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Segment-specific inverted repeats found adjacent to conserved terminal sequences in wound tumor virus genome and defective interfering RNAs

(Reoviridae/plant virus/deletion mutation/macromolecular sorting)

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ABSTRACT **Defective interfering (DI) RNAs are often associated with transmission-defective isolates of wound tumor virus (WTV), a plant virus member of the Reoviridae. We report here the cloning and characterization of WTV genome segment S5 [2613 base pairs (bp)] and three related DI RNAs (587–776 bp). Each DI RNA was generated by a simple internal deletion event that resulted in no sequence rearrangement at the deletion boundaries. Remarkably, although several DI RNAs have been in continuous passage for more than 20 years, their nucleotide sequences are identical to that of corresponding portions of segment S5 present in infrequently passaged, standard, transmission-competent virus. The positions of the deletion breakpoints indicate that the minimal sequence information required for replication and packaging of segment S5 resides within 319 bp from the 5′ end of the (+)-strand and 205 bp from the 3′ end of the (+)-strand. The terminal portions of segment S5 were found to contain a 9-bp inverted repeat immediately adjacent to the conserved terminal 5′-hexanucleotide and 3′-tetranucleotide sequences shared by all 12 WTV genome segments. The presence of a 6- to 9-nucleotide segment-specific inverted repeat immediately adjacent to the conserved terminal sequences was found to be a feature common to all WTV genome segments. These results reveal several basic principles that govern the replication and packaging of a segmented double-stranded RNA genome.**

Viruses classified as members of the family Reoviridae have been isolated from a wide range of hosts including vertebrates, insects, mollusks, and plants (1). Several members of this virus group are of medical and economic importance; e.g., rotaviruses are a major cause of infant mortality in many parts of the world, and cytoplasmic polyhedrosis viruses are potential insect-control agents (1). Other members provide valuable model systems for molecular, pathogenicity, and epidemiologic studies (1). The genomes of these viruses consist of 10–12 segments of double-stranded RNA (1). Available evidence suggests that each virus particle contains one copy of each segment comprising the genome (2–5). The mechanism responsible for the selective sorting of individual genome segments remains an intriguing unsolved problem presumably involving complex protein–RNA and RNA–RNA interactions. Characterization of functional remnants of genome RNAs generated by deletion events has provided insights into the mechanism of genome replication and encapsidation for members of other virus families (6–9). Although there exists little information regarding remnant RNAs associated with the genome of mammalian reoviruses, there have been several reports describing remnant RNAs associated with isolates of wound tumor virus (WTV), the type member of the genus *Phytoreovirus* (10–12).

WTV replicates both in plant hosts and in several species of leafhoppers, which act as vectors. Maintenance of WTV exclusively in vegetatively propagated plant hosts results in virus populations that are defective in the ability to replicate in, and be transmitted by, the insect vector (10, ¶). The genomes of these transmission-defective isolates often contain deletion remnants of specific genome segments (10). Once generated, a remnant RNA is capable of displacing the segment from which it was derived. In this regard, WTV remnant RNAs are analogous to defective interfering (DI) RNAs of influenzaviruses, a group of viruses that also possess a segmented RNA (single-stranded, negative polarity) genome (6). In this communication, we present the nucleotide sequence analysis of three WTV DI RNAs, their progenitor genome segment, and the terminal nucleotide sequences of all 12 WTV genome segments.‖ These results, combined with those obtained from previous characterizations of WTV-associated DI RNAs, provide an indication of basic mechanisms operating during replication and assembly of a double-stranded RNA genome.

MATERIALS AND METHODS

Virus Isolates. Standard transmission-competent WTV [inoculum RB (13)] was maintained by infrequent passage in vector cell monolayers [*Agallia constricta*, line AC20 (14)], as described previously (15). Transmission-defective WTV isolates, originally obtained from L. M. Black, were maintained by vegetative propagation of systemically infected sweet clover plants. Detailed information concerning the origin and previous characterizations of these virus isolates can be found in refs. 10, 11, 12, and 16.

Preparation of DI RNAs. Double-stranded RNA used for cDNA synthesis and cloning of DI RNAs was prepared directly from virus-induced root tumors harvested from sweet clover plants systemically infected with transmission-defective isolates as described previously (12). To separate the DI RNAs [587–776 base pairs (bp)] from any residual full-length segment S5 (2613 bp) in the genome RNA population, the RNA was sedimented through a 5–20% sucrose

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Abbreviations: WTV, wound tumor virus; DI, defective interfering.

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¶Black, L. M., Wolcyrz, S. & Whitcomb, R. F. (1958) Seventh International Congress of Microbiology Symposium, Stockholm, p. 255 (abstr.).

‖The sequence of WTV genome segment S5 is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03020).

gradient in 25 mM Tris, pH 7.5/0.1 M NaCl/1 mM EDTA/1% $NaDodSO_4$ at 30,000 rpm for 13 hr at 25°C in a Beckman SW41 rotor. The double-stranded RNA segments were recovered from 0.5-ml gradient fractions by ethanol precipitation and analyzed by polyacrylamide gel electrophoresis and silver staining (17).

cDNA Cloning and Sequence Analysis. cDNA clones of WTV segment S5 were prepared as described by Asamizu *et al.* (18) from genome RNA isolated from purified standard, transmission-competent virus. cDNA copies of S5-related DI RNAs were synthesized in reactions containing oligodeoxynucleotides (18-mers) complementary to the 3'-terminal sequences of both strands of segment S5 as primers and gradient-enriched double-stranded DI RNA (denatured) as template, also as described by Asamizu *et al.* (18). Oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer and used directly for sequencing after deprotection according to the protocol supplied by the manufacturer.

RESULTS

The genome profiles of standard and several transmission-defective WTV isolates are shown in Fig. 1. The three designated DI RNAs (arrows) were shown to be related to segment S5 by molecular hybridization (12). In addition, the DI RNA indicated by the asterisk in lane 4 was previously shown to be derived from segment S5 as a result of an internal deletion event (12). To determine the precise relationship between segment S5 and the related DI RNAs, cDNA clones of the DI RNAs were generated and their nucleotide sequences were compared with those of cDNA clones of the segment S5 progenitor RNA.

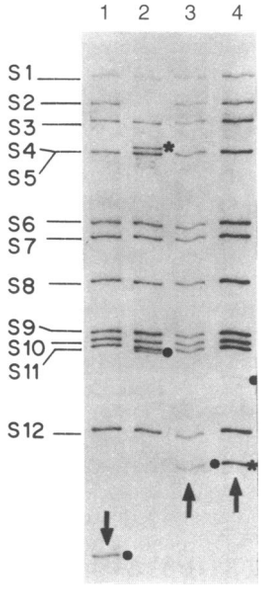

FIG. 1. Polyacrylamide gel electrophoretic analysis of [^{32}P]pCp-labeled genome segments associated with several transmission-defective isolates of WTV. The migration positions of segments associated with standard, transmission-competent virus are indicated at left. DI RNAs are indicated by dots. Asterisks indicate DI RNAs previously characterized (12). Arrows indicate the three S5-related DI RNAs fully characterized in this report. The four transmission-defective isolates are −S5(60) (lane 1), −S2(70) (lane 2), 10%S1(60) (lane 3), and 10%S1(49) (lane 4). Nomenclature is described in detail in several publications (e.g., refs. 11 and 12). Note in lane 2 that segment S2 has been completely replaced by a related DI RNA (12). RNAs were isolated from purified virus particles and labeled as described in ref. 12.

Sequence Analysis and Characterization of Cloned cDNA Copies of Segment S5. The complete nucleotide sequence of WTV segment S5 is presented in Fig. 2. The segment consists of 2613 bp and contains the terminal nucleotide sequences [(+)-strand] 5' GGUAUU . . . and . . . UGAU 3' previously reported to be conserved among the 12 WTV genome segments (18). The sequence also contains one long open reading frame, which begins at nucleotides 26–28, extends 2412 nucleotides, and terminates with two consecutive stop codons at nucleotides 2438–2443. Computer analysis predicts that the 804-codon-long open reading frame encodes a polypeptide of molecular weight 91,074.

The polypeptide product of segment S5 was previously identified as the 76,000 molecular weight component of the outer protein coat of WTV, P5 (16, 23, 24). To eliminate the possibility that the disparity in the reported (76,000) and the computer-predicted (91,074) molecular weights reflects an error in the sequence analysis, the following *in vitro* translation studies were performed. Homopolymer flanking sequences, added to the segment S5 cDNA sequence during cDNA synthesis and cloning (18), were removed by a recently developed strategy that allows the simultaneous removal of homopolymer tails and tailoring of the resulting termini (21). The tailored, full-length S5 cDNA clone was subcloned into a pGEM transcription vector (Promega Biotec) and the resulting *in vitro*-generated transcript was used to program a reticulocyte lysate translation system. As expected, the *in vitro*-synthesized translation product comigrated with *in vivo*-synthesized WTV polypeptide P5 (Fig. 3). The approximate location of the S5 translation termination codon was mapped by translational analysis of 3'-truncated pGEM-derived S5 transcripts generated by site-specific, oligonucleotide-directed RNase H digestions (25). The results (Fig. 3) were completely consistent with the sequence information presented in Fig. 2.

Sequence Analysis of Segment S5-Related DI RNAs. Sequence analysis of cloned, full-length cDNA copies of the DI RNA associated with the genome of transmission-defective isolate 10%S1(49) confirmed that the RNA is an internally deleted remnant of segment S5. It is 776 bp long and contains a single deletion breakpoint 319 bp from the 5' terminus of the (+)-strand of segment S5 and 457 bp from the 3' end of the same strand (Fig. 4). The DI RNA associated with the genome of isolate −S5(60) is 587 bp in length. It also contains a single breakpoint, but this is located 382 bp from the 5' terminus of the (+)-strand of segment S5 and only 205 bp from the 3' end of the same strand—i.e., ≈78% of the internal portion of segment S5 has been deleted (Fig. 4). Surprisingly, the nucleotide sequences determined for both DI RNAs were found to be identical to that determined for the corresponding domains of the standard segment S5 cDNA clones (Fig. 2). The deletion breakpoint for the DI RNA associated with the genome of transmission-defective isolate 10%S1(60) was found to be located at the same position as in the 10%S1(49)-associated DI RNA. However, the nucleotide sequence of this RNA differed from that of the standard segment S5 and other DI RNAs at two positions: A·T rather than C·G at position 21 from the 5' end of the (+)-strand and A·T rather than G·C at position 69 from the 3' end of the same strand. In each case, there was no rearrangement of the nucleotide sequence adjacent to the deletion breakpoint (Fig. 4). That is, each DI RNA was apparently generated by a single internal deletion event.

Sequence Analysis of the Terminal Portions of WTV Genome Segments. Inspection of the nucleotide sequence of segment S5 revealed an exact 9-bp inverted repeat immediately adjacent to the conserved terminal nucleotide sequences shared by all 12 WTV genome segments (Fig. 5). Inspection of the previously determined sequence of WTV genome segment S12 (18) also revealed an imperfect (1 base differ-

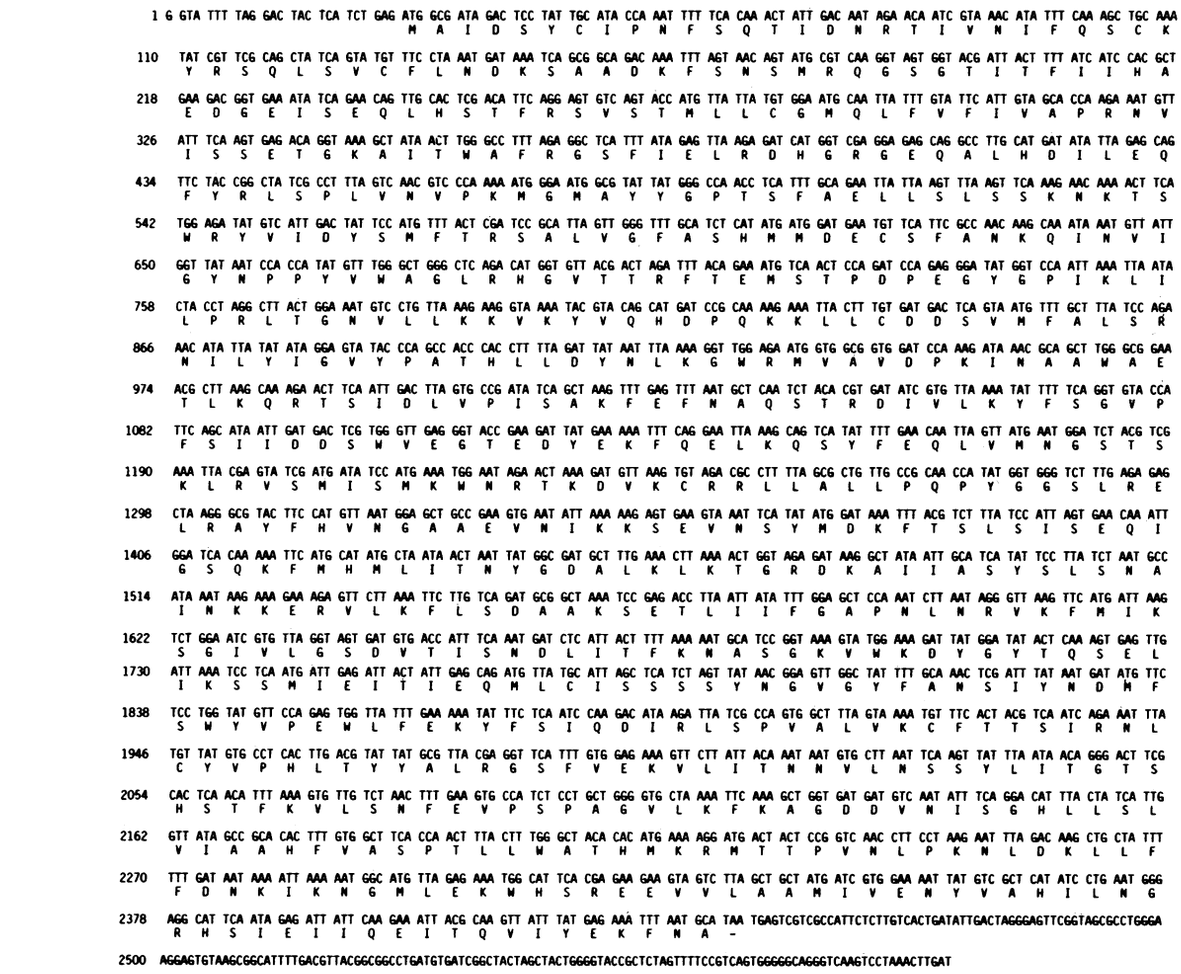

FIG. 2. Nucleotide and predicted amino acid sequences of the mRNA-sense DNA strand of cloned WTV genome segment S5. The nucleotide sequence was determined from multiple independent clones by the dideoxy chain-termination method (19). A combination of strategies was used, including sequencing of M13 subclones (20), synthetic oligonucleotide primer-directed sequencing of intact recombinant plasmids (21), and use of phasing primers to obtain terminal nucleotide sequences (22).

ence) inverted repeat of 10 nucleotides (Fig. 5). Although the inverted repeats were located in the same relative positions in the two genome segments, they differed significantly in sequence. This observation prompted us to determine the terminal nucleotide sequences for the remaining 10 WTV genome segments. As shown in Fig. 5, each of the 12 WTV genome RNAs contains a 6- to 10-bp segment-specific perfect (segments S10, S9 and S5) or near-perfect inverted repeat located immediately adjacent to the conserved terminal sequences.

DISCUSSION

The apparent ability of members of the family Reoviridae to selectively package a genome complement composed of 10–12 individual double-stranded RNA segments within a single particle poses an intriguing biological puzzle involving a complex set of macromolecular recognition and sorting events. Although there have been numerous speculations as to the mechanisms involved, the paucity of direct physical data related to specific events has hampered the advancement of testable hypotheses. In this regard, the results presented in this report, coupled with those obtained from previous analyses of WTV-associated DI RNAs, provide an emerging view of the principles that govern the replication and packaging of a segmented double-stranded RNA genome. This information is summarized in the form of several statements that describe the nature of the mechanisms involved in these events.

(*i*) *Sequence information required for replication and packaging of a genome segment is located within the terminal domains.* The results presented in this report provide evidence that the sequence domains within the internal portion of a genome segment are not required for these functions. Furthermore, the location of the sequence domains that are involved in transcription, replication, and packaging of segment S5 must reside within 319 bp from the 5′ end of the (+)-strand and 205 bp from the 3′ end.

(*ii*) *Packaging of a genome equivalent of terminal structures is favored over packaging of a genome equivalent of nucleotide bases.* There appear to be more constraints placed on packaging a full complement of 12 individual WTV terminal structures than on packaging a full complement of genome mass. This is indicated by the observation that genome RNA isolated from purified transmission-defective WTV particles contain an equimolar concentration of the 12 termini (10, 11) even though the major form of a particular set of terminal structures (e.g., S5) may be a DI RNA that is only 20% the length of the progenitor segment.

(*iii*) *Packaging of one pair of terminal structures excludes the packaging of a second copy of the same pair of terminal structures.* Reddy and Black (10, 11) showed that an increase in the molar concentration of a DI RNA in the genome population resulted in a concomitant reduction in the molar

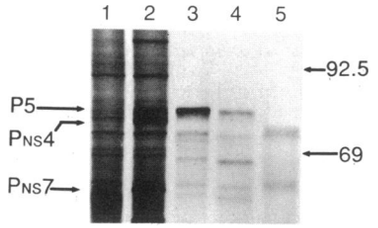

FIG. 3. *In vitro* expression of the WTV segment S5 cDNA clone. A full-length cDNA clone of segment S5 was tailored and subcloned in a pGEM transcription vector, and the resulting transcripts were translated in a reticulocyte lysate as described by Xu *et al* (21). [^{35}S]Methionine-labeled lysates of mock-infected and WTV-infected AC20 cells were prepared as described (15). Lane 1: lysate of mock-infected cells. Lane 2: lysate of infected cells. Lane 3: *in vitro* lysate programed by full-length pGEM-derived S5 transcript. In addition, truncated transcripts were translated to physically map the position of the S5 termination codon. The truncated transcripts were generated by site-specific oligonucleotide-directed RNase H digestions and analyzed by electrophoresis in 5% polyacrylamide/urea gels before use (25). Lane 4: *in vitro* lysate programed by S5 transcript truncated at nucleotide 2379, which should yield a polypeptide 20 amino acids shorter (and hence ≈2250 daltons smaller) than the S5 translation product, P5, if the termination codon is located at positions 2438–2440 in Fig. 2. Lane 5: *in vitro* lysate programed by S5 transcript truncated at nucleotide 2240, which should yield a polypeptide 67 amino acids shorter (and hence ≈7500 daltons smaller) than P5. The migration positions of molecular weight standards ($M_r \times 10^{-3}$) are indicated at right, while the positions of several virus-specific polypeptides, including P5, are indicated at left.

concentration of the progenitor RNA segment. That is, a DI RNA interferes only with the replication and/or packaging of the genome segment from which it was derived and not with that of unrelated genome segments. This "exclusion mechanism" has two important corollaries: (*a*) Each segment must contain at least two operational recognition sequence domains ("sorting signals"): one that specifies that it is a viral and not a cellular RNA (perhaps the conserved terminal sequences, Fig. 5) and a second that specifies that it is a particular RNA segment (perhaps the segment-specific inverted repeats, Fig. 5). (*b*) Packaging of the 12 individual WTV segments must involve 12 different and specific protein–RNA and/or RNA–RNA interactions.

It is well documented that RNA genomes can evolve at a rapid pace (28, 29). Thus, it was surprising to find the deduced nucleotide sequences of two DI RNAs and the progenitor segment to be identical with the exception of the positions of the deletion breakpoints (Figs. 2 and 4). It is likely that the DI RNAs were generated within several years after the year of introduction of the transmission-competent inoculum into the plant host, which was 1949 for isolate 10%S1(49) and 1960 for isolate −S5(60) (10). Certainly, both DI RNAs were detectable in genome RNA preparations analyzed in 1971 (10). Therefore, the sequences of these RNAs have had the opportunity to diverge during continuous replication within separate, systemically infected, vegetatively propagated plant hosts for a period of at least 15 and perhaps as many as 35 years. The genome RNA from which the full-length segment S5 sequence was deduced was obtained from a transmission-competent virus preparation that had been infrequently passaged in both plant hosts and insect vectors or their cultured cells (14) [all laboratory WTV stocks are derived from one original field isolate (30)]. Thus, either replication of phytoreovirus genomes proceeds with an unexpectedly high degree of fidelity or mechanisms exist that ensure that a "wild-type" segment S5 sequence predominates within the genome population (31).

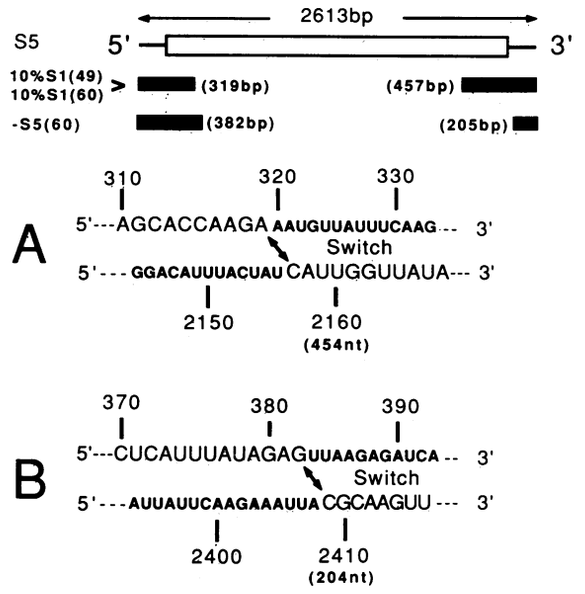

FIG. 4. Sequence information retained in S5-related DI RNAs. The sequences of cDNA clones of DI RNAs were determined by analyzing several independent full-length clones of each DI RNA, using an appropriate set of synthetic oligonucleotide primers. The deletion boundary in the S5-related DI RNA present in virus isolate 10%S1(49) was also determined by direct sequencing of the denatured DI RNA using primers located on either side of the suspected deletion boundary (26). The coding region of segment S5 is indicated by the open bar. The solid bars indicate the portions of segment S5 retained in the DI RNAs associated with isolates 10%S1(49), 10%S1(60) and −S5(60). The positions of the deletion boundaries relative to the 5′ and 3′ termini of the (+)-strand are indicated within parentheses inside the solid bars. (*A* and *B*) The sequence context around the deletion boundaries of the DI RNAs associated with isolate 10%S1(49) and −S5(60), respectively. The nucleotide numbering system corresponds to that shown in Fig. 2. Distances in terms of nucleotides (nt) from the 3′ end of the (+)-strand are given in parentheses.

The organization of sequence domains within the terminal portions of segmented double-stranded RNA genomes suggests that this region is intimately involved in the interactions required for molecular sorting of individual segments. As indicated previously, all genome segments of a particular member of the Reoviridae share common 5′ and 3′ terminal sequences consisting of 4–8 nucleotides (listed in ref. 18). In the case of the WTV genome, segment-specific sequences analogous to inverted repeats have now been identified immediately adjacent to the common terminal sequences (Figs. 4 and 5). Inspection of sequences published for the genome RNAs of other members of the Reoviridae [e.g., the terminal sequences of human reovirus serotype 3 (32)] indicates that segment-specific inverted repeats within the terminal sequence domains are a common feature of genomes of this virus group.

Recently, Stoeckle *et al.* (27) reported segment-specific sequences of inverted complementarity (equivalent to the inverted repeats in WTV genome RNAs; Fig. 5) within the terminal noncoding regions of influenza B virus. The genomes of influenzaviruses are composed of single-stranded RNA segments of negative polarity, and, as in the Reoviridae, the individual RNAs comprising a specific genome share common terminal sequences (Fig. 5). As noted by Stoeckle *et al.* (27), segment-specific sequences of inverted complementarity can be identified within the published terminal sequences of influenza A and C genome RNAs, and these sequence domains are located adjacent to the conserved terminal sequences in each case. The striking simi-

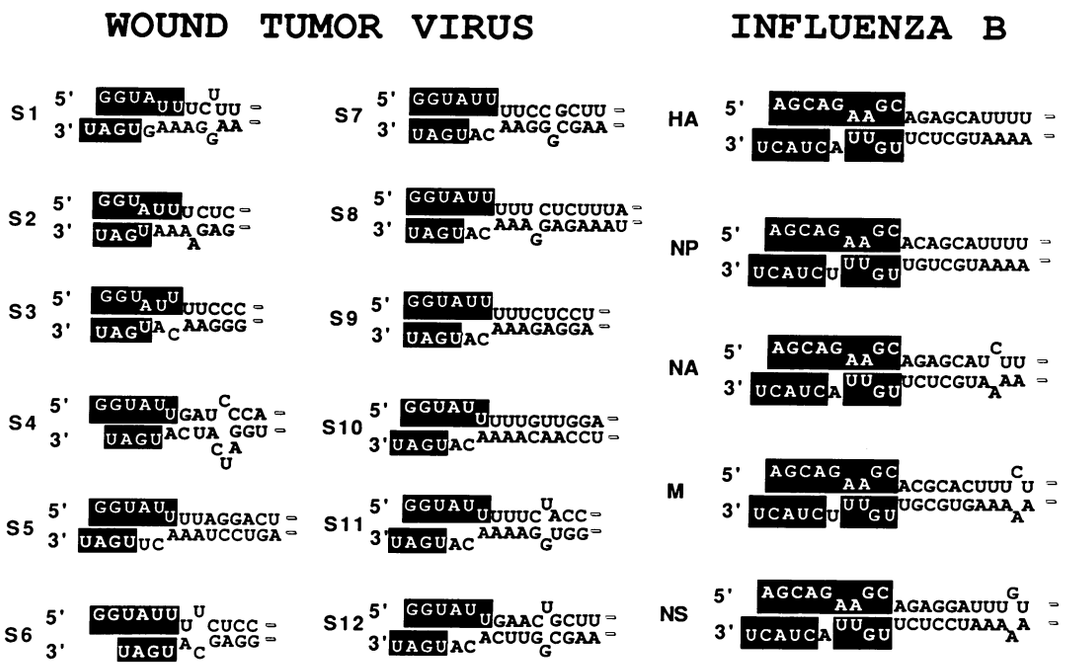

FIG. 5. Terminal sequence domains of WTV genome segments. (*Left* and *Center*) The terminal nucleotide sequences (+ sense only) of all 12 WTV genome segments. The conserved terminal 5′-hexanucleotide and 3′-tetranucleotide sequences shared by all 12 WTV genome segments are shown in white on black. The segment-specific inverted repeats are orientated to indicate potential base-pairing interactions. Terminal sequence of individual genome segments were determined in several ways. Initially, sequences were obtained by "wandering-spot" analysis of individual genome segments, as described (18). This sequence information was confirmed and extended for genome segments S12 through S4 by sequence analysis of full-length cDNA clones, using phasing primers and other appropriate synthetic oligonucleotide primers. Only sequence information pertinent to this report is included, due to space limitations. (*Right*) For purposes of comparison, available sequence information for terminal domains of influenza B genome segments are presented. The sequence information was taken from Stoeckle *et al.* (27) and reformatted to illustrate similarities to WTV terminal structures. Conserved terminal sequences are shown white on black. HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, membrane protein; NS, nonstructural protein.

larity in organization of the terminal domains of the segmented double-stranded RNA genomes of the Reoviridae and the segmented single-stranded RNA genomes of the influenza-viruses provides compelling evidence that both the conserved terminal sequences and segment-specific domains of inverted complementarity (inverted repeats) play a significant role in the sorting and assembly of segmented RNA genomes.

1. Joklik, W. K., ed. *The Reoviridae* (1983) (Plenum, New York).
2. Nonoyama, M., Watanabe, Y. & Graham, A. F. (1970) *J. Virol.* **6,** 226–236.
3. Spendlove, R. S., McClain, M. E. & Lennette, E. H. (1970) *J. Gen. Virol.* **8,** 83–94.
4. Kimura, I. & Black, L. M. (1972) *Virology* **49,** 549–561.
5. Reddy, D. V. R. & Black, L. M. (1973) *Virology* **54,** 557–562.
6. Nayak, D. P., Chambers, T. M. & Akkina, R. K. (1985) *Curr. Top. Microbiol. Immunol.* **114,** 103–151.
7. Re, G. G. & Kingsbury, D. W. (1986) *J. Virol.* **58,** 578–582.
8. Levis, R., Weiss, B. G., Tsiang, M., Huang, H. & Schlesinger, S. (1986) *Cell* **44,** 137–145.
9. DePolo, N. J., Giachetti, C. & Holland, J. J. (1987) *J. Virol.* **61,** 454–464.
10. Reddy, D. V. R. & Black, L. M. (1974) *Virology* **61,** 458–473.
11. Reddy, D. V. R. & Black, L. M. (1977) *Virology* **80,** 336–346.
12. Nuss, D. L. & Summers, D. (1984) *Virology* **133,** 276–288.
13. Reddy, D. V. R. & Black, L. M. (1972) *Virology* **50,** 412–421.
14. Chiu, R. J. & Black, L. M. (1967) *Nature (London)* **215,** 1076–1078.
15. Nuss, D. L. & Peterson, A. J. (1980) *J. Virol.* **34,** 532–541.
16. Nuss, D. L. & Peterson, A. J. (1981) *Virology* **114,** 399–404.
17. Peterson, A. J. & Nuss, D. L. (1986) *J. Virol.* **59,** 195–202.
18. Asamizu, T., Summers, D., Motika, M. B., Anzola, J. V. & Nuss, D. L. (1985) *Virology* **114,** 398–409.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
20. Messing, J., Crea, R. & Seeburg, P. H. (1981) *Nucleic Acids Res.* **9,** 309–321.
21. Xu, Z., Anzola, J. V. & Nuss, D. L. (1987) *DNA* **6,** 505–513.
22. Elleman, T. C., Hoyne, P. A., Dyall-Smith, M. L., Holmes, I. H. & Azad, A. A. (1983) *Nucleic Acids Res.* **11,** 4689–4701.
23. Nuss, D. L. (1983) in *Double-Stranded RNA Viruses*, eds. Bishop, D. H. L. & Compans, R. W. (Elsevier, Amsterdam), pp. 415–423.
24. Reddy, D. V. R. & MacLeod, R. (1976) *Virology* **20,** 274–282.
25. Chattopadhyay, D. & Banerjee, A. K. (1987) *Cell* **49,** 407–414.
26. Bassel-Duby, R., Spriggs, D. R., Tyler, L. L. & Fields, B. N. (1986) *J. Virol.* **60,** 64–67.
27. Stoeckle, M. Y., Shaw, M. W. & Chopin, P. W. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2703–2707.
28. Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. & VandePol, S. (1982) *Science* **215,** 1577–1585.
29. Reanney, D. C. (1982) *Annu. Rev. Microbiol.* **36,** 47–73.
30. Black, L. M. (1944) *Proc. Ann. Philos. Soc.* **88,** 132–144.
31. Domingo, E., Sabo, D., Taniguchi, T. & Weissmann, C. (1978) *Cell* **13,** 735–744.
32. Antczak, J. B., Chmelo, R., Pickup, D. J. & Joklik, W. K. (1982) *Virology* **121,** 307–319.