KOR. JOUR. MICROBIOL. September, 1991 p. 221-225 Vol. 29, No. 4

Analysis of RNA Transcripts Generated by Bluetongue Virus Core

Yang, Jai Myung and JaRue S. Manning*
Department of Genetic Engineering, Sung Kyun Kwan University, Suwon, Korea
*Department of Microbiology, University of California, Davis, California, U.S.A.

Bluetongue virus core에 의해 생산된 RNA 전사체 분석

양 재 명 · JaRue S. Manning*
성균관대학교 유전공학과, *캘리포니아대학교 대이비스

ABSTRACT: The RNA transcripts produced from *in vitro* transcription reaction of BTV core were analyzed on agarose-urea gel. Fast migrating abortive RNAs, in addition to full length species of RNA, were observed. Fast migrating RNAs extracted from agarose-urea gel were hybridized to all 10 segments of genomic ds RNA, while slow migrating RNAs extracted from agarose-urea gel were hybridized only to the large and medium size genomic ds RNA. These results indicate that fast migrating RNA transcripts are most likely the products of abortive transcription.

KEY WORDS □ Bluetongue virus; transcriptase; abortive transcription; agarose-urea gel

INTRODUCTION

Bluetongue is a disease of domestic and wild ruminants caused by bluetongue virus (BTV), a member of family Reoviridae. The capsid of BTV is composed of four major and three minor polypeptides, which contains ten genomic segments of ds RNA size ranged from 0.5×10^6 to 2.8×10^6 daltons (Verwoerd *et al.*, 1972). The ten segments of RNA are roughly distributed into three groups namely, large (segment 1, 2, and 3), medium (segment 4, 5, and 6), and small (segment 7, 8, 9, and 10), based on their size (Verwoerd and Huismans, 1972; Van Dijk and Huismans, 1980).

Treatment of the virion with chymotrypsin in the presence of $MgCl_2$ removes both P_2 and P_5 and the resultant BTV core exhibits RNA dependent RNA polymerase (transcriptase) activity *in vitro* (Van Dijk and Huismans, 1980). The hybrid RNA mixture of *in vitro* transcribed RNAs and denatured genomic RNAs were analyzed on gel electrophoresis but RNA transcripts themselves were not studied.

In vitro transcription of closely related reovirus is well studied (Joklik, 1981). Electron microscopic study suggests that the reovirus core is able to transcribe all 10 genomic ds RNA (Bartlett *et al.*, 1974) and the transcripts are spontaneously released or extruded from the intact core (Luftig, *et al.*, 1972). When the RNA transcripts were analyzed on agarose urea gel electrophoresis, 8 to 10 RNA species of the appropriate molecular weights were separated (Dryana and Fields, 1982).

In the presence of required Mg^{2+} ion, all 10 RNA segments are transcribed at the same rate. Four and two times as many small and medium size RNA transcripts, respectively, are synthesized as large size RNA transcripts implying that the frequency of transcription initiation is not the same for each genomic RNA but is inversely proportional to the RNA size (Skehel and Joklik, 1969).

In this study the RNA transcripts produced from *in vitro* transcription reaction of the BTV core were analyzed. It was observed that the BTV core produced both complete full length and abortive nascent RNA transcripts. The possible cause for the generation of abortive nascent RNA transcripts was discussed.

MATERIALS AND METHODS

Virus and cell

BHK 21 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO). BTV (kindly provided by B. Osburn, University of California, Davis) grown in BHK 21 cells was harvested at 24 to 48 hr postinfection and purified as described by Poli *et al.* (1982).

Preparation of BTV core

BTV at a concentration of 210 μg/m*l* was treated with 0.1 M Tris-HCl (pH 8.0), 0.6 M $MgCl_2$, and 32 μg/m*l* of chymotrypsin. The mixture was incubated for 1 hr at 37℃, diluted 1:3 with 2 mM Tris-HCl (pH 8.8), and pelleted through 40% sucrose cushion in a Beckman SW 50.1 rotor at 40,000 rpm for 45 min. The core was resuspended in 2 mM Tris-HCl (pH 8.8) buffer at a concentration of 4 μg/u*l* and stored in 4℃.

Transcriptase assay

The transcription reaction was done as described by Van Dijk and Huismans (1980) with a minor modification. The reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 1.7 mM each of GTP, CTP, and ATP, 20 μCi of [α-^{32}P] UTP (specific activity 410 μCi/mmole, Amersham), 6 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mg/m*l* pyruvate kinase, 2 mM dithiothreitol (DTT), 0.25 mM S-adenosyl-L- Methionine (SAM), 7.5 mM phosphoenol pyruvate (PEP), 100 units of RNasin (Promega Biotech.), and 20 μg of BTV core. Unlabeled UTP was also added to each reaction mixture to the various final concentration. After 5 hr of incubation at 28℃, RNA was purified as described in the next section.

RNA purification

The unincorporated [α-^{32}P]-UTP was removed by spin dialysis (Maniatis *et al.*, 1982). RNA transcripts were purified by centrifuging the reaction mixture in a Beckman SW 50.1 rotor at 40,000 rpm for 45 min to remove core. The supernatant was phenol extracted, washed with chloroform, ethanol precipitated, pelleted, vacuum dried, and resuspended in 0.1 X TE.

Electrophoresis

RNA transcripts were separated by electrophoresis in a slab gel containing 1.5% agarose, 7 M urea, 36 mM Tris-HCl (pH 7.8), 30 mM NaH_2PO_4, and 1 mM EDTA. The gel solidified at 4℃ overnight was run for 16 hr at a constant voltage of 50 V, rinsed with deionized water to remove urea, dried, and exposed to Kodak XR-5 film for autoradiography. BTV genomic RNA was run in 1% agarose gel prepared in TEA buffer (0.04 M Tris-acetate (pH 8.0), 21 mM EDTA) at 1.5 V/cm for 16 h.

Polyacrylamide gel electrophoresis

BTV was disrupted by boiling for 3 min in electrophoresis sample buffer (0.06 M Tris-HCl (pH 6.8), 4% SDS, 40% glycerol, 3% dithiothreitol, 0.005% bromophenol blue). Samples were applied to 10% acrylamide gel (acrylamide/bisacrylamide weight ratio, 37.5:1) and subjected to electrophoresis as described previously (Laemmli, 1970).

Northern blot analysis

BTV genomic RNA, separated in 1.0% agarose gel, was denatured in 0.4 N NaOH for 15 min, neutralized in 12 mM Tris (pH 7.5), 6 mM sodium acetate, and 0.3 mM EDTA for 30 min, and transferred to nylon membrane (New England Nuclear) by electroblotting. The membrane was prehybridized in 10 m*l* of 50% formamide, 1% SDS, 1 M sodium chloride, and 10% dextran sulfate overnight at 42℃. ^{32}P labeled RNA transcripts and denatured salmon sperm DNA (150 μg/m*l*) were added into the bag. The bag was resealed and incubated at 42℃ overnight. The membrane was washed twice with 100 m*l* of 2 X SSC (1 X SSC : 0.15 M NaCl-0.015 M sodium citrate) at room temperature for 5 min, twice with 200 m*l* of 2 X SSC and 1% SDS at 65℃ for 30 min, twice with 100 m*l* of 0.1 X SSC at room temperature for 30 min, dried, and exposed to Kodak XR-5 film for autoradiography.

RESULTS

Preparation of BTV core

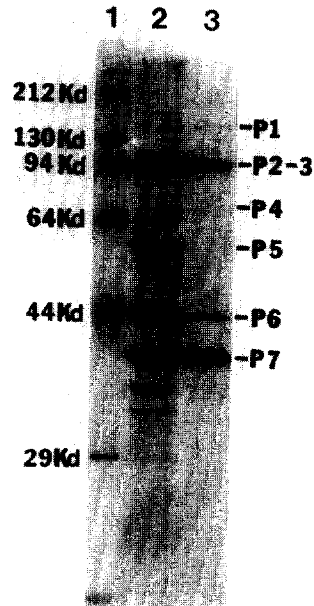

Fig. 1. *10% SDS polyacrylamide gel electrophoresis of BTV core. BTV core was prepared as described in Materials and Methods. (1) molecular weight standard marker; (2) BTV; (3) BTV digested with 8 1g of chymotrypsin.*

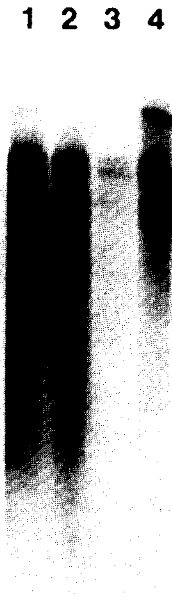

Fig. 2. *1.5% agarose-7 M urea gel electrophoresis of the RNA transcripts produced from BTV core. The in vitro* transcription and RNA purification were done as described in Materials and Methods except that unlabeled UTP was added into each reaction mixture to the final concentration of 0 mM (lane 1), 0.034 mM (lane 2), 0.17 mM (lane 3) and 1.7 mM (lane 4).

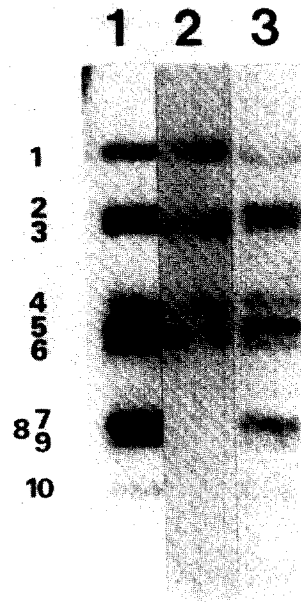

Fig. 3. *Northern blot analysis of the RNA transcripts produced from BTV core. Transcription reaction, purification and separation of the RNA transcripts were done as described in Fig. 2 legend except that 120 Ci of [α-^{32}P] labeled ATP, CTP, GTP and UTP were also added and total reaction volume was increased to 1.2 ml. Fast and slow migrating RNAs were extracted from preparative gel by crushing the gel into small pieces and soaked in 0.1 X TE buffer at room temperature overnight with constant agitation. ^{32}P labeled whole RNA transcripts (lane 1), slow migrating RNA transcripts (lane 2) and fast migrating RNA transcripts (lane 3) were hybridized back to the denatured BTV genomic RNA as described in Materials and Methods.*

BTV grown in BHK 21 cells was purified by sucrose density gradient (Poli *et al.*, 1982). Purified BTV before and after digestion with chymotrypsin in the presence of 600 mM $MgCl_2$ was run in 10% SDS polyacrylamide gel electrophoresis. Lane 3 of Fig. 1 shows that chymotrypsin removed the two major polypeptides (P_2 and P_5) from the viral capsid yielding the core. The resultant BTV core was pelleted through sucrose cushion and its transcriptase activity was assayed *in vitro*.

***In vitro* transcription of BTV core**

BTV core was added into the transcription reaction mixture and incubated for 5 hr at 28℃. RNA transcripts labeled with [α-^{32}P] UTP were partially purified by spin dialysis and centrifuged for 45 min at 40 K rpm in a Beckman SW 50.1 rotor to remove core. The supernatant containing the RNA transcripts was analyzed in agarose-urea gel electrophoresis. Although full length RNA bands were clearly visible, the resolution of the small size RNAs was rather poor (Fig. 2, lane 1).

The intensity of the large size RNA appeared to be equal compared to the medium and small size RNA. This is most likely due to the frequency of transcription initiation being inversely proportional to the RNA size as observed in reovirus *in vitro* transcription. The addition of unlabeled UTP to the final concentration of 1.7 mM improved the resolution of individual band, decreased the production of fast migrating RNA, and increased the intensity of large size RNA band (Fig. 2, lane 4).

Northern blot hybridization

When whole RNA transcripts produced from BTV core *in vitro* were hybridized to the denatured genomic ds RNA by Northern blot analysis, all 10 segments of RNA were visible with similar band intensity (Fig. 3, lane 1) indicating that overall ^{32}P-UTP incorporation into each RNA species was in similar level. This result together

with the above suggests that short length abortive nascent RNAs, in addition to full length species of all three class RNA, were also produced from the *in vitro* transcription reaction.

To prove that the short length abortive nascent RNAs were generated from the BTV core, the RNA transcripts were separated in an agarose-urea gel. Slow migrating and fast migrating RNAs were extracted from the gel and hybridized to the denatured genomic ds RNA using Northern blot technique. As seen in Fig. 3, slow migrating RNAs were only hybridized to the large and some of the medium size RNAs (Fig. 3, lane 2) while the fast migrating RNAs were hybridized to all 10 RNA segments (Fig. 3, lane 3) indicating that the fast migrating RNAs were composed of full length species of small size RNA and short length abortive transcripts of the large, medium, and small size RNA.

DISCUSSION

In this study we confirmed and extended observation (Van Dijk and Huismans, 1980) on *in vitro* RNA transcription from BTV core prepared by chymotrypsin treatment. RNA transcripts analyzed in an agarose-urea gel, showed the presence of both fast migrating RNAs as well as full length species of RNA. Fast migrating RNAs extracted from agarose-urea gel were hybridized to all 10 genomic ds RNA, while slow migrating RNAs extracted from agarose-urea gel were only hybridized to the large and medium size genomic ds RNA. These results indicate that the fast migrating RNAs are the products of abortive transcription or/and degradation products of the transcripts.

Unlike BTV core, abortive transcription does not appear to occur in reovirus core since 8 to 10 species of RNA transcripts with similar intensity were clearly observed (Dryana and Fields, 1982). The transcription initiation of reovirus appears to be a complex process. The initiator oligonucleotides (less than five residues) are made in high molar excess relative to complete mRNA because of reiterative initiation (Yamakawa *et al.*, 1981). However, once the initiation complex reads through first few residues, the complex appears to be stable during elongation process since pulse labeled mRNA could be chased with unlabeled NTPs after several cycles of washing without generating significant amount of abortive nascent RNA (Nichols *et al.*, 1972). Similar finding was also observed in psoralen inactivated reovirus core (Nakashima *et al.*, 1979).

It is not clear why BTV core exhibits greater abortive transcription compared to reovirus core. Several possible explanations are 1). BTV RNA transcripts may not be stable compared to that of reovirus. When reaction time was reduced from 5 hours to various intermediate time, it was found that, although total acid precipitable counts were reduced, the RNA band resolution was greatly improved as reaction time was reduced (data not shown) suggesting that RNA transcripts appeared to be degraded under the given reaction condition. 2) Since BTV is unstable at high salt concentration, an extended reaction time changes intact core into loose conformation which leads to the failure of tight association between transcriptase and template RNA. The resultant dissociation of transcriptase from template RNA would release nascent abortive RNA. 3) The optimum temperature of *in vitro* transcription for BTV and reovirus is 28℃ (Van Dijk and Huismans, 1980) and 48℃ (Joklik, 1981), respectively. The temperature would be another measure of the stability of the transcriptase and template complex. Additional work should be done to answer all the above possible explanations.

적 요

*Bluetongue virus core*에 의해 *in vitro*에서 생산된 RNA 전사체를 아가로스우레아 젤로 분석하였다. 완전한 길이의 전사체 외에 빠르게 이동하는 짧은 길이의 전사체가 관찰되었다. 이들은 모든 genomic RNA와 교잡하나 느리게 이동하는 긴 전사체는 길이가 길거나 중간인 genomic RNA와만 교잡한다. 이 실험 결과는 빠르게 이동하는 짧은 전사체가 abortive 전사에 의한 산물임을 가리킨다.

REFERENCES

1. **Bartlett, N.M., Gillies, S.C., Bullivant, S. and Bellamy, A. R.** 1974. Electron microscopic study of reovirus reaction cores. *J. Virol.* **14**, 315-326.
2. **Dryana, D. and Fields, B.N.** 1982. Activation and characterization of the reovirus transcriptase: Genetic Analysis. *J. Virol.* **41**, 110-118.
3. **Joklik, W.K.** 1981. Structure and function of the reovirus genome. *Microbiol. Rev.* **45**, 483-501.
4. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
5. **Luftig, R.B., Kilham, S., Hay, A.J., Zweerink, H.J. and Joklik, W.K.,** 1972. An ultrastructure study of virions and cores of reovirus type 3. Virology 48, 170-181.
6. **Maniatis, T., Fritsch, E.F. and Sambrook, J.** 1982.

Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N. Y.

7. **Nakashima, K., Lafiandra, A.J. and Shatkin, A.J.** 1979. Differential dependence of reovirus-associated enzyme activities on genome RNA as determined by psoralen photosensitivity. *J. Biol. Chem.*, **254**, 8007-8014.
8. **Nichols, J.L., Hay, A.J. and Joklik, W.K.** 1972. 5'-terminal nucleotide sequence of reovirus mRNA synthesized *in vitro*. *Nature* (London) New Biol. 235, 105-107.
9. **Poli, G., Stott, J., Liu, Y.S. and Manning, J.S.** 1982. Bluetongue virus: comparative evaluation of enzyme-linked immunosorbent assay, immunodiffusion and serum neutralization for detection of viral antibodies. *J. Clin. Microbiol.*, **15**. 159-162.
10. **Skehel, J.J. and Joklik, W.K.** 1969. Studies on the in vitro transcription of reovirus RNA catalyzed by reovirus cores. Virology 39, 822-831.
11. **Van Dijk, A.A. and Huismans, H.** 1980. The *in vitro* activation and further characterization of the bluetongue virus-associated transcriptase. *Virology*, **104**, 347-356.
12. **Verwoerd, D.W., Els, H.J., De Villiers, E. and Huismans, H.**, 1972. Structure of the bluetongue virus capsid. *J. Virol.*, **10**, 783-794.
13. **Verwoerd, D.W. and Huismans, H.** 1972. Studies on the *in vitro* and *in vivo* transcription on the BTV genome. Onderstepoort *J. Vet. Res.* **39**, 185-192.
14. **Yamakawa, M., Furuichi, Y., Nakashima, K., Lafiandra, A.J. and Shatkin, A.J.** 1981. Excess synthesis of viral mRNA 5'-terminal oligonucleotides by reovirus transcriptase. *J. Biol. Chem.* **256**, 6507-6514.

(Received March 11, 1991)
(Accepted April 4, 1991)