agarose gel electrophoresis. A part of the RF RNA labelled at 5′ ends of both plus and minus strands were hybridized in excess with unlabelled virion RNA and then separated by CF11 cellulose chromatography into single-stranded radioactive plus strand of RF RNA and double-stranded RNA containing radioactive minus strand of RF RNA.

Two dimensional mobility shift analysis. The terminal nucleotide sequences were determined by mobility shift analysis (Jay *et al.*, 1974; Gillum *et al.*, 1975; Wengler and Wengler, 1981) on the following specimens; (1) RF RNA labelled at the 3′ ends of both plus and minus strands, (2) RF RNA labelled at the 5′ ends of both plus and minus strands, (3) labelled plus strand chased from RF RNA, and (4) labelled minus strand remaining on RF RNA after chasing. The first dimension of mobility shift analysis was performed by cellulose acetate electrophoresis at pH 3.5, 5 % acetic acid, 0.5 % pyridine, 1 mmol/l Na_2 EDTA, and the second dimension by homochromatography on DEAE cellulose-coated thin layer chromatography (TLC) in 30 mmol/l homomix (3 % sodium salt of crude RNA from yeast, 7 mol/l urea, 30 mmol/l KOH).

Results

The 3′-end labelled and RNase T2 digested RF RNA was analysed by cellulose coated TLC with a solvent system (isobutylic acid 577) concentrated ammonium 38(H_2O 385). The result showed the spot of Up, indicating that the 3′ terminal nucleosides for both plus and minus strands were uridine. The 3′-end labelled RF RNA was alkali-digested and subjected to mobility shift analysis. The result in Fig. 1 showed the sequence of 5′... $UCU_{OH}3'$. Although the sample was not separated into plus and minus strands because of the low labelling efficiency of the 3′-end, the result indicated that the 3 nucleotides at the 3′-ends of both plus and minus strands were identical.

The 5′-end labelled and nuclease Pl digested RF RNA showed only the spot of pA on TLC, indicating that the 5′-end nucleotide is A for both plus and minus strands. The RF RNA could be labelled at the 5′-end with (γ-^{32}P) ATP and T4 polynucleotide kinase after alkaline phosphatase treatment alone, indicating that the 5′-ends of both plus and minus strands of RF RNA were not capped.

The results of mobility shift analysis on the 5′-end labelled RF RNA and its separated plus and minus strands, are presented in Figs. 2—4. The results showed that the plus strand sequence was 5′AGAAGUUUAUCUGUGUGAA...3′, and the minus strand sequence was 5′AGAUCCUGUGUUCUUCCUCA...3′ (Fig. 5).

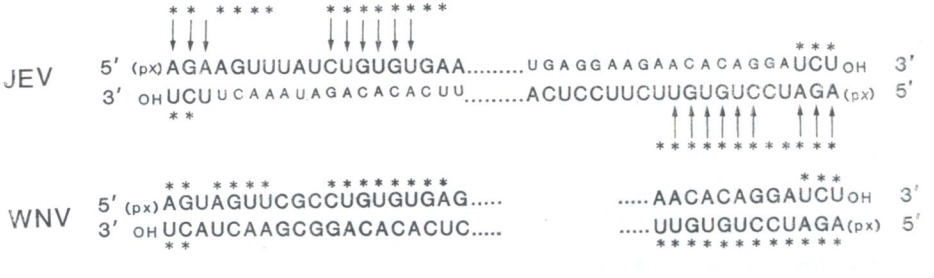

Fig. 5.

Terminal nucleotide sequences of JE virus RF RNA and the comparison with the sequences of WN virus RF RNA.

Identical nucleotides in the plus and minus strands are indicated with an arrow. The nucleotides common to JE and EN virus RF RNAs as reported by Wengler and Wengler (1981) are marked by asterisks. The sequences presented by small letters were derived from the sequence of the complementary strand.

Discussion

The terminal sequences of JE virus RF RNA are similar to those of WN virus reported by Wengler and Wengler (1981) with identical 5′ terminal 12 nucleotides of minus strands. The terminal dinucleotides, 5′AG3′, and somewhat internal hexanucleotides 5′CUGUGU3′ are conserved among 3 flaviviruses, i. e. the JE, WN, and yellow fever viruses (Rice *et al.*, 1985). These homologous sequences may have some crucial roles in the replication and/or transcription of flaviviruses.

Flavivirus genomic RNAs were reported to have no polyA tract at their 3′ ends (Wengler *et al.*, 1978). When the 3′ end labelled JE virus genomic RNA was purified by 15 % PAGE, digested with RNase T2 and analysed by TLC, the result showed Up and Ap spots. However, the Ap spot was supposed to have originated from the 3′ termini of contaminating tRNAs. Therefore, the 3′ terminal nucleoside of JE virus genomic RNA could probably be uridine, which is compatible with the data of JE virus genomic RNA obtained by cDNA clones (Sumiyoshi *et al.* in preparation) or direct sequencing (Takegami *et al.*, 1986).

Previous reports on flaviviruses such as WN and dengue-2 have shown a type 1 cap structure $m^7GpppAm$ at the 5′ terminus (Wengler *et al.*, 1978; Cleaves and Dubin, 1979). Since the 5′ terminal base of JE virus RF RNA was A, the 5′ terminal adenosine in genomic RNA of JE virus may probably be methylated by post transcriptional modification. However, this question needs further elucidation.

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Legends to Figures (Plates VIII—XI):

Fig. 1. Mobility shift analysis of 3′-end labelled RF RNA. JE virus RF RNA was 3′-end labelled, purified by PAGE and digested by heating at 95 °C for 3—15 min. in 50 mmol/l $NaHCO_3$, 0.5 mmol/l EDTA before analysis

Figs. 2—4. Mobility shift analysis of 5′-end labelled RF RNA. A part of the 5′-end labelled and purified RF RNA was analysed (2). The remainder was hybridized with excess genomic RNA and separated by CF11 cellulose chromatography into single-stranded radioactive plus strand from RF RNA in (3), and double-stranded RNA containing radioactive minus strand from RF RNA in (4) before analysis. Each sample was alkali digested and analysed as described in the legend to Fig. 1.