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EVIDENCE OF 5′-TERMINAL MODIFICATION IN THE KEMEROVO VIRUS DOUBLE-STRANDED RNA SEGMENTS AND ITS REMOVAL BY TREATMENT WITH ALKALINE PHOSPHATASE

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Summary. — Only one strand of each double-stranded (ds) RNA segment of the Kemerovo virus genome was 5′end-labelled using γ-^{32}P-ATP and T 4 polynucleotide kinase after preceding dephosphorylation of 5′ends by calf intestinal alkaline phosphatase. This suggests a 5′-terminal modification of the one of complementary strands in the ds RNA segments.

Key words: *Kemerovo virus*; *double-stranded RNA*; *5′-terminal modification*; *alkaline phosphatase*

Introduction

The presence of a cap stucture at 5′ends of the plus RNA strands of double-stranded RNA (dsRNA) segments has been referred to for viruses of family *Reoviridae* such as reovirus, cytoplasmic polyhedrosis virus or rotavirus (Furuichi and Miura, 1973; Furuichi *et al.*, 1975; Imai *et al.*, 1983). The R 10 strain of Kemerovo virus (KV) is a member of the genus *Orbivirus* of the family *Reoviridae* (Verwoerd *et al.*, 1979). Genomic RNA of KV consists of ten dsRNA segments (Slávik *et al.*, 1984). In order to investigate whether the 5′-terminal modification in dsRNA segments of KV was present or not, dephosphorylation by calf intestinal alkaline phosphatase (CIP) and labelling of 5′ends using γ-^{32}P-ATP and T4 polynucleotide kinase were performed. The electrophoretic separation of plus and minus RNA strands was achieved in agarose gel as described by Smith *et al.* (1981).

Materials and Methods

Preparation of KV dsRNAs. dsRNA was isolated from partially purified KV (Bačík and Čiampor, 1989) as follows: the band of viral material from CsCl density gradient (density of 1.32 g × cm^{-3}) was diluted to the final volume of 2.5 ml by addition of solutions of sodium dodecyl sulphate (SDS) and sodium acetate to the concentration of 1% and 0.5 mol/l, respectively. After adding an equal volume of water-saturated phenol, the sample was shaken for 30 min at 60 °C and cooled in an ice-bath. The sample was centrifuged at 5 000 rev/min for 5 min (K-23 centrifuge MLW, G. D. R.) to separate the phases. The water phase was again extracted with phenol at room temperature for 3 min and then with the same vol of chloroform — isoamyl-

alcohol (24 : 1) mixture. RNA was precipitated from the water phase by 3 vol of ethanol at −20 °C.

Dephosphorylation of the 5' ends of dsRNA. Two different methods were used. The first was essentially the same as described by Donis-Keller *et al.* (1977), using dsRNA as substrate instead of yeast ribosomal RNA, and CIP (type VII-L, 1480 units/mg, Sigma), instead of bacterial alkaline phosphatase. In brief, 50 μg dsRNA were treated with 1 unit of CIP in 100 μl of 100 mmol/l Tris-HCl pH 8.0, at 37 °C for 30 min. The second method used was specific for blunt ends of nucleic acids (Maniatis *et al.*, 1982). For this procedure 50 μg of dsRNA in 100 μl of CIP buffer (0.05 mol/l Tris-HCl pH 9.0, 1 mmol/l $MgCl_2$, 0.1 mmol/l $ZnCl_2$, 1 mmol/l spermidine) were incubated with 1 unit of CIP for 15 min at 37 °C and another 15 min at 56 °C; the whole procedure was repeated again. After dephosphorylation, 100 μl reaction mixture was diluted to the vol of 250 μl by the addition of EDTA-Na_2, sodium acetate and SDS to the concentration of 2 mmol/l, 0.5 mol/l and 1% respectively; then 250 μl of water-saturated phenol was added, the sample was incubated twice at 70 °C for 1 min and cooled in an ice-bath. After vigorous mixing the phases were separated by centrifugation and the water phase was again extracted with a mixture of chloroform-isoamylalcohol. dsRNA was precipitated from the water phase with 3 vol of ethanol at −20 °C.

Labelling the 5' ends of dsRNA. dsRNA dephosphorylated at 5'ends was labelled using γ-^{32}P-ATP and T4 polynucleotide kinase as described by Maniatis *et al.* (1982) for blunt ends of nucleic acids. Unincorporated radiolabelled substrates were then removed by the "spun-column" technique using Sephadex G-50 (Maniatis *et al.*, 1982). After that, the reaction mixture was extracted twice with phenol and once with a mixture of chloroform-isoamylalcohol. dsRNA was recovered from the water phase by ethanol precipitation.

Analysis of ^{32}P-labelled RNAs. Procedure of agarose-urea gel electrophoresis was used (Smith and Furuichi, 1980; Smith *et al.*, 1981). For separation of dsRNA segments, the samples were applied in 7 mol/l urea onto the gel. The separation of complementary RNAs of dsRNA segments was achieved after denaturing the dsRNAs in 7 mol/l urea; the RNA samples were heated in boiling water bath for 20 sec and quickly cooled on ice prior to loading the gel. The RNAs were electrophoresed for 16 hr at 130—160 V, 14—18 mA and 4 °C, until the bromophenol blue dye front migrated about 15 cm. Bands containing ^{32}P-labelled RNAs were detected by autoradiography of wet gels at 4 °C using X-ray film Medix-Rapid (Foma, Hradec Králové).

Results

Evidence of 5'-terminal modification of KV dsRNA segments

Dephosphorylation by phosphatase as described by Donis-Keller *et al.* (1977) was applied on KV dsRNA segments to free 5'ends prior to ^{32}P-labelling, using γ-^{32}P-ATP and T4 polynucleotide kinase. Then, ten KV dsRNA segments (Slávik *et al.*, 1984) were resolved in agarose-urea gel into 7 bands (Fig. 1*a*). The dsRNA segments 4,5 and 6 and 7 and 8 were not detected by autoradiography as separate bands. After heat-denaturation of the dsRNA segments 10 bands of RNA with higher electrophoretic mobilities than dsRNA segments were detected (Fig. 1*b*). This means, that complementary RNAs of each dsRNA segment were separated from each other but only the unblocked 5'ends of the RNA strands from dsRNA segments were labelled and detected by autoradiography.

Removal of the 5'-terminal modification

KV dsRNA segments dephosphorylated and 5'ends-labelled according to Maniatis *et al.* (1982) were resolved in seven bands by agarose-urea gel electrophoresis (Fig. 2*a*). After heat-denaturation the resulting complemen-

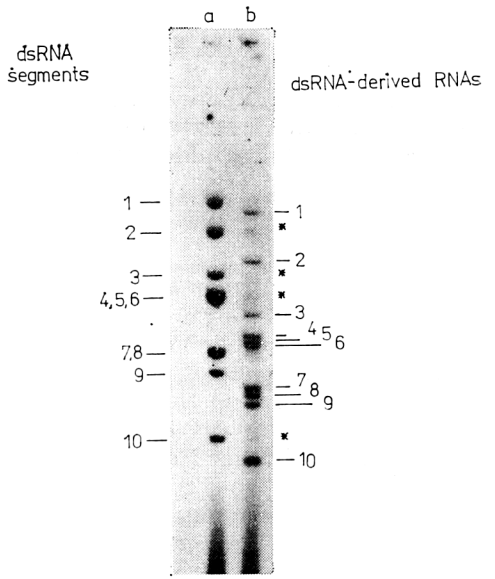

Fig. 1
Separation of the KV genome RNAs by electrophoresis in 1.75% agarose gel in the presence of 7 mol/l urea
KV dsRNA was treated with alkaline phosphatase according to Donis-Keller *et al.* (1977) prior to the 5′end-labelling. Lane a: native dsRNA segments of KV; lane b: dsRNA-derived RNAs obtained after heat-denaturation in 7 mol/l urea.

tary RNA strands migrated more quickly in agarose gels than the original dsRNA segments (Fig. 2*b*); some of them were comparable with those detected before (Fig. 1*b*). But the bands of RNA detected and marked as a_1 and a_2, and also h_1 and h_2 (Fig. 2*b*) were not detected when dephosphorylation according to Donis-Keller *et al.* (1977) was performed prior to the ^{32}P-labelling of 5′ends (Fig. 1*b*). This means that following dephosphory-

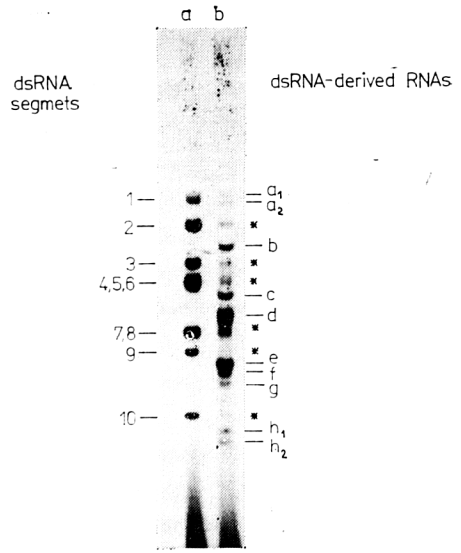

Fig. 2
Electrophoretic separation of KV genome RNAs in agarose-urea gel
dsRNA segments were dephosphorylated by alkaline phosphatase according to Maniatis *et al.* (1982), prior to the 5′end-labelling. Lane a: native dsRNA segments; lane b: dsRNA-derived RNAs obtained after heat-denaturation in 7 mol/l urea. Some incompletely denatured dsRNA segments are marked by asterisks.

lation of blunt 5′ends as described by Maniatis *et al.* (1982), both complementary RNA strands of the dsRNA segments were ^{32}P-labelled by γ^{32}P-ATP using the T4 polynucleotide kinase. The structure blocking the 5′terminus of one RNA strand in the dsRNA segments was removed during the treatment with alkaline phosphatase.

Discussion

Smith *et al.* (1981) when labelling the 5′ends of reovirus dsRNA segments with γ-^{32}P-ATP and T4 polynucleotide kinase after dephosphorylation with CIP (Boehringer, Mannheim) according to Miura *et al.* (1974) reported that the minus RNA strands of reovirus dsRNAs became labelled only. Mertens and Sangar (1985) found that using CIP (Sigma) and the same methods of dephosphorylation and ^{32}P-labelling of BTV segments as above, both complementary RNA strands (plus and minus) of each dsRNA segments were equally labelled at the 5′terminus. The authors supposed that a blocking structure at the 5′terminus of plus strand had been removed probably during the phosphatase treatment.

In our experiments with KV dsRNA segments, two procedures of dephosphorylation of 5′ends by CIP were used, but the same procedure of ^{32}P-labelling in both cases. Using mild conditions during CIP treatment (Donis-Keller *et al.*, 1977), followed by ^{32}P-phosphorylation only one strand of each dsRNA segment became ^{32}P-labelled. Using more stringent dephosphorylation (Maniatis *et al.*, 1982), ^{32}P-labelling of both complementary strands of dsRNA segments was found. In the latter method of dephosphorylation, not only Mg^{2+} ions, but also Zn^{2+} ions were added to the reaction mixture. Fernley and Walker (1967) reported that the same enzyme — calf intestinal alkaline phosphatase was responsible for phosphomonoesterase, pyrophosphatase, and adenosine triphosphatase activities; in the presence of excess of Mg^{2+} ions, the phosphomonoesterase activity was enhanced, while pyrophosphatase and adenosine triphosphatase activities were strongly inhibited.

Our results indicate that the pyrophosphatase activity of CIP preparations might be influenced also by the presence of Zn^{2+} ions in addition to Mg^{2+}. Treatment of dsRNA segments with CIP can be chosen according to the purpose, using either the method described by Donis-Keller *et al.* (1977) or that by Maniatis *et al.* (1982).

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