Synthesis of infectious transcripts of blueberry scorch carlavirus in vitro

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Blueberry scorch carlavirus (BBScV) is a filamentous virus with a polyadenylated, positive-sense RNA genome. A near full-length cDNA clone of BBScV was constructed by assembly of clones from a cDNA library. To generate a full-length cDNA clone, the 5' terminus was mutagenized by PCR to introduce nucleotides present in the wild-type virus and not in the near full-length clone, and then fused directly to the T7 bacteriophage RNA polymerase promoter at the 5' terminus. Capped and uncapped BBScV transcripts were synthesized in vitro from the full-length cDNA clone. Capped transcripts were infectious, producing systemic symptoms identical to those caused by the wild-type virus following inoculation onto Chenopodium quinoa leaves. Uncapped transcripts were substantially less infectious than capped transcripts. This represents the first report of infectious transcripts for a member of the carlavirus group.

Blueberry scorch virus (BBScV) is a member of the carlavirus group of positive-sense ssRNA viruses. Partial nucleotide sequences of several carlaviruses have been published (MacKenzie et al., 1989; Foster et al., 1990; Haylor et al., 1990; Memelink et al., 1990; Monis & de Zoeten, 1990; Foster & Mills, 1991, 1992; Levay & Zavriev, 1991; Meehan & Mills, 1991; Henderson et al., 1992); however only the BBScV (Cavileer et al., 1994) and potato virus M (PVM) (Zavriev et al., 1991) genomes have been sequenced in their entirety. The BBScV genome is 8514 nucleotides in length excluding the poly(A) tail at the 3' terminus. The 5' terminus is thought to contain a cap structure by primer extension analysis of viral RNA.

The complete nucleotide sequence of BBScV has revealed that it contains six open reading frames (ORFs), with the coding potential for proteins homologous to the six deduced PVM products (Zavriev et al., 1991; Cavileer et al., 1994). ORF 1 encodes a deduced protein of 223 KDa, containing putative methyl transferase, helicase and polymerase domains typical of those found in proteins involved in RNA replication (Koonin & Dolja, 1993). These proteins in carlaviruses are similar to proteins encoded by the genomes of potexviruses and tymoviruses (Morozov et al., 1990; Rozanov et al., 1992). ORFs 2, 3 and 4 overlap to form the 'triple gene block' encoding proteins with deduced sizes of 25 KDa, 11 KDa and 7 KDa respectively, similar to products encoded by the genomes of potexviruses, hordeiviruses and the furovirus, beet necrotic yellow vein virus (BNYVV), that have been shown to be involved in cell-to-cell movement of the virus (Petty & Jackson, 1990; Beck et al., 1991; Gilmer et al., 1992). ORFs 5 and 6 overlap and encode the coat protein (33 KDa) and a putative cysteine-rich protein (16 KDa) respectively.

The synthesis of infectious transcripts in vitro has been reported for members of a number of virus groups (reviewed by Boyer & Haenni, 1994). In this study we present the construction of the first full-length cDNA clone of a carlavirus genome and the synthesis of biologically active transcripts in vitro.

Complementary DNA clones representing all but the 5'-terminal 26 nucleotides of the BBScV genome (NJ-2 strain) were previously mapped and sequenced (Cavileer et al., 1994). A full-length cDNA clone deficient in the 5' and 3' termini was constructed in pGEM-7Zf(+) (Promega) using the cDNA clones and restriction sites shown in Fig. 1(a). The 3' terminus containing the poly(A) tail was amplified by PCR from total cDNA, synthesized from randomly primed BBScV RNA, using an oligo(dT)n primer. The PCR product containing a poly(A) tail of 50 residues was subcloned into the near full-length cDNA clone.

PCR amplification was used to remove the non-viral bases downstream from the bacteriophage T7 RNA polymerase promoter and to add the 26 5'-terminal viral bases not present in the near full-length clone. The second nucleotide from the 5' terminus was unclear in primer extension analysis; therefore, the primer was synthesized with a mixed site, a T or G, at this position (Fig. 1b). The mutagenized 5' terminus PCR product (approximately 15 kb) was cloned into the TA vector pCRII (Invitrogen). Full-length cDNA clones were subsequently assembled at the EcoRI site of pBluescript.
were generated by the addition of the cap analogue, and incubated at 37°C for 2h according to the manufacturer's protocol (Promega). Capped transcripts with a T or G at position 2 from the 5’ terminus were not infectious in any of the three experiments. The presence of additional ribonucleotides at the 3’ terminus is downstream from the bac 5’ terminus are shown boxed. (c) Orientation of the full-length cDNA clone and restriction sites used in the pBluescript II KS(-) vector.

Fig. 1. Construction of a full-length cDNA clone of BBScV. (a) cDNA clones and the respective restriction sites used to construct a near full-length cDNA clone. (b) 5’-Terminal sequence of the viral RNA and the oligonucleotidic primer used to mutagenize the 5’ terminus of the near full-length cDNA clone. The T7 bacteriophage RNA polymerase promoter and the mixed site, a G or T, at the second nucleotide from the 5’ terminus is shown boxed. (c) Orientation of the full-length cDNA clone and restriction sites used in the pBluescript II KS(-) vector.

II KS(-) vector (Stratagene) (Fig. 1c). The bacteriophage T7 RNA polymerase fused to the 5’ terminus of the BBScV positive strand is downstream from the bacteriophage T3 RNA polymerase promoter in this plasmid. Full-length cDNA clones are linearized at the NolI restriction site located in the multiple cloning site of the vector 50 residues downstream from the end of the poly(A) tail. An additional bacteriophage T7 RNA polymerase promoter present in this vector does not appear to lead to instability. The cDNA clones containing a T or G at position 2 from the 5’ terminus were designated pBS.T4 or pBS.G17 respectively.

For infectivity studies, transcripts were synthesized from the full-length cDNA clones following linearization with NotI. Typically, 2 µg of linearized plasmid DNA was used in a 50 µl reaction with T7 RNA polymerase and incubated at 37°C for 2h according to the manufacturer’s protocol (Promega). Capped transcripts were generated by the addition of the cap analogue, m7G(5')ppp(5')G (0·5 mm) (New England Biolabs), to the reaction. After 15 min at 37°C, the rGTP concentration in these reactions was increased to 0·6 mm from the initial concentration of 25 nm. Capped and uncapped transcripts derived from pBS.T4 and pBS.G17, and RNA purified from BBScV virions were analysed by gel electrophoresis. As shown in Fig. 2, transcripts synthesized in vitro were comparable in size to RNA extracted from virions.

Transcription reactions (50 µl) or BBScV (20 µg virions) were diluted with 150 µl 50 mm-sodium phosphate buffer pH 7·2, 0·1% bentonite, 0·1% celite and mechanically inoculated onto leaves of the systemic host, *Chenopodium quinoa.* There are no local lesion hosts known for BBScV. Transcripts were inoculated at an approximate concentration of 1·0 µg/leaf. Ten to 12 days following inoculation with pBS.T4 transcripts, systemic symptoms identical to those produced by wild-type virus infection were observed. As shown in Table 1, pBS.T4 transcripts were far more infectious when capped, a similar result to those reported for *in vitro* synthesized infectious transcripts of the potexviruses, potato virus X (Hemenway et al., 1990), white clover mosaic virus (WC1MV) (Beck et al., 1990), clover yellow mosaic virus (CYMV) (Holy & AbouHaidar, 1993), papaya mosaic virus (PMV) (Sit & AbouHaidar, 1993) and the furovirus BNYVV (Zeigler-Graf et al., 1988). This result supports the conclusion that native BBScV RNA is capped as predicted from primer extension analysis (Cavileer et al., 1994). Dilutions of the capped pBS.T4 transcripts to approximately 50 ng/leaf were infectious in *C. quinoa.* Transcripts from clone pBS.G17, which have a G rather than a T as the penultimate nucleotide at the 5’ terminus, were not infectious in any of the three experiments.

The BBScV infectious transcripts contain poly(A) tails of 50 A residues. Requirement for the presence and optimal length of the poly(A) tail for infectivity is unknown at this stage. *In vitro* synthesized transcripts of the potexviruses, WCIMV, CYMV and PMV have shown that as the length of the poly(A) tail is decreased, reduction in infectivity is observed (Guilford et al., 1991; Holy & AbouHaidar, 1993; Sit & AbouHaidar, 1993). The presence of additional ribonucleotides at the 3’ terminus of the transcript did not appear to affect infectivity of the BBScV transcripts, a result similar to those reported for other poly(A)-containing virus genomes (Zeigler-Graf et al., 1988; Eggen et al., 1989; Beck et al., 1990).

Although several studies have been performed on the genome expression of potexviruses, tymoviruses, BNYVV and barley stripe mosaic virus, which share features in common with carlaviruses, few details on carlaviruses genome expression are understood. The ability to synthesize biologically active carlaviruses transcripts *in vitro* is an important tool for the further understanding of the virus's replication process.
in vitro will enable us to examine expression of a carlavirus genome and the roles of virus-encoded proteins in replication and infection.

We gratefully acknowledge the financial support of the USDA (Grant 58-3615-9-066) and the New Jersey Agriculture Experiment Station (Project 11192).

Table 1. Infectivity of BBSv transcripts

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Infected/ inoculated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBS. T4 capped</td>
<td>17/19</td>
</tr>
<tr>
<td>pBS. T4 uncapped</td>
<td>1/19</td>
</tr>
<tr>
<td>pBS. G17 capped</td>
<td>0/19</td>
</tr>
<tr>
<td>pBS. G17 uncapped</td>
<td>0/19</td>
</tr>
<tr>
<td>Virions</td>
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<tr>
<td>Buffer</td>
<td>0/19</td>
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* Data are the total results of three separate experiments.

References


(Received 21 December 1993; Accepted 10 March 1994)