VIROLOGY 214, 100–109 (1995)

Deletion Analysis of the Promoter for the Cucumber Necrosis Virus 0.9-kb Subgenomic RNA

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3; and Agriculture and Agri-Food Canada, Pacific Agriculture Research Centre, Vancouver, British Columbia V6T 1X2, Canada

Received August 14, 1995; accepted September 25, 1995

Sequences comprising the core promoter for the cucumber necrosis virus (CNV) 0.9-kb subgenomic RNA have been determined using deletion analysis and site-directed mutagenesis. The deletion studies indicate that the promoter lies within a region located 20 nucleotides upstream and 6 nucleotides downstream and including the subgenomic start site. Sequences further upstream or downstream of the core promoter do not appear to strongly affect promoter activity or viral RNA accumulation. Results of site-directed mutagenesis studies indicate that nucleotides immediately surrounding the subgenomic start site regulate promoter activity. Comparison of sequences within the CNV promoter region with the corresponding region of other tombusviruses shows that the tombusvirus promoter shares a region of near complete identity in 14 of the 26 core promoter nucleotides. Little similarity exists between the CNV 0.9-kb subgenomic RNA promoter and the region surrounding the transcription initiation site for the CNV 2.1-kb subgenomic RNA. Likewise, limited similarity occurs with the 5′ region of CNV genomic RNA. Sequences similar to the ICR2-like motifs found in the promoters of several alphavirus-like (supergroup III) plant and animal viruses are not apparent. This study represents the first analysis of a subgenomic promoter from a member of supergroup II of positive-strand RNA viruses.

INTRODUCTION

The production of subgenomic RNAs is one strategy by which internally located open reading frames (ORFs) of multicistronic eukaryotic RNA viruses may be expressed and regulated during replication. Two mechanisms for the synthesis of subgenomic RNAs have been proposed: the first, discontinuous leader RNA-primed transcription, is thought to occur during the production of coronavirus subgenomic RNAs (Spaan *et al.,* 1983; Lai *et al.,* 1984; Lai, 1990) and the second, internal initiation of transcription on minus (−) strand template RNA has been shown to occur *in vitro* for the plant viruses brome mosaic virus (BMV; Miller *et al.,* 1985) and alfalfa mosaic virus (AlMV; van der Kuyl *et al.,* 1990) and *in vivo* for turnip yellow mosaic virus (Gargouri *et al.,* 1989). This latter mechanism first requires transcription of a genomic-length (−) strand template from (+) sense genomic RNA. The viral RNA-dependent RNA polymerase, along with any associated viral or host factors is believed to then bind to internally located promoter regions on the (−) strand template to produce one or more subgenomic RNAs. The subgenomic RNAs are 3′ coterminal with genomic RNA but now contain otherwise internally located ORFs at their 5′ termini.

The promoter regions on the (−) strand template responsible for directing the synthesis of subgenomic RNAs have been well studied in several members of supergroup III (the alphavirus-like supergroup — see Koonin and Dolja, 1993) of the positive-strand RNA viruses, most notably Sindbis virus (Levis *et al.,* 1990; Raju and Huang, 1991; Hertz and Huang, 1992) and the plant brome mosaic and related tricornaviruses (Marsh *et al.,* 1988; French and Ahlquist, 1988; Allison *et al.,* 1989; van der Kuyl *et al.,* 1990; Pacha and Ahlquist, 1992; Boccard and Baulcombe, 1993; Smirnyagina *et al.,* 1994)). The subgenomic promoters of these viruses do not share extensive nucleotide sequence similarity but do contain similar sequence motifs which include the subgenomic promoter elements, suggesting possible parallels in subgenomic RNA transcription among members of the group (Marsh *et al.,* 1988; French and Ahlquist, 1988; Smirnyagina *et al.,* 1994). In contrast to those members of the alphavirus-like supergroup, subgenomic promoter regions of applicable members of supergroup II (the flavivirus-like supergroup — see Koonin and Dolja, 1993), which includes the animal flavi- and pestiviruses, as well as the plant barley yellow dwarf luteovirus and the diantho-, necro-, carmo-, and tombusvirus groups, have not been characterized.

Cucumber necrosis virus (CNV), a member of the tombusvirus group, is a small spherical virus containing one molecule of a 4.7-kb single-stranded (+) sense RNA genome. The CNV genome is multicistronic containing ORFs for at least five and possibly six proteins (see Fig. 1A) (Rochon and Tremaine, 1989; Boyko and Karasev, 1992). Infection by CNV genomic RNA results in the synthesis of two 3′ coterminal subgenomic RNAs of 2.1 and 0.9 kb (Johnston and Rochon, 1990; Rochon and Johnston,

[1] To whom correspondence and reprint requests should be addressed. Fax: 604-666-4994. Email: rochon@pargva.agr.ca.

0042-6822/95 $12.00

1991); a possible third subgenomic RNA of ca. 0.35 kb is currently under investigation (D. M. Rochon, unpublished data). The CNV 2.1-kb subgenomic RNA directs the synthesis of p41 which is the viral coat protein and the 0.9-kb subgenomic RNA directs the synthesis of both p20 (the absence of which leads to the rapid *de novo* generation of defective interfering RNAs; Rochon, 1991) and p21 (which may function in cell-to-cell movement; J. C. Johnston and D. M. Rochon, unpublished observations) from nested ORFs (Johnston and Rochon, 1990). In addition to the production of subgenomic RNAs, CNV utilizes at least two other strategies for the expression of internally located ORFs; these include readthrough translation of an amber codon for expression of p92, the putative RNA-dependent RNA polymerase (Rochon and Tremaine, 1989), and leaky ribosomal scanning for production of p20 from the 0.9-kb subgenomic RNA (J. C. Johnston and D. M. Rochon, manuscript submitted for publication). In this paper, we have used deletion analysis to delineate the 5′ and 3′ borders of the core promoter for the CNV 0.9-kb subgenomic RNA. In addition, site-directed mutagenesis was used to investigate the importance of nucleotides immediately flanking the 0.9-kb subgenomic RNA initiation site.

MATERIALS AND METHODS

Plasmid construction

All plasmids were originally constructed from pK2/M5 (Rochon and Johnston, 1991), a full-length CNV cDNA clone in Bluescribe (Stratagene) phagemid, using standard recombinant DNA techniques (Sambrook *et al.,* 1989). The construction of XpK2/M5 which contains two introduced *Xho*I restriction enzyme sites at CNV nucleotides 3417 and 3733 in the coat protein protruding domain coding sequence was previously described (McLean *et al.,* 1993). The generation of PD(−) containing a deletion of the region between the *Xho*I sites has also been described as has the construct CP(−) corresponding to an *in planta* derived deletion mutant of PD(−) which lacks almost the entire ca. 1-kb coat protein coding region (McLean *et al.,* 1993).

The plasmid pK2/M5BamHI was generated by oligonucleotide-directed *in vitro* mutagenesis which was carried out essentially as described by Kunkel *et al.* (1987). pSCHinc1.55, a subclone containing a region corresponding to CNV nucleotides 2566 to 4116 (Johnston and Rochon, 1990), was used to produce a single-stranded DNA template for *in vitro* mutagenesis. The phosphorylated mutagenic oligonucleotide, 5′ATTAGGGGCTTCTGGA*TCC*TAACCAATTCATGGATACTGAATACGAAC3′ (corresponding to CNV nucleotides 3771 to 3818; the introduced *Bam*HI site is underlined and the modified nucleotides are italicized), was then used to introduce a *Bam*HI restriction enzyme recognition site at nucleotide position 3784 which was confirmed by restriction enzyme digestion. A 447 nucleotide *Bgl*II–*Nco*I fragment containing this site was subcloned into similarly digested pK2/M5 and the entire subcloned region was verified by DNA sequencing.

Two deletion construct series were generated: the pK2/M5 X series was constructed from pK2/M5XhoI which contains a single introduced *Xho*I restriction enzyme site at CNV nucleotide position 3733. pK2/M5XhoI was generated from p13XpK2/M5 (which contains a *Xho*I site at position 3733 in pK2/M5; McLean *et al.,* 1993) by restriction enzyme digestion with *Bgl*II and *Nco*I to yield a 447-nucleotide fragment. This fragment was purified following agarose gel electrophoresis using the Qiaex gel extraction kit (Qiagen) and ligated into similarly digested pK2/M5. To generate a series of deletions, pK2/M5XhoI was linearized with *Xho*I and then treated with 0.05 U Bal 31 exonuclease (BRL) per microgram of DNA at 25° which resulted in the removal of ca. 50 bp per termini in 10 min. During the 30-min reaction time, aliquots of the reaction were stopped in 50 m*M* EDTA and the separate Bal 31-treated samples were phenol/chloroform extracted, ethanol precipitated, resuspended, and treated with *Asu*II (CNV nucleotide 4331) to yield fragments of between ca. 450 and 600 nucleotides. The samples were then gel-purified using Qiaex matrix and ligated into *Xho*I linearized pK2/M5XhoI which had been treated with mung bean nuclease, digested with *Asu*II, and dephosphorylated with calf intestinal phosphatase followed by gel purification. Ligation reactions were transformed into *Escherichia coli* DH5α cells, the resulting colonies grown in LB medium and the DNA extracted and screened by restriction enzyme digestion. Treatment with *Nde*I and *Kpn*I resulted in the generation of fragments of interest of between ca. 210 and 260 nucleotides which were separated on a 4% GTG Agarose (NuSieve) gel allowing the selection of appropriate plasmids for further screening by DNA sequencing. A series of 15 plasmids carrying deletions of between 4 and 74 nucleotides (designated pK2/M5XΔ4 through $-$XΔ74) were finally chosen for further analysis. The pK2/M5 N series was generated by digestion of pK2/M5 with *Nco*I followed by treatment with Bal 31 as described above. Further digestion with *Bgl*II yielded fragments of between ca. 300 and 450 nucleotides which were purified as above and ligated into *Nco*I, mung bean nuclease, and *Bgl*II, CIP-treated, and gel-purified pK2/M5 vector DNA. Ligation, DNA extraction, and screening were also carried out as above and a series of 9 plasmids designated pK2/M5NΔ10 through -NΔ55, carrying deletions of between 10 and 55 nucleotides, were selected.

Plasmid pK2/M5NcoI-HpaI was generated by digestion of pK2/M5 with *Nco*I followed by mung bean nuclease treatment, digestion with *Hpa*I, and religation to yield a mutant lacking a 286-nucleotide region encompassing CNV nucleotides 3830 to 4116. pK2/M5NcoI-AsuII was similarly constructed by digestion of pK2/M5

with *Nco*I and *Asu*II followed by mung bean nuclease treatment and religation to produce a mutant lacking a 504-nucleotide region corresponding to CNV nucleotides 3830 to 4334.

In vitro transcription and transcript inoculation

Full-length transcripts from pK2/M5, pK2/M5XhoI, pK2/M5BamHI, and the pK2/M5 X series and N series deletion constructs were synthesized using *Sma*I-linearized templates and the bacteriophage T7 RNA polymerase (BRL) as described by Rochon and Johnston (1991). Approximately 5 μg of each uncapped transcript was used to inoculate either cucumber cotyledon protoplasts or *Nicotiana clevelandii* leaves. For time course studies using different transcripts, the amount of each transcript was estimated by agarose gel electrophoresis and ethidium bromide staining of a dilution series of transcript RNA. For protoplast inoculation, *in vitro* transcribed RNA was resuspended in sterile H_2O and used to polyethylene glycol-transfect 1×10^6 protoplasts which were prepared by the method of Wieczorek and Sanfacon (1995). Transfected protoplasts were incubated under alternating 12-hr light and dark conditions (at 25 and 20°, respectively) for the time periods indicated. For plant inoculation, sodium phosphate buffer (pH 7.0) was added to RNA still contained in the transcription reaction and this was used to rub-inoculate three *N. clevelandii* leaves as described previously (Rochon and Johnston, 1991). Total RNA was then extracted from systemically infected leaves at 6 to 18 days postinoculation as indicated.

RNA extraction and Northern blot analysis

RNA was purified from infected protoplasts by first collecting the protoplasts by centrifugation for 5 min at 225 *g* and extracting the RNA with phenol/chloroform and TNE buffer (100 m*M* Tris–HCl, pH 7.5, 100 m*M* NaCl, 10 m*M* EDTA, 5 m*M* β-mercaptoethanol, and 0.1% SDS) as described by Rochon and Johnston (1991). Total nucleic acid was precipitated with ethanol, resuspended in sterile H_2O, and then one-tenth of the sample was used for Northern blot analysis. The RNA was then denatured in methyl mercuric hydroxide-containing sample mix and separated by electrophoresis through a denaturing 1% agarose gel (unless percentage indicated as otherwise) as previously described (Rochon and Johnston, 1991). ^{32}P-labeled DNA probes containing sequences corresponding to the 3′ terminal 370 nucleotides of the CNV genome were generated by nick-translation (Sambrook *et al.,* 1989). Radiolabeled RNA probes for the detection of virion sense RNA were prepared by *in vitro* transcription of *Eco*RI-linearized pK2/M5RI-6 (a plasmid which contains sequences corresponding to the entire CNV genome with the exception of the second nucleotide; Rochon and Johnston, 1991) using the bacteriophage T3 promoter (Sambrook *et al.,* 1989).

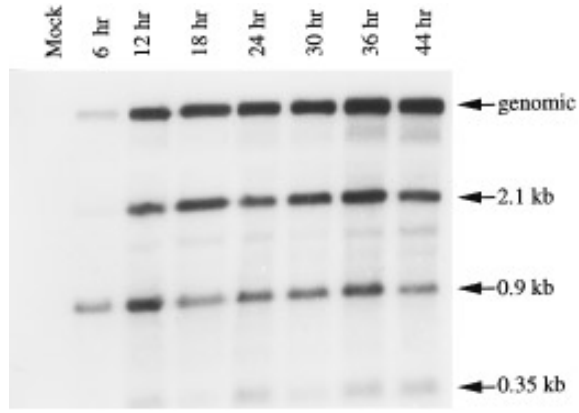

FIG. 1. Time course of the accumulation of CNV subgenomic RNAs in protoplasts. Cucumber protoplasts were inoculated with equal amounts of WT CNV transcripts for the indicated times and one-tenth of each sample was analyzed by Northern blotting using a ^{32}P-labeled RNA probe complementary to the entire CNV genome. Bands corresponding to CNV genomic RNA and the 2.1-, 0.9-, and 0.35-kb subgenomic RNAs are indicated.

RESULTS

Time course of CNV subgenomic RNA production in protoplasts

The relative abundance of genomic and subgenomic-length RNA species in cucumber protoplasts inoculated with wild-type (WT) transcripts of a full-length CNV cDNA clone (pK2/M5; Rochon and Johnston, 1991) was examined at 6 to 44 hr postinoculation by Northern blot analysis using a CNV-specific probe (Fig. 1). Although both the 2.1- and 0.9-kb subgenomic RNAs were observed at all time points tested, the 0.9-kb subgenomic RNA was most abundant (relative to genomic RNA and 2.1-kb subgenomic RNA) at earlier time points (i.e., 6 and 12 hr postinoculation), whereas the 2.1-kb subgenomic RNA has a higher relative abundance at later time points (i.e., 18 to 44 hr postinoculation). The early accumulation of the 0.9-kb subgenomic RNA and the later accumulation of the 2.1-kb subgenomic RNAs are consistent with the postulated and known roles of their translation products in cell-to-cell movement and virus assembly/long distance movement, respectively.

Deletions far upstream of the CNV 0.9-kb subgenomic RNA do not affect genomic or 0.9-kb subgenomic RNA accumulation

In a previous paper, we noted that a deletion in the coding region of the CNV coat protein protruding domain [mutant PD(−)], which lies upstream of the CNV 0.9-kb subgenomic RNA start site (see Fig. 2A), does not noticeably affect 0.9-kb subgenomic RNA production (Sit *et al.,* 1995). This deletion is 316 nucleotides and ends at an introduced *Xho*I site 51 nucleotides upstream from the 0.9-kb subgenomic RNA transcription initiation site. This

observation subsequently suggested to us that it should be possible to delineate the 5′ border of 0.9-kb subgenomic RNA promoter by making progressively longer deletions toward the subgenomic RNA start site from a similarly positioned *Xho*I site in CNV transcripts. Before initiating these studies, we wanted to confirm that the deletion in PD(−) indeed does not affect 0.9-kb promoter function in cucumber protoplasts over several time points. Cucumber protoplasts were inoculated with WT CNV and PD(−) transcripts and the levels of 0.9-kb subgenomic RNA (relative to genomic RNA) were analyzed by Northern blotting at 12, 24, and 40 hr postinoculation. Fig. 2B shows that the levels of 0.9-kb subgenomic RNA in PD(−) infected protoplasts are similar to those in WT CNV-infected protoplasts at each time point analyzed. The 40-hr sample of PD(−) is faint in this experiment due to a problem during loading of the sample. In other experiments the level of the viral RNA species and 0.9-kb subgenomic RNA was similar to the 40-hr WT level. These studies therefore indicate that the 0.9-kb subgenomic RNA promoter in PD(−) is not appreciably affected by the large upstream deletion.

The above studies were also conducted using CP(−) transcripts. CP(−) is a CNV mutant which lacks nearly the entire coat protein coding region of CNV. It was derived *de novo* from PD(−) during infection in whole plants. The location of the deletions in CP(−) are shown in Fig. 2A. It can be seen that the 3′ border of the deletion is the same as that of PD(−) but that the 5′ border is far upstream near the 5′ terminus of the coat protein gene. In addition, a small internal portion of the coat protein coding region is retained in CP(−). It can be seen in Fig. 2B that the relative amount of 0.9-kb subgenomic RNA in CP(−)-infected cucumber protoplasts is not substantially affected compared to that observed in WT CNV-infected protoplasts. In addition, the overall levels of CP(−) viral RNA appear to be higher (possibly due to increased replication and/or lack of encapsidation). These results suggest that the 0.9-kb subgenomic RNA core promoter begins no farther than 51 nucleotides upstream from the subgenomic RNA start site and further suggest that strong auxiliary promoter elements do not lie within the deleted portions of CP(−) and PD(−). In addition, these studies show that mutations which affect coat protein synthesis (and thus viral RNA encapsidation) do not appear to inhibit the ability of genomic RNA to be stably replicated.

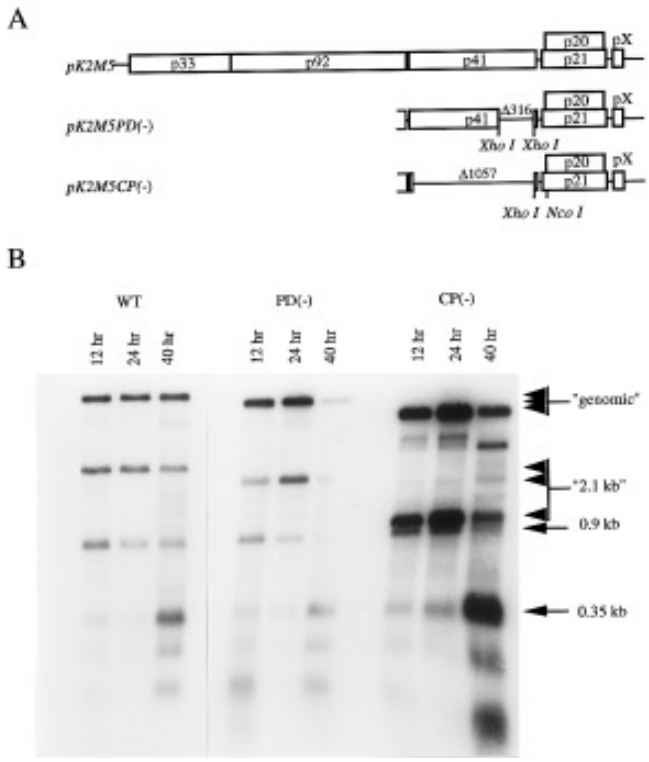

FIG. 2. Characterization of large-scale deletion mutants used to analyze the 5′ border of the CNV 0.9-kb subgenomic RNA promoter. (A) Diagrammatic representation of pK2/M5PD(−) and pK2/M5CP(−) deletion constructs used to generate transcripts to delineate the 5′ border. The structure of the WT CNV genome is shown in the top portion of the diagram and relevant portions of the two deletion mutants are shown below. Restriction enzyme cleavage sites used to generate pK2/M5PD(−) are shown along with the sizes of the deletions in nucleotides for both pK2/M5PD(−) and pK2/M5CP(−). (B) Accumulation of PD(−) and CP(−) subgenomic RNAs in cucumber protoplasts. Cucumber protoplasts were inoculated with equal amounts of WT, PD(−), or CP(−) transcripts for the indicated times and one-tenth of each sample was analyzed by Northern blotting using a ^{32}P-labeled RNA probe complementary to the entire CNV genome. Bands corresponding to CNV genomic RNA and the 2.1-, 0.9-, and 0.35-kb subgenomic RNAs are indicated. The multiple arrowheads indicate the different sizes of the "genomic" and "2.1-kb subgenomic" RNAs affected by the 316- and 1057-nucleotide deletions in PD(−) and CP(−), respectively (see A).

Deletion analysis of the 5′ border of the 0.9-kb subgenomic RNA promoter

The above analysis of PD(−) and CP(−) RNA accumulation in protoplasts indicates that the promoter for the 0.9-kb subgenomic RNA lies downstream of the deleted region, the 3′ border of which corresponds to an introduced *Xho*I restriction enzyme recognition site at CNV nucleotide position 3733 (McLean *et al.,* 1993). This *Xho*I site, located 51 nucleotides upstream of the 0.9-kb subgenomic RNA start site (Rochon and Johnston, 1991), was used as a convenient site from which to make further downstream deletions. A schematic representation of the deletion constructs used to map the 5′ border (with respect to virion sense RNA) of the 0.9-kb subgenomic promoter is shown in Fig. 3A.

For initial analyses, transcripts were synthesized from selected mutants (pK2/M5XΔ4, Δ18, Δ22, Δ41, Δ64, and Δ74), transfected into cucumber protoplasts and the resulting levels of subgenomic RNA (relative to genomic RNA) were determined by Northern blot analysis. Figure 3B demonstrates that 0.9-kb subgenomic RNA levels are not substantially affected by deletions of up to 22 nucleo-

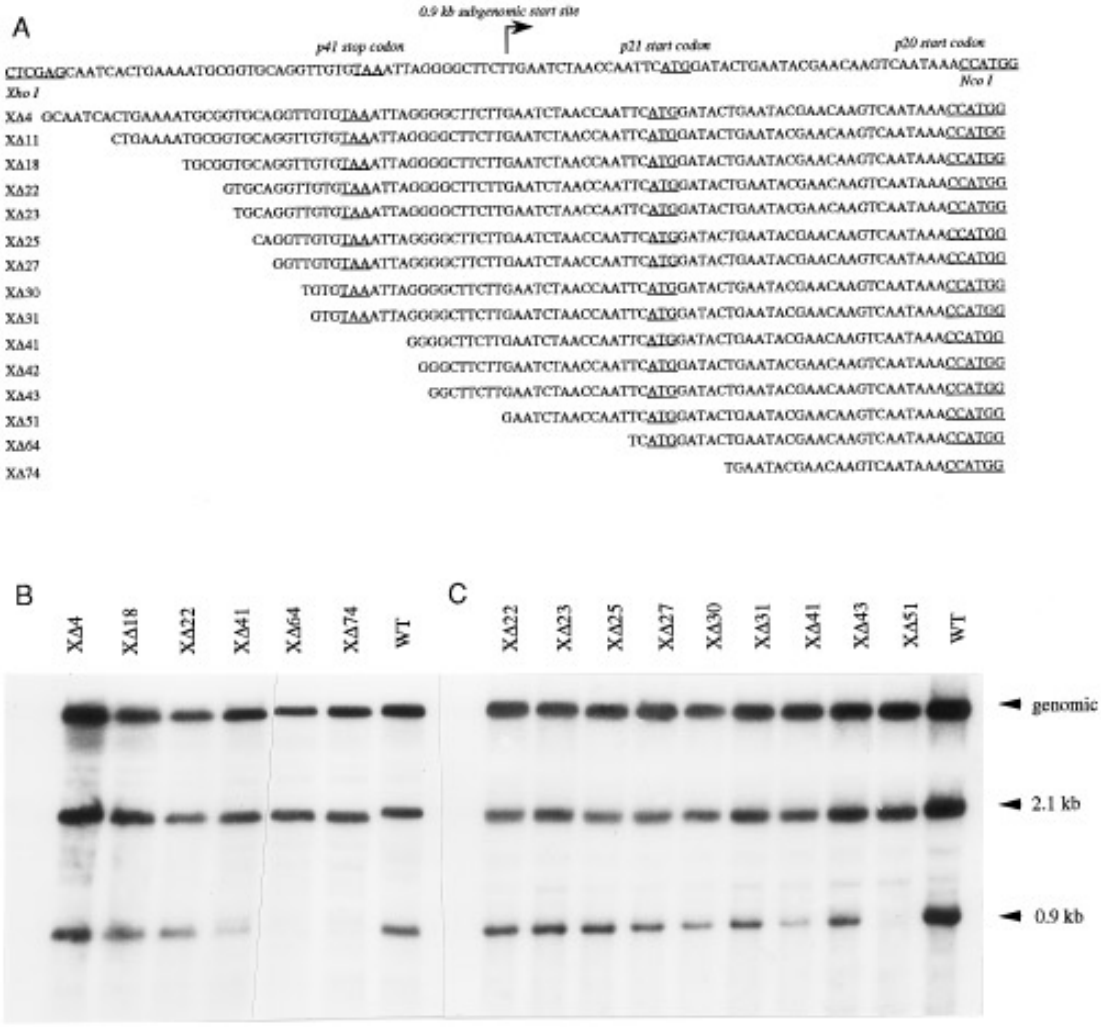

FIG. 3. Characterization of the deletion mutants used to refine the 5′ border of the CNV 0.9-kb subgenomic RNA promoter. (A) CNV sequences remaining in the pK2/M5 X series following digestion of *Xho*I cleaved template with Bal 31 exonuclease are shown. Sequences surrounding the WT CNV 0.9-kb subgenomic RNA are shown in the top line. The 0.9-kb subgenomic start site as well as the location of the p41 (coat protein) stop codon and the p21 and p20 start codons are indicated. (B) Accumulation of pK2/M5 X series 0.9-kb subgenomic RNAs in cucumber protoplasts. Cucumber protoplasts were inoculated with equal amounts of the indicated transcripts and one-tenth of the sample was analyzed by Northern blotting using a ^{32}P-labeled nick-translated DNA probe corresponding to the CNV 3′ terminus. (B and C) Different experiments in which protoplasts were transfected with the indicated deletion mutant for 24 hr. Each of the transcripts including XΔ11 (see A) were also tested at 12 hr postinoculation with similar results (not shown). Bands corresponding to CNV genomic RNA and the 2.1-, 0.9-, and 0.35-kb subgenomic RNAs are indicated.

tides downstream of the *Xho*I site. However, a deletion of 41 nucleotides is associated with decreased levels of 0.9-kb subgenomic RNA and deletions of 64 nucleotides or more appear to abolish 0.9-kb subgenomic RNA production.

For subsequent more refined promoter analyses, transcripts carrying deletions of between 22 and 51 nucleotides downstream of the *Xho*I site were analyzed as above. Figure 3C indicates that deletions of up to 31 nucleotides do not noticeably affect the level of 0.9-kb subgenomic RNA, but as before, a deletion of 41 nucleotides is associated with reduced 0.9-kb subgenomic RNA levels. In addition, a deletion of 51 nucleotides appears to completely inhibit 0.9-kb subgenomic RNA synthesis. The reduced levels of subgenomic RNA associated with XΔ41 suggests that the promoter for 0.9-kb subgenomic RNA lies upstream of the 3′ border of XΔ41. However, the levels of 0.9-kb subgenomic RNA appear to be unaffected in XΔ43 which contains two additional deleted nucleotides compared to XΔ41. To examine this apparent anomaly in more detail, the levels of 0.9-kb subgenomic RNA were analyzed at two different time points (24 and 36 hr postinoculation) following inoculation with transcripts of mutants XΔ41, XΔ42, and XΔ43 (data not shown). The levels of subgenomic RNA were found to be considerably reduced in XΔ41, nearly absent in XΔ42-infected protoplasts, but again detectable in XΔ43. In addition, it was noted that the band corresponding to the 0.9-kb subgenomic appeared to be heterogeneous in size suggesting that transcription initiation may be af-

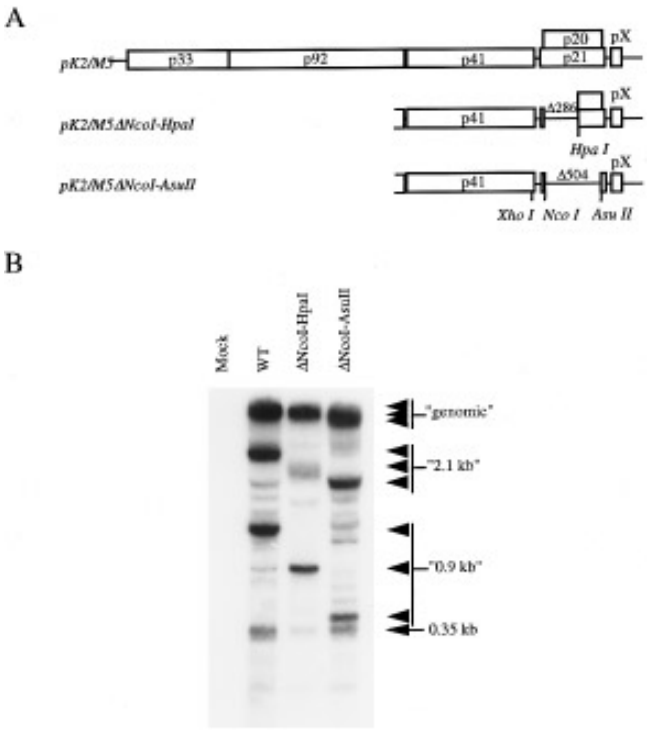

FIG. 4. Characterization of large-scale deletion mutants used to analyze the 3′ border of the CNV 0.9-kb subgenomic RNA promoter. (A) Diagrammatic representation of pK2/M5ΔNcoI-HpaI and pK2/M5ΔNcoI-AsuII constructs used to generate transcripts to delineate the 3′ border. The structure of the CNV genome is shown in the top portion of the diagram and relevant portions of the two deletion mutants are shown below. Restriction enzyme cleavage sites used to generate the two mutants (pK2/M5ΔNcoI-HpaI and pK2/M5ΔNcoI-AsuII) are shown with the number of nucleotides deleted indicated. (B) Accumulation of M5ΔNcoI-HpaI and M5ΔNcoI-AsuII subgenomic RNAs in cucumber protoplasts. Cucumber protoplasts were inoculated with equal amounts of the indicated transcripts and then analyzed 24 hr postinfection by Northern blotting from a 2% agarose gel using a nick-translated cDNA probe corresponding to the 3′ terminus of CNV RNA. Bands corresponding to CNV genomic RNA and the 2.1-, 0.9-, and 0.35-kb subgenomic RNAs are indicated. The multiple arrowheads indicate the different sizes of the "genomic" and "2.1-kb" and "0.9-kb" subgenomic RNAs affected by the 286- and 504-nucleotide deletions in ΔNcoI-HpaI and ΔNcoI-AsuII, respectively (see A).

fected. The possible influence of sequences or structures upstream of the deletion site when placed in conjunction with the 0.9-kb subgenomic promoter region will be discussed further. Taken together, these deletion studies suggest that the 5′ border of the core promoter for the 0.9-kb subgenomic RNA lies between 10 and 20 nucleotides upstream of the start site for transcription.

Deletion analysis of the 3′ border of the 0.9-kb subgenomic RNA promoter

To determine whether large-scale deletions downstream of the 0.9-kb subgenomic RNA start site affect promoter function, transcripts were synthesized from constructs carrying deletions in the p20 and p21 coding regions. ΔNcoI-HpaI and ΔNcoI-AsuII (see Fig. 4A) carry deletions of 286 and 504 nucleotides, respectively, downstream of the *Nco*I site at CNV nucleotide position 3830 (which forms part of the p20 start codon and is located 50 nucleotides downstream of the transcription start site). Figure 4B shows that both ΔNcoI-HpaI- and ΔNcoI-AsuII-infected protoplasts accumulate near WT levels of the deleted forms of the "0.9-kb subgenomic RNA" (i.e., 0.6 and 0.4 kb, respectively). In other experiments (not shown), the deleted forms of the 0.9-kb subgenomic RNA also accumulated to close to WT levels demonstrating that the 3′ border of the 0.9-kb subgenomic RNA core promoter lies within 50 nt downstream of the start site for transcription. In addition, the observation that both of these mutants accumulate to WT levels in protoplasts suggests that RNA accumulation is not drastically affected by the absence of either p21 or p20. Reports by others have similarly indicated the lack of requirement for p21 and p20 in protoplast infections by other tombusviruses (Dalmay *et al.,* 1993; Scholthof *et al.,* 1993).

A schematic diagram of the deletion constructs used to further define the 3′ border of the 0.9-kb subgenomic RNA promoter is shown in Fig. 5A. The *Nco*I site at the 5′ border of the deletion constructs described above was used as the site from which to make further deletions toward the 0.9-kb subgenomic RNA start site located 50 nucleotides upstream. Transcripts carrying deletions of between 10 and 55 nucleotides were used to inoculate cucumber protoplasts and the resulting subgenomic RNA levels were determined by Northern blot analysis. Figure 5B demonstrates that deletions of up to 44 nucleotides do not noticeably affect 0.9-kb subgenomic RNA levels but that a deletion of 55 nucleotides completely inhibits 0.9-kb subgenomic RNA synthesis. These results indicate that the 3′ border of the 0.9-kb subgenomic RNA core promoter extends no further than 6 nucleotides downstream and including the transcription start site.

Mutational analysis of the core promoter for the 0.9-kb subgenomic RNA

To investigate the effect of mutations immediately surrounding the 0.9-kb subgenomic transcription initiation site (see Fig. 5A), a *Bam*HI site was introduced into pK2/M5 resulting in the alteration of nucleotides in the −1, +3, and +4 positions. These changes led to the substitution of a G for a U at position −1 (nucleotide 3784), a U for an A at position +3 (nucleotide 3787), and a C for a U at position +4 (nucleotide 3788) in CNV RNA. Northern blot analysis of cucumber protoplasts transfected with transcripts of this mutant (M5BamHI; see Fig. 6) showed a significantly reduced level of 0.9-kb subgenomic RNA compared to WT levels. This suggests the involvement of any or all of the mutated nucleotides in the regulation of 0.9-kb subgenomic RNA synthesis.

In previous work we noted that mutations in the AUG codons which initiate translation of p21 and p20 resulted

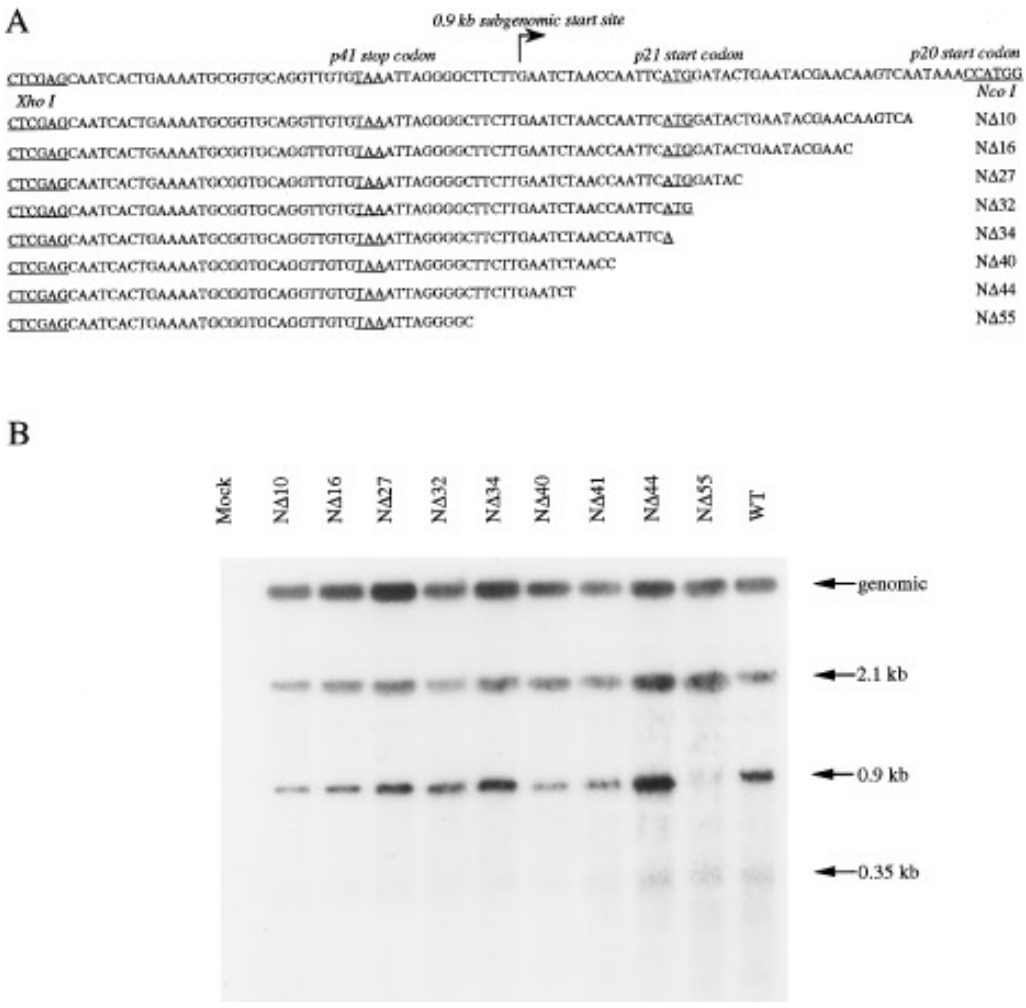

FIG. 5. Characterization of deletion mutants used to refine the 3′ border of the CNV 0.9-kb subgenomic RNA promoter. (A) CNV sequences remaining in the pK2/M5 N series following digestion of *Nco*I cleaved template with Bal 31 exonuclease are shown. Sequences surrounding the WT CNV 0.9-kb subgenomic RNA are shown in the top line. The 0.9-kb subgenomic start site as well as the location of the p41 (coat protein) stop codon and the p21 and p20 start codons are indicated. (B) Accumulation of pK2/M5 N series 0.9-kb subgenomic mRNA in cucumber protoplasts. Cucumber protoplasts were inoculated with the indicated transcripts and then analyzed 24 hr postinfection by Northern blotting using a nick-translated cDNA probe corresponding to the 3′ terminus of CNV RNA. Bands corresponding to CNV genomic RNA and the 2.1-, 0.9-, and 0.35-kb subgenomic RNAs are indicated.

in phenotypic changes upon infection of *N. clevelandii* plants; i.e., the p21 AUG codon mutant did not accumulate to detectable levels and the p20 mutant accumulated to high levels but the symptoms were dramatically attenuated (Rochon and Johnston, 1991). Transcripts of the pK2/M5BamHI mutant were inoculated onto *N. clevelandii* leaves to determine if the lower level of subgenomic RNA synthesis observed in protoplasts would affect the symptoms produced in whole plants. Plants developed symptoms but the symptoms were delayed and considerably attenuated in comparison with WT infected plants (data not shown). In addition, analysis of viral RNA from systemically infected leaves 18 days postinoculation indicated that the 0.9-kb subgenomic RNA accumulates (Fig. 6) but not to the same high levels as seen in WT infections. Thus, the mutations surrounding the subgenomic RNA start site affects subgenomic RNA levels in protoplasts as well as in plants and the lower levels appear to result in reduced synthesis of the corresponding p21 and p20 proteins.

DISCUSSION

Deletion mapping of the promoter region for the CNV 0.9-kb subgenomic RNA has established the location of the core promoter to be within a 26-nucleotide region surrounding the subgenomic RNA initiation site (+1). The 5′ border of the promoter is situated within a short AU-rich region between nucleotides −10 and −20 and the 3′ border extends no further than 6 nucleotides downstream of the transcription start site (see Fig. 7A). This region was determined to be essential for subgenomic RNA production and from examination of coat protein deletion mutants, sequences upstream of this "core" pro-

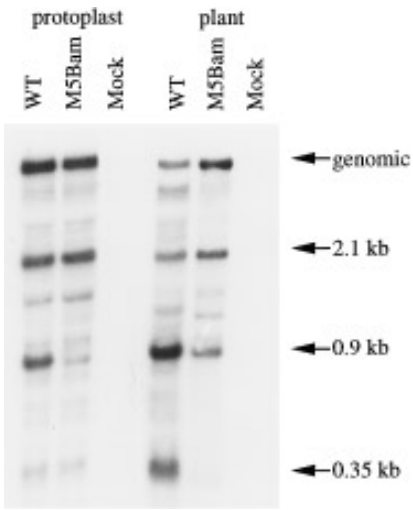

FIG. 6. Effects of mutations surrounding the 0.9-kb subgenomic RNA transcription start site on subgenomic RNA levels in protoplasts and plants. Cucumber protoplasts or plants were inoculated with WT or M5Bam transcripts and then analyzed by Northern blotting using a nick-translated ^{32}P-labeled cDNA probe corresponding to the CNV 3′ terminus. Protoplasts were analyzed 24 hr postinoculation. WT CNV- and M5Bam-infected plants were analyzed 6 or 18 days postinoculation, respectively.

moter region do not appear to dramatically influence the strength of the promoter. For comparison, subgenomic RNA production in the alphavirus-like BMV requires a minimum of 20 bases upstream and 16 bases downstream of the subgenomic RNA initiation site. However, WT levels of RNA production require sequences extending to at least 74 nucleotides upstream and includes a poly(A) sequence immediately upstream of the −20 to +16 core promoter; further upstream sequences including the ICR2-like motif (see below) influence RNA3 accumulation (French and Ahlquist, 1987, 1988; Marsh *et al.,* 1988). Likewise, the promoter for the related cucumovirus, cucumber mosaic virus (CMV) is located between 70 nucleotides upstream (which includes the ICR2-like motif) and 20 nucleotides downstream of the initiation site (Boccard and Baulcombe, 1993). The promoter for AlMV is located between nucleotides −8 and −55 relative to the initiation site (plus an enhancer element between nucleotides −55 and −136; Van der Kuyl *et al.,* 1990) with additional downstream sequences required for full *in vivo* activity (Van der Kuyl *et al.,* 1991). One exception to the observation that alphavirus-like subgenomic promoters lie primarily upstream of the transcription initiation site is noted for beet necrotic yellow vein virus RNA 3sub which is situated largely downstream, extending only to position −16 in the 5′ direction and to between +100 and +208 in the 3′ direction (Balmori *et al.,* 1993).

Extensive analysis of the intercistronic regions of several members of the alphavirus-like supergroup has revealed sequence motifs analogous to the downstream portions of internal control regions (ICR2 or box B regions) of RNA polymerase III promoters located within tRNA genes suggesting fundamental similarities between certain members of this group (Marsh *et al.,* 1988; French and Ahlquist, 1988; Smirnyagina *et al.,* 1994). The CNV 0.9-kb subgenomic RNA core promoter was examined for elements or features in common with the ICR2-like motifs found in the *cis*-acting replication sequences of several members of the alphavirus-like supergroup and obvious similarities were not apparent. The 0.9-kb subgenomic promoter also shares little homology with other putative *cis*-acting sequences within the CNV genome (i.e., sequences at the 5′ terminus of genomic RNA and those surrounding the 2.1-kb subgenomic RNA; see Fig. 7B). The lack of similarity between the 0.9-kb subgenomic RNA promoter and the region surrounding the transcription initiation site for the 2.1-kb subgenomic RNA may reflect their independent regulation by different *trans*-acting factors within the replicase complex as has been suggested to be the case for the tobacco mosaic virus subgenomic RNAs (Lehto *et al.,* 1990). Some homology is predicted to occur between subgenomic RNA promoters and sequences at the 5′ terminus of the genome since the viral replicase (with its associated factors) is expected to recognize and interact with specific (−)

```
A
                                      ****** ********    **
                        * ** **  *  * *  ****** ********   **
CNV     0.9   ggugcagguuGUGUAAAUUAGGGGCUUCUUGAAUCUaac
                                                ^
TBSV    0.9   UAAUUUAGUGUGUCCUGCGAGGGGCCUCUUGAACAAGAC
                                                ^
CymRSV  1.0   GUAGUUGCAUUGCACAGGAAGGGGCUUCUUGAACCUAAC
                                                ^
AMCV    0.9   UAAUUUAGUGAGUCCUGUGAGGGGCCUCUUGAACUAGAC
                                                ^

B
CNV     5'                                      AGAAAUUCU
                                                ^***  ***
CNV     0.9   ggugcagguuGUGUAAAUUAGGGGCUUCUUGAA--UCU
               *  ***  *   * * *  *  *   * ***
CNV     2.1   AGCCCAGCAUCCUUGACUCCGCCGUAGCAUGACCAAGC
                                                ^
```

FIG. 7. Sequences surrounding the CNV 0.9-kb subgenomic promoter and comparison with other putative promoters. (A) The CNV 0.9-kb subgenomic promoter and comparison to sequences surrounding the subgenomic start site of the analogous region of other tombusviruses. The subgenomic start site for each viral RNA is indicated with a caret. Sequences which comprise the CNV core promoter as defined in this study are shown in capital letters. The underlined sequences correspond to the stop codon for the coat protein. Double asterisks indicate identity between all four sequences and single asterisks identity at three of four positions. (B) Comparison of the CNV 0.9-kb promoter with sequences surrounding the CNV 2.1-kb coat protein subgenomic RNA start site and sequences at the 5′ terminus of CNV genomic RNA. The caret corresponds to the start sites for the 0.9-kb (Rochon and Johnston, 1991) and 2.1-kb subgenomic RNA (unpublished data) and the position of the CNV genomic RNA 5′ nucleotide. Asterisks indicate nucleotide identity between the 0.9-kb promoter and either of the other two sequences. The italicized AG in the CNV 5′ sequence are the presumed first and second nucleotides based on analyses of dimer junctions in CNV DI RNAs (Finnen and Rochon, 1995).

strand signals for (+) strand RNA synthesis (Pacha *et al.,* 1990; Pogue *et al.,* 1990). Similarities between the transcription start sites of the subgenomic mRNAs and the 5′ end of genomic RNA within individual viruses have been noted for other members of the flavivirus-like supergroup, e.g., BYDV-PAV (Kelly *et al.,* 1994) and maize chlorotic mottle virus (Lommel *et al.,* 1991) as well as the alphavirus-like BMV (Marsh and Hall, 1987; Marsh *et al.,* 1989), cowpea chlorotic mottle virus (Allison *et al.,* 1989), CMV (Boccard and Baulcombe, 1993), AIMV (Van der Kuyl *et al.,* 1990), and tobacco rattle virus (TRV; Cornelissen *et al.,* 1986; Goulden *et al.,* 1990).

The core promoter for CNV 0.9-kb subgenomic RNA synthesis contains significant sequence homology to analogous regions in the genomes of other members of the tombusvirus group (see Fig. 7A). The regions surrounding the 0.9/1.0-kb subgenomic RNA transcription initiation site of tomato bushy stunt (Hillman *et al.,* 1989), cymbidium ringspot (Grieco *et al.,* 1989), and artichoke mottle crinkle virus (Tavazza *et al.,* 1994) each contain a 14-nucleotide AGGGGC$^{U}/_{C}$UCUUGAA element which is identical or near-identical (with the exception of one nucleotide) to nucleotides −11 to +3 relative to the transcription start site of CNV. The 5′ border of this region of near-identity between the viral sequences is located one nucleotide upstream of the region remaining after the XΔ41 deletion, the smallest deletion to significantly alter 0.9-kb subgenomic RNA accumulation (see Fig. 3B). This observation suggests that the core promoter may be even smaller than the 26-nucleotide region determined by deletion analysis; however, further experiments would be required to substantiate this suggestion. The importance of the core promoter was further demonstrated by the drastically reduced levels of 0.9-kb subgenomic RNA directed by mutants carrying nucleotide substitutions in the −1 and +3 (and +4) positions within this region.

Comparison of sequences remaining after the XΔ41 deletion and the XΔ43 deletion (the latter of which appears to restore 0.9-kb subgenomic RNA production; see Fig. 3B) reveals no obvious homology between the area upstream of the deletion site and the 0.9-kb subgenomic RNA promoter region aside from a G in the −20 position relative to the initiation site which is present in XΔ43 but not in XΔ41. However, it is still possible that the partial restoration of 0.9-kb subgenomic RNA promoter activity for XΔ43 could be explained by a fortuitous juxtaposition of sequence upstream of the deleted region with those contained in the 0.9-kb subgenomic RNA promoter, or alternatively, by an alteration in secondary structure due to the deletion.

In addition to contributing to the delineation of the 0.9-kb subgenomic RNA core promoter, the large-scale deletion mutants used in this study also demonstrate the dispensable nature of the CNV p41 coat protein, p20, and the p21 movement protein for replication and accumulation of genomic and subgenomic RNAs in protoplasts. This contrasts with the requirement of coat protein in systemic movement (Sit *et al.,* 1995) and p21 in cell-to-cell movement in plants (unpublished observations). The absence of coat protein and movement protein genes might be expected to affect RNA accumulation since their products either encapsidate (in the case of coat protein) or possibly bind viral RNA (if p21 is indeed analogous to other cell-to-cell movement proteins; see Citovsky and Zambryski, 1993) and therefore function to protect the RNA. However, these and other experiments in which the AUG codons for p20 and p21 were changed to non AUG codons (unpublished data) show that, in the absence of these proteins, overall genomic and subgenomic RNA accumulation is not drastically reduced and the ratio of genomic to subgenomic RNA does not appear affected in protoplasts over the time periods used.

ACKNOWLEDGMENTS

J.C.J. was supported by a I. W. Killam predoctoral fellowship and by NSERC Operating Grant 0GP0043840. We thank David Theilmann for his critical review of the manuscript.

REFERENCES

Allison, R. F., Janda, M., and Ahlquist, P. (1989). Sequence of cowpea chlorotic mottle virus RNAs 2 and 3 and evidence of a recombination event during bromovirus evolution. *Virology* 172, 321–330.

Bailey, J. M., and Davidson, N. (1976). Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. *Anal. Biochem.* 70, 75–85.

Balmori, E., Gilmer, D., Richards, K., Guilley, H., and Jonard, G. (1993). Mapping the promoter for subgenomic RNA synthesis on beet necrotic yellow vein virus RNA3. *Biochimie* 75, 517–521.

Boccard, F., and Baulcombe, D. (1993). Mutational analysis of *cis*-acting sequences and gene function in RNA3 of cucumber mosaic virus. *Virology* 193, 563–578.

Boyko, V. P., and Karasev, A. V. (1992). Tombusvirus genome may encode the sixth small protein near its 3′ terminus. *Virus Genes* 6, 143–148.

Citovsky, V., and Zambryski, P. (1993). Transport of nucleic acids through membrane channels: Snaking through small holes. *Annu. Rev. Microbiol.* 47, 167–197.

Cornelissen, G. J. C., Linthorst, H. J. M., Brederode, F. T., and Bol, J. F. (1986). Analysis of the genome structure of tobacco rattle virus strain PSG. *Nucleic Acids Res.* 14, 2157–2169.

Dalmay, T., Rubino, L., Burgyan, J., Kollar, A., and Russo, M. (1993). Functional analysis of cymbidium ringspot virus genome. *Virology* 194, 697–704.

Finnen, R. L., and Rochon, D. M. (1995). Characterization and biological activity of DI RNA dimers formed during cucumber necrosis virus coinfections. *Virology* 207, 282–286.

French, R., and Ahlquist, P. (1987). Intercistronic as well as terminal sequences are required for efficient amplification of brome mosaic virus. *J. Virol.* 61, 1457–1465.

French, R., and Ahlquist, P. (1988). Characterization and engineering of sequences controlling *in vivo* synthesis of brome mosaic virus subgenomic RNA. *J. Virol.* 62, 2411–2420.

Gargouri, R., Joshi, R. L., Bol, J. F., Astier-Manifacier, S., and Haenni, A-L. (1989). Mechanism of synthesis of turnip yellow mosaic virus coat protein subgenomic RNA *in vivo. Virology* 171, 386–393.

Goulden, M. G., Lomonossoff, G. P., Davies, J. W., and Wood, K. R.

(1990). The complete nucleotide sequence of PEBV RNA2 reveals the presence of a novel open reading frame and provides insights into the structure of tobraviral subgenomic promoters. *Nucleic Acids Res.* 18, 4507–4512.

Grieco, F., Burgyan, J., and Russo, M. (1989). Nucleotide sequence of the 3′ terminal region of cymbidium ringspot virus RNA. *J. Gen. Virol.* 70, 2533–2538.

Hertz, J. M., and Huang, H. V. (1992). Utilization of heterologous alphavirus junction sequences as promoters by Sindbis virus. *J. Virol.* 66, 857–864.

Hillman, B. I., Hearne, P., Rochon, D., and Morris, T. J. (1989). Organization of tomato bushy stunt virus genome: Characterization of the coat protein gene and the 3′ terminus. *Virology* 169, 42–50.

Johnston, J. C., and Rochon, D. M. (1990). Translation of cucumber necrosis virus RNA *in vitro. J. Gen. Virol.* 71, 2233–2241.

Kelly, L., Gerlach, W. L., and Waterhouse, P. M. (1994). Characterization of the subgenomic RNAs of an Australian isolate of barley yellow dwarf luteovirus. *Virology* 202, 565–573.

Koonin, E. V., and Dolja, V. V. (1993). Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* 28, 375–430.

Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotype selection. *In* "Methods in Enzymology" (R. Wu, Ed.), Vol. 154, pp. 367–382. Academic Press, New York.

Lai, M. M. (1990). Coronavirus — Organization, replication and expression of genome. *Annu. Rev. Microbiol.* 44, 303–333.

Lai, M. M., Baric, R. S., Brayton, P. R., and Stohlman, S. A. (1984). Characterization of leader RNA sequences on the virion and mRNAs of mouse hepatitis virus, a cytoplasmic RNA virus. *Proc. Natl. Acad. Sci. USA* 81, 3626–3630.

Lehto, K., Grantham, G. L., and Dawson, W. O. (1990). Insertion of sequences containing the coat protein subgenomic RNA promoter. *Virology* 174, 145–157.

Levis, R., Schlesinger, S., and Huang, H. V. (1990). Promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. *J. Virol.* 64, 1726–1733.

Lommel, S. A., Kendall, T. L., Xiang, Z., and Nutter, R. C. (1991). Identification of the maize chlorotic mottle virus capsid protein cistron and characterisation of its subgenomic messenger RNA. *Virology* 181, 382–385.

Marsh, L. E., and Hall, T. C. (1987). Evidence implicating a tRNA heritage for the promoters of (+) strand RNA synthesis in brome mosaic virus and related viruses: The evolution of catalytic function. *Cold Spring Harbor Symp. Quant. Biol.* 52, 331–341.

Marsh, L. E., Dreher, T. W., and Hall, T. C. (1988). Mutational analysis of the core promoter and modulator sequences of the BMV RNA3 subgenomic promoter. *Nucleic Acids Res.* 16, 981–995.

Marsh, L. E., Pogue, G. P., and Hall, T. C. (1989). Similarities among plant virus (+) and (−) RNA termini imply a common ancestry with promoters of eukaryotic tRNAs. *Virology* 172, 415–427.

McLean, M. A., Hamilton, R. I., and Rochon, D. M. (1993). Symptomatology and movement of a cucumber necrosis virus mutant lacking the coat protein protruding domain. *Virology* 193, 932–939.

Miller, W. A., Dreher, T., and Hall, T. (1985). Synthesis of brome mosaic virus subgenomic RNA *in vitro* by internal initiation on (−) sense genomic RNA. *Nature* 313, 68–70.

Pacha, R. F., and Ahlquist, P. (1992). Substantial portions of the 5′ and intercistronic noncoding regions of cowpea chlorotic mottle virus RNA3 are dispensible for systemic infection but influence viral competitiveness and infection pathology. *Virology* 187, 298–307.

Pacha, R. F., Allison, R. F., and Ahlquist, P. (1990). *cis*-Acting sequences required for *in vivo* amplification of genomic RNA3 are organized differently in related bromoviruses. *Virology* 174, 436–443.

Pogue, G. P., Marsh, L. E., and Hall, T. C. (1990). Point mutations in the ICR2 motif of brome mosaic virus RNAs debilitate (+) strand replication. *Virology* 178, 152–160.

Raju, R., and Huang, H. V. (1991). Analysis of Sindbis virus promoter recognition *in vivo,* using novel vectors with two subgenomic mRNA promoters. *J. Virol.* 65, 2501–2510.

Rochon, D. M. (1991). Rapid de novo generation of defective interfering RNA from cucumber necrosis virus mutants which do not express the 20 kDa non-structural protein. *Proc. Natl. Acad. Sci. USA* 88, 11153–11157.

Rochon, D. M., and Johnston, J. C. (1991). Infectious transcripts from cloned cucumber necrosis virus cDNA: Evidence for a bifunctional subgenomic mRNA. *Virology* 181, 656–665.

Rochon, D. M., and Tremaine, J. H. (1989). Complete nucleotide sequence of the cucumber necrosis virus genome. *Virology* 169, 251–259.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scholthof, H. B., Morris, T. J., and Jackson, A. O. (1993). The capsid protein gene of tomato bushy stunt virus is dispensable for systemic movement and can be replaced for localized expression of foreign genes. *Mol. Plant-Microbe Interact.* 6, 309–322.

Sit, T. L., Johnston, J. C., ter Borg, M., Frison, E., McLean, M. A., and Rochon, D. (1995). Mutational analysis of the cucumber necrosis virus coat protein gene. *Virology* 206, 38–48.

Smirnyagina, E., Hsu, Y-H., Chua, N., and Ahlquist, P. (1994). Second-site mutations in the brome mosaic virus RNA3 intercistonic region partially suppress a defect in coat protein mRNA transcription. *Virology* 198, 427–436.

Spaan, W., Delius, H., Skinner, M., Armstrong, J., Rotteir, P., Smeekens, S., van der Zeijst, B. A. M., and Siddell, S. G. (1983). Coronavirus mRNA synthesis involves fusion of non-contiguous sequences. *EMBO J.* 2, 1839–1844.

Tavazza, M., Lucioli, A., Calogero, A., Pay, A., and Tavazza, R. (1994). Nucleotide sequence, genomic organization and synthesis of infectious transcripts from a full-length clone of artichoke mottle crinkle virus. *J. Gen. Virol.* 75, 1515–1524.

Van der Kuyl, A. C., Langereis, K., Houwing, C. J., Jaspars, E. M. J., and Bol, J. F. (1990). *cis*-Acting elements involved in replication of alfalfa mosaic virus RNAs *in vitro. Virology* 176, 346–354.

Van der Kuyl, A. C., Neeleman, L., and Bol, J. F. (1991). Deletion analysis of cis- and trans-acting elements involved in replication of alfalfa mosaic virus RNA 3 in vivo. *Virology* 183, 687–694.

Vrati, S., Mann, D. A., and Reed, K. C. (1987). Alkaline Northern blots: transfer of RNA from agarose gels to Zeta-Probe membrane in dilute NaOH. *Mol. Biol. Rep.* 1, 1–4.

Wieczorek, A., and Sanfacon, H. (1995). An improved method for the generation and transfection of protoplasts from *Cucumis sativus* cotyledons. *Plant Cell Rep.* 14, 603–610.