Infectivity of plasmids containing brome mosaic virus cDNA linked to the cauliflower mosaic virus 35S RNA promoter

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Full-length biologically active cDNAs of brome mosaic virus genomic RNAs 1, 2 and 3 were constructed by joining cDNA fragments. The cDNAs were constructed so that, at the 5' ends, unique SnaBI sites were present at the site of initiation of transcription. The cDNAs were inserted between a modified cauliflower mosaic virus (CaMV) 35S RNA promoter and terminator regions derived from CaMV DNA, and cloned into pUC18. When a mixture of the plasmid DNAs was inoculated onto Chenopodium hybridum leaves, local lesions appeared 5 to 6 days later. However, no symptoms appeared in similarly inoculated barley plants. Plasmid cDNAs with extra sequences at the 5' end were infectious but RNAs transcribed from cDNAs with similar sequences were not.

Introduction

Brome mosaic virus (BMV) has a tripartite genome of positive-sense RNAs, designated RNA 1, RNA 2 and RNA 3 in order of decreasing Mr (Lane, 1981); the nucleotide sequence of each RNA is known (Ahlquist et al., 1981, 1984a). In vitro transcripts synthesized by bacterial RNA polymerase from cDNAs of BMV RNAs linked to the Pr promoter were infectious to barley plants (Ahlquist et al., 1984b). Moreover, it has been reported that infectious RNA can be transcribed by unknown promoters from cDNA copies of viral RNA inserted into bacterial plasmids (Taniguchi et al., 1978; Racaniello & Baltimore, 1981; Cress et al., 1983; van Emmelo et al., 1987). We have constructed plasmids containing BMV cDNA linked to the cauliflower mosaic virus (CaMV) 35S RNA and T7 promoters and tested their infectivity. In this paper, we compare the infectivities of cDNA plasmids and in vitro transcripts of cDNA linked to the T7 promoter, and discuss the significance of such a cDNA plasmid system in virus research.

Methods

cDNA construction of genomic RNAs. BMV (ATCC66) RNA was extracted from the purified virus as described by Okuno & Furusawa (1979). The full-length cDNAs of BMV RNAs 1, 2 and 3 were constructed from cDNA fragments corresponding to each BMV RNA as shown in Fig. 1. Plasmids pBl#4021, pBl#4161 and pBl#6161 were obtained from Dr. M. Horikoshi and both pBl#5161 and pBl#5161 were synthesized by the method of Gubler & Hoffman (1983) using synthetic oligonucleotide primers. Almost all of the central part of each RNA sequence was represented in pBl#4021 and pBl#5161 for RNA 1, pBl#4161 and pBl#6161 for RNA 2 and pBl#875 for RNA 3. To obtain cDNA clones containing a T7 polymerase sites were annealed and the resulting DNA fragment, which contained the T7 promoter sequence and an XmaI recognition site, was inserted into...
the HindIII and XbaI sites of pUC18, creating pUCT7. pUCT7 was cut with NsiI, made blunt-ended with T4 DNA polymerase and cut with EcoRI. The resulting plasmid was ligated with the SnaBl/EcoRI fragment of the cDNA insert from pBB1, 2 or 3, creating pBTF1, 2 and 3, respectively (Fig. 2).

Construction of pCap35. The SalI and PstI sites of pUC9 were eliminated by HindII and PstI cleavage, treatment with T4 DNA polymerase and religation. The HindIII (nucleotide 7013 on the sequence map)--BglII (nt 7670) fragment of pCaMV10 (Howarth et al., 1981), containing the CaMV 35S promoter and terminator regions, was ligated into the SnaBl/BamHI-cut modified pUC9 vector to create pC2. The plasmid pC2 was digested with HphI and made blunt-ended with T4 DNA polymerase. After additional digestion with EcoRI and HindIII, the 425 bp and 249 bp fragments were recovered from an agarose gel and ligated into HindII/EcoRI-cut and SnaBl/HindIII-cut pUC19 to create pC2P and pC2T, respectively. The EcoRI (nt 5647 to nt 6106) (Howarth et al., 1981) fragment was cloned into EcoRI-cut pUC19, creating pCaEc4. The HindIII (nt 8249) (Howarth et al., 1981)–PstI fragment of pCaEc4, containing a 0.2K fragment of CaMV DNA and the polylinker sequence between the PstI and KpnI sites of pUC19, were cloned into the PstI/HindIII site of pC2P, creating pC33. The EcoRI–KpnI fragment of pC33, containing the promoter region and the polylinker sequence, was ligated with the large fragment of EcoRI/KpnI-cut pC2T, containing the termination region, creating pCAM35. The HindIII/EcoRI-cut fragment of pCAM35 was sub-cloned into the HindIII/EcoRI site of pUC19, creating pCAM35EP.

To introduce a SalI site at the site of initiation of transcription of the 35S RNA promoter, a 25 base synthetic oligonucleotide 5′-pd(GTAAAGGCTTCTCATAATGGAATGAA)-3′ (GTAGGCGGCTTCTCATAATGGAATGAA), complementary to the promoter sequence with two mismatched bases and generating a SalI site, was annealed to ssDNA generated from pCAM35EP. dsDNA was synthesized using the Klenow fragment of Escherichia coli DNA polymerase I. After digestion with PvuII, the DNA fragment was denatured by heating and separated by urea-PAGE (Maniatis et al., 1982). ssDNA (458 nt) containing the modified sequence was recovered and second-strand DNA was synthesized by DNA polymerase using an M13 reverse sequence primer (Takara Shuzo). A 431 bp fragment of the EcoRI-cut DNA was cloned into the EcoRI/SmaI site of pUC18, creating pCap35.

Construction of pBICBR1, 2 and 3 and pUCBR1, 2 and 3. The small fragment of EcoRI/HindIII-cut pBI101 (Clontech Laboratories) was replaced with the small fragment of EcoRI/HindIII-cut pCAM35, creating pBIC35. This plasmid was cut with EcoRI, made blunt-ended with T4 DNA polymerase and then ligated with SalI linkers. The plasmids were cut with SalI and self-ligated, thus removing the promoter region. The resultant plasmid, pBICT(-E), was cut with SmaI and then ligated with EcoRI linkers 5′ pd(GGAATTCC), creating pBICTE. pCap35 was cut with EcoRI, blunt-ended with T4 DNA polymerase, ligated with SalI linkers and then cut with SalI and BamHI, the resulting small fragment was inserted into SalI/BamHI-cut pBICTE, creating pBICPC35. This plasmid has SalI, BamHI, EcoRI and KpnI sites between the modified 35S promoter and terminator regions. Each cDNA fragment of SnaBl/EcoRI-cut pBB1, pBB2 and pBB3 was inserted into SalI/EcoRI-cut pBICP35, creating pBICBR1, 2 and 3, respectively (Fig. 2). The small fragment of pBICP35, cut with SalI, blunt-ended with T4 DNA polymerase and then cut with HindIII,
Table 1. Infectivity of plasmid DNA

<table>
<thead>
<tr>
<th>Expt</th>
<th>Inoculum</th>
<th>Concentration (µg/µl)</th>
<th>Infectivity*</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>pUCBR1+2+3</td>
<td>3</td>
<td>1268</td>
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<tr>
<td></td>
<td></td>
<td>0.06</td>
<td>633</td>
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<td>0.12</td>
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<td></td>
<td></td>
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<td>6</td>
</tr>
<tr>
<td>2</td>
<td>BMV RNA</td>
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<td>4003</td>
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<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>836</td>
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<td></td>
<td></td>
<td>0.002</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>pUCBR2+3 plus pUCBRIR1</td>
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<td>785</td>
</tr>
<tr>
<td></td>
<td>pUCBR1+3 plus pUCBRIR2</td>
<td>1</td>
<td>897</td>
</tr>
<tr>
<td></td>
<td>pUCBR1+2 plus pUCBRIR3</td>
<td>1</td>
<td>902</td>
</tr>
<tr>
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<td>149</td>
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<tr>
<td></td>
<td>pUCBR1+2+3</td>
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<td>1631</td>
</tr>
<tr>
<td></td>
<td>Transcript of pBTCRI + pBTC2+3</td>
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<td>50</td>
</tr>
<tr>
<td></td>
<td>Transcript of pBTC1+2+3</td>
<td>0.01</td>
<td>16875</td>
</tr>
</tbody>
</table>

* Total number of lesions in 15 (expt. 1 and 3) and six (expt. 2) half leaves of C. hybridum.

Infectivity. Transcript RNA synthesized from EcoRI-linearized pBTC1 and pBTC2 by T7 RNA polymerase in the presence of 5'-GpppG was inoculated onto the first leaves of barley (Hordeum vulgare L. cv. Goshikokon) or Carborundum-dusted leaves of Chenopodium hybridum. Local lesions were counted 8 days post-inoculation. Plasmid DNA of pBTC1 and pBTC2 was extracted from E. coli, mixed and inoculated onto C. hybridum at a final concentration of 1 µg/µl. Necrotic lesions formed on the inoculated leaves 6 days later. To prepare large amounts of plasmid DNA from E. coli the 35S promoter and terminator regions containing BMV cDNA were subcloned in pUC18 to create pUCBR1, 2 and 3. These plasmids were infectious to C. hybridum. The infectivity of such plasmids disappeared after treatment with DNase but not RNase. This confirmed that the infectivity was due to the plasmid DNA. No systemic symptoms appeared in 20 barley plants inoculated with plasmids at a concentration of 1 µg/µl. Neither pBB1, 2 and 3 nor pBTC1, 2 and 3 were infectious to either plant species.
Properties of pUCBR1, 2 and 3

A mixture of pUCBR1, 2 and 3 had about 1% of the infectivity of BMV RNA (Table 1). Although viral RNA having a non-viral sequence of more than 5 bases at the 5’ end was not infectious (Janda et al., 1987) or poorly infectious (Table 1), plasmids which had an extra 12 bases (CCTACGGGGATC) at the 5’ ends of the cDNA inserts were infectious and induced local lesion formation in C. hybridum leaves. In contrast, mixtures of pUCBR1 with pUCBR2+3, pUCBR1R2 with pUCBR1+3 and pUCBR1R3 with pUCBR1+2 had specific infectivities of about 50% that of the homologous mixtures of pUCBR1, 2 and 3 (Table 1). Local lesions induced by plasmid inocula were indistinguishable from those induced by BMV in appearance and in their virus content. However, lesions induced by DNA plasmid inocula became apparent about 24 h later than those induced by virus RNA. The delay is possibly explained by the time required for plasmid DNAs to be transferred to cell nuclei and for host RNA polymerase to synthesize transcripts.

Discussion

It is known that cDNAs of the genomic RNA of poliovirus (Racaniello & Baltimore, 1981), satellite tobacco necrosis virus (van Emmelo et al., 1987), Qβ phage (Taniguchi et al., 1978) and potato spindle tuber viroid (Cress et al., 1983) inserted into bacterial plasmids are infectious, although the infectious RNAs seem to be transcribed from the cDNAs by an unknown promoter. In our experiments, bacterial plasmids containing the cDNAs of BMV RNAs (pUCBR1, 2 and 3) were infectious to C. hybridum when the cDNA was joined upstream to the promoter sequence of CaMV 3S RNA. This indicates that the mechanically inoculated plasmid DNA was introduced into C. hybridum cells and that BMV RNAs were produced by transcription of the cDNAs from the promoter in the cells; no virus was detected after inoculation with pBTF1, 2 and 3, which contained the bacteriophage T7 promoter instead of the CaMV 3S promoter. Nuclease sensitivity tests showed that the infectivity was not due to viral RNA contaminating the pUCBR1, 2 and 3 DNAs. It is not surprising that a DNA copy of an RNA virus genome inserted into a plasmid with a functional promoter for eukaryotic cells can initiate the infection process. However, the virus sense RNA strands would probably have extra non-viral sequences at their 3’ ends and, in the case of pUCBR1R1, 2 and 3, also at the 5’ end. Precisely how these molecules replicate is not clear; the extra sequence might be randomly cleaved off by nuclease or not removed at all. In the latter case, replication might occur by the recognition of an internal initiation site by the viral replicase. In contrast, transcripts with more than five bases at the 5’ end were not infectious (Janda et al., 1987). Although we have not yet characterized the primary transcripts, the first transcripts with extra sequences at either or both ends would be quickly altered so that the terminal sequences were like those of wild-type BMV, because the infectivity of an extract of a local lesion induced by plasmid DNA was almost the same as that of an extract of a lesion induced by BMV RNA.

The plasmid DNA system used in these experiments has several advantages over an RNA system using transcripts as inoculum. These are (i) transcripts are continuously provided from cDNA in the nucleus of the inoculated cells, (ii) the DNA system does not require that the 5’ and 3’ ends of the inserted cDNA sequence be as precise as when synthesizing in vitro transcripts and (iii) with plasmid DNA there is no requirement for the use of cap structures.

References

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