A unique virus complex causes *Ageratum* yellow vein disease

Keith Saunders*, Ian D. Bedford*, Rob W. Briddon*, Peter G. Markham*, Sek Man Wong†, and John Stanley*‡

*Department of Virus Research, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom; and †Department of Biological Sciences, National University of Singapore, Lower Kent Ridge Road, Singapore 117543, Republic of Singapore

Communicated by George Bruening, University of California, Davis, CA, April 3, 2000 (received for review January 10, 2000)

***Ageratum conyzoides* L., a weed species widely distributed throughout southeast Asia, frequently exhibits striking yellow vein symptoms associated with infection by *Ageratum* yellow vein virus (AYVV), a member of the *Geminiviridae* (genus *Begomovirus*). Most begomoviruses have bipartite genomes (DNAs A and B), but only a DNA A has been identified for AYVV. We demonstrate that yellow vein disease of *A. conyzoides* results from co-infection by AYVV DNA A (2,741 nt) and a circular DNA that is approximately half its size (1,347 nt) that we designate DNA β. Apart from the sequence TAATATTAC, common to all geminiviruses and containing the initiation site of rolling circle replication, DNA β shows negligible sequence homology either to AYVV DNA A or to DNA B associated with bipartite begomoviruses. DNA β depends on DNA A for replication and is encapsidated by DNA A-encoded coat protein and so has characteristics of a DNA satellite. However, systemic infection of *A. conyzoides* by DNA A alone is sporadic and asymptomatic, and DNA A accumulation is reduced to 5% or less of its accumulation in the presence of DNA β. Therefore, DNA A and DNA β together form a previously unrecognized disease-inducing complex. Our data also demonstrate that the nanovirus-like DNA 1 component associated with infected *A. conyzoides* plays no essential role in the disease and represents a satellite-like DNA. Furthermore, the satellite DNA previously found associated with tomato leaf curl virus is probably a defective DNA β homologue.**

The majority of members of the *Geminiviridae*, genus *Begomovirus*, have a genome comprising two similar-sized DNA components (DNA A and DNA B). DNA A encodes a replication-associated protein (Rep), coat protein, and proteins that participate in the control of replication and gene expression. DNA B encodes proteins required for nuclear trafficking and cell-to-cell movement of the viral DNA (reviewed in ref. 1). In contrast, only a single genomic component that resembles DNA A has been isolated for several begomoviruses including tomato yellow leaf curl virus, tomato leaf curl virus (TLCV), *Ageratum* yellow vein virus (AYVV), and cotton leaf curl virus (2–6). Cloned DNA A components of tomato yellow leaf curl virus and TLCV are infectious in tomato, the crop species from which they were first isolated, as well as in permissive laboratory hosts such as *Nicotiana benthamiana* and *N. tabacum*. In addition, the progeny of tomato yellow leaf curl virus DNA A is transmissible by the begomovirus whitefly vector, *Bemisia tabaci*, implying that the single genomic component is sufficient for maintenance of the disease in this host. Because bipartite begomoviruses require both genomic components for systemic infectivity in these and other hosts, it is evident that monopartite begomoviruses can in some way compensate for the lack of a DNA B component.

Weed species are believed to act as reservoir hosts for many economically important plant viral diseases. *Ageratum conyzoides* L., a weed that occurs throughout southeast Asia, frequently exhibits yellow vein symptoms that are associated with infection by AYVV (7). Like tomato yellow leaf curl virus and TLCV, AYVV DNA A is infectious in tomato and *Nicotiana* spp., in which it induces leaf curl symptoms. However, it is unable to induce typical yellow vein symptoms when reintroduced into *A. conyzoides*. Rigorous attempts to isolate a DNA B component have been unsuccessful, and it was concluded that another factor must contribute to the disease (5). Several recombinant DNAs of approximately half the size of the DNA A, designated by using the prefix "def," have been isolated from infected *A. conyzoides* and characterized. They contain the intergenic region and flanking sequences of DNA A fused to sequences of unknown origin (referred to as non-DNA A sequences) (8). Subsequently, the non-DNA A sequences of recombinant def19 were shown to be homologous to a region of a unique viral DNA, referred to as DNA 1, which had been isolated from cotton leaf curl virus-infected cotton (9). This facilitated the isolation of a DNA 1 homologue from AYVV-infected *A. conyzoides* (10). DNA 1 is related to nanovirus DNA components that encode Reps (11). It can replicate autonomously, but relies on DNA A for coat protein (9) and for systemic infection of *N. benthamiana* (10). However, co-inoculation of AYVV DNA A and DNA 1 did not produce a symptomatic infection in *A. conyzoides*, and so the aetiology of yellow vein disease remained unresolved. Nonetheless, the data suggested that the characterization of other AYVV recombinants may uncover additional begomovirus and/or nanovirus-like components. Here, the non-DNA A sequences of recombinant def17 (8) have been used to isolate and characterize a novel viral DNA component that, together with DNA A, is shown to cause yellow vein disease of *A. conyzoides*.

Materials and Methods

Source and Maintenance of Virus Isolate. *A. conyzoides* plants showing yellow vein symptoms were collected from Singapore, and the virus was maintained in *A. conyzoides* by transmission using *B. tabaci* (Gennadius) in insect-proof cages (12).

Construction and Characterization of Cloned Viral DNAs. The construction of clones pHNIC419 and pHNBin419 containing partial repeats of AYVV DNA A in pIC19H and the binary vector pBin19, respectively, has been described (5). The construction of clones pBinAYVdef17 and pBinAYVV1/7, containing partial repeats of a defective DNA A recombinant (referred to here as def17) and DNA1, respectively, in the binary vector pBin19, has been described (8, 10).

The preparation of viral supercoiled DNA (scDNA) from symptomatic *A. conyzoides* leaf material harvested in Singapore during July, 1995 (sample 1) has been described (8). Viral scDNA was similarly isolated from a second batch of leaf material (sample 2) harvested from the same location in Singa-

Abbreviations: AYVV, *Ageratum* yellow vein virus; TLCV, tomato leaf curl virus; Rep, replication-associated protein; ss, single-stranded; scDNA, supercoiled DNA; p.i., postinoculation.

Data deposition: The nucleotide sequence reported in this paper has been deposited in the European Molecular Biology Laboratory Nucleotide Sequence Database (accession No. AJ252072).

‡To whom reprint requests should be addressed. E-mail: john.stanley@bbsrc.ac.uk.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

pore during April, 1999. A viral DNA fragment of ≈800 bp was PCR-amplified from sample 1 by using primers V3996 and V3518 corresponding to def17 (European Molecular Biology Laboratory Nucleotide Sequence Database accession no. Y14167) nucleotides 854–880 (virion-sense) and 343–367 (complementary-sense), respectively. The amplified DNA was cloned into pGEM-T Easy (Promega), and six clones were chosen at random and sequenced. Sequences were derived by using an Applied Biosystems PRISM Big Dye Terminator, Cycle Sequencing, Ready Reaction Kit (Perkin–Elmer), and either T7 and SP6 sequencing primers or primers based on established sequence. Sequence reactions were resolved by using an Applied Biosystems 377 automated sequencer. Sequences were analyzed by using version 7 of the program library of the Genetics Computer Group (13).

An aliquot of scDNA from sample 2 was digested with *Spe*I and was cloned into pBluescript SK(+) (Stratagene) to produce clone pBS-AYVVβ/5 containing a full-length copy of a viral DNA component (DNA β). Abutting primers V4539 and V4540, corresponding to nucleotides 1,167–1,193 (virion-sense) and 1,139–1,166 (complementary-sense) of DNA β, were used to PCR-amplify full-length DNA β from scDNA sample 1. The amplified DNA was cloned into pGEM-T Easy, and clones pGEM-AYVVβ/3 and pGEM-AYVVβ/6 were selected and sequenced.

To produce a dimer of DNA β for inoculation purposes, a copy of the *Spe*I insert of pBS-AYVVβ/5 was cloned into *Spe*I/*Xba*I-digested pBluescript SK(+), and a second copy was subsequently cloned into the unique *Spe*I site of the resulting clone to produce pBS-AYVV2β. The DNA β dimer was excised by using *Sal*I and *Sac*I and was cloned into pBin19 to produce pBin-AYVVβ. To clone both DNA A and DNA β in the same binary vector, the DNA A partial repeat in pHNIC419 (5) was excised by using *Hin*dIII and was cloned into pBin-AYVVβ to produce pBin-AYVVAβ.

Whole Plant Inoculation and Replication Assays. *A. conyzoides* and *N. benthamiana* plants were agroinoculated with combinations of DNA A, DNA β, DNA 1, and def17 as described (5) by using *Agrobacterium tumefaciens* strain GV3850 (14) transformed with the binary vector constructs described above. The ability of viral components to replicate was assayed by agroinoculating *N. benthamiana* leaf disks by using *A. tumefaciens* strain LBA4404 (15) as described (16). Leaf disks were harvested 3, 5, and 7 days postinoculation (p.i.).

Insect Transmission. Nonviruliferous *B. tabaci* K biotype originating from Pakistan were maintained on *Hibiscus rosa-sinensis* in cages within a controlled environment growth room kept at 25°C with supplementary lighting to give a 12-h photoperiod (12). For virus transmission, 100 nonviruliferous insects were maintained in a cage containing *A. conyzoides* plants infected with either DNA A and DNA β or DNA A alone, and healthy seedlings were introduced the following day.

Viral DNA Detection. Nucleic acids were isolated from plants and leaf disks (17) and were fractionated by agarose gel electrophoresis in TNE buffer (40 mM Tris acetate, pH 7.5/20 mM Na acetate/2 mM EDTA). DNA was transferred to Hybond-N (Amersham Pharmacia) and was hybridized to randomly labeled probes (18) specific for DNA A, DNA β, and DNA 1. Viral DNAs were detected in asymptomatic plants by dot-blot analysis (19).

Identification of Virion-Sense DNA Strand. A clarified extract of systemically infected plant material in RIPA buffer (50 mM Tris·HCl, pH 8.0/150 mM NaCl/1% Nonidet P-40/0.5% Na deoxycholate/0.1% SDS) was treated with polyclonal antiserum raised against purified virions of African cassava mosaic virus that had been cross-absorbed against a healthy *N. benthamiana* extract (20). Immuno-complexes were precipitated by using Pansorbin Cells (Calbiochem) and were washed extensively with RIPA buffer. Viral DNAs were extracted from the complexes (17) and were analyzed, either directly or after digestion with mungbean nuclease (GIBCO/BRL) (8), by blot hybridization using strand-specific probes synthesized *in vitro* using the T3 and T7 promoters of pBS-AYVVβ.

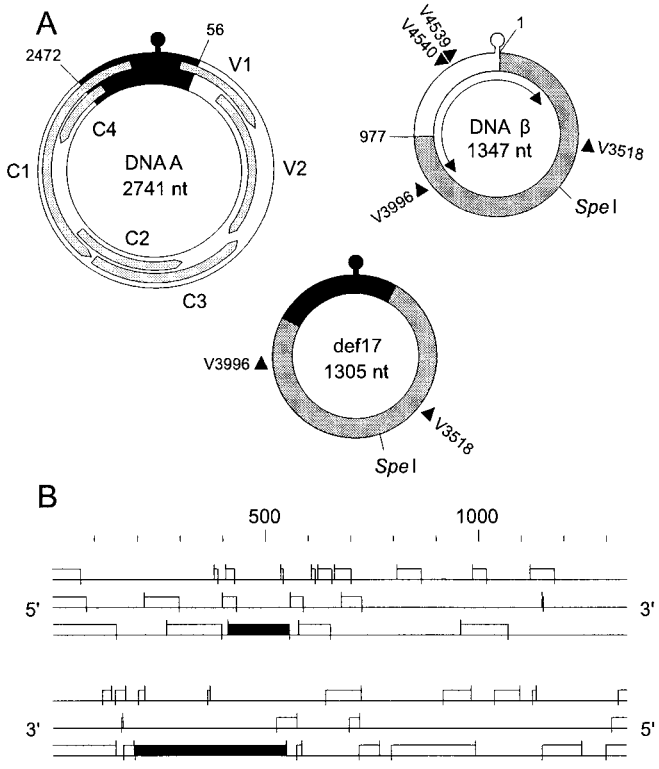

Fig. 1. Structural relationship between AYVV DNA A, DNA β and recombinant def17. (*A*) The position and orientation of DNA A virion-sense (V) and complementary-sense (C) genes is shown in relation to the stem-loop sequence containing the conserved nonanucleotide TAATATT↓AC. Regions of the recombinant def17 derive from DNA A (black) and DNA β (gray) sequences, the coordinates of which are indicated. Nucleotide numbering starts from the putative nick site (↓) within the conserved nonanucleotide. The double-headed arrow demarcates the region of DNA β showing homologies to TLCV satellite DNA. The positions of primers used to PCR-amplify DNA β fragments and the *Spe*I site used to clone DNA β are indicated. (*B*) The program FRAMES (13) was used to predict DNA β ORFs, starting from an in-frame ATG triplet, that occur in the virion-sense (top) and complementary-sense (bottom) strands. The program TESTCODE predicted two potential coding regions (black ORFs). Nucleotide numbering of DNA β follows the accepted convention for geminivirus DNAs and begins immediately downstream of the putative nick site within the conserved nonanucleotide.

Results and Discussion

A Unique Circular DNA Associated with *A. conyzoides* Yellow Vein Disease. On the assumption that the non-DNA A sequences of recombinant def17 originate from a unique circular DNA, primers V3518 and V3996 (Fig. 1*A*) were used to PCR-amplify a fragment from purified viral scDNA (sample 1). Sequence analysis of six clones derived from the amplified fragment indicated that only one resembled def17, the remainder having almost identical non-DNA A sequences but lacking the DNA A region found within the recombinant. Subsequently, a full-length copy of the DNA was cloned directly from scDNA (sample 2) by

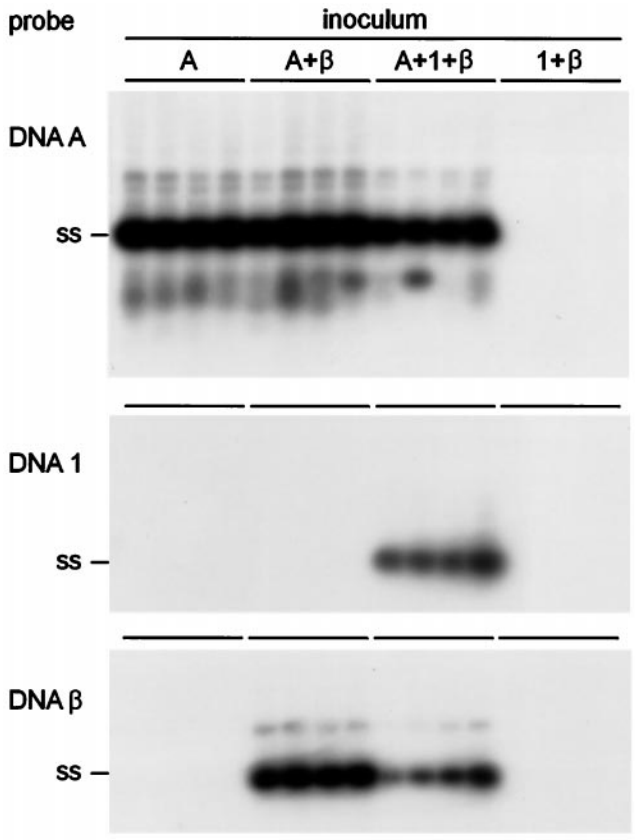

Fig. 2. Detection of viral DNAs in agroinoculated *N. benthamiana* plants. Samples (5 μg of nucleic acids) were extracted 15 days p.i. from individual plants agroinoculated with combinations of DNA A, DNA β, and DNA 1 as indicated. Samples were fractionated by using agarose gels in TNE buffer, and blots were screened by using probes specific for DNA A (*Top*), DNA 1 (*Middle*), and DNA β (*Bottom*). The position of single-stranded DNA (ss) is shown.

Fig. 3. Infectivity of DNA A and DNA β in *A. conyzoides*. Plants were inoculated either with DNA A (*Upper*) or with DNA A and DNA β (*Lower*). DNA A and DNA β produce yellow vein symptoms indistinguishable from those occurring in naturally infected plants. Plants were photographed 3 weeks after inoculation.

using an *Spe*I site that is known to occur only once in def17, to produce pBS-AYVVβ. Sequence analysis indicated that the pBS-AYVVβ insert is 1,347 nt in length (Fig. 1*A*). We refer to this component as DNA β to emphasize that it is structurally distinct from previously characterized begomovirus components, yet in some respects may be functionally equivalent to DNA B. Analysis of a fragment amplified by using primers V4539 and V4540 (described below) showed that sequences adjacent to the *Spe*I site were contiguous, confirming the circular nature of DNA β. DNA β is comparable in size to def17 (1,305 nt), and DNA β nucleotides 1–977 show 95% identity to def17 nucleotides 57–1,035. The remaining def17 nucleotides derive from DNA A (8). The data demonstrate that *A. conyzoides* plants exhibiting yellow vein disease contain a unique circular DNA and suggest that def17 has been produced by recombination between this DNA and AYVV DNA A.

The Aetiology of *A. conyzoides* Yellow Vein Disease. It has been established that AYVV DNA A is associated with infected *A. conyzoides* plants, but attempts to isolate a component equivalent to DNA B have been unsuccessful (5, 8). When introduced into plants by agroinoculation, DNA A produces a symptomatic infection in *N. benthamiana* and can also systemically infect *A. conyzoides*, although plants remain asymptomatic (5, 10). To observe the effect of DNA β on infectivity in *N. benthamiana*, groups of six seedlings were agroinoculated with combinations of DNA A, DNA β, and DNA 1 by using clones pHNBin419, pBin-AYVVβ, and pBin-AYVV1/7. All plants co-inoculated with DNA A and DNA β produced systemic symptoms of severe downward leaf curl and chlorotic mottling, with mild upward leaf roll also developing on older infected leaves. The leaf curl symptom is reminiscent of infection with DNA A and def17, but contrasts with the severe upward leaf roll associated with DNA A alone in this host (8). However, unlike co-infection with def17 (8), DNA β did not adversely affect the accumulation of DNA A in systemically infected tissues (Fig. 2). Although DNA 1 appeared to have little effect on the accumulation of DNA A in this host (10), the level of DNA β was significantly reduced in the presence of this nanovirus-like component (Fig. 2). This may be because of competition between these DNAs for encapsidation by DNA A-encoded coat protein (discussed below). Agroinoculation of DNA β (data not shown) or a combination of DNA 1 and DNA β (Fig. 2) failed to produce a systemic infection, demonstrating that DNA A alone is required for amplification of DNA β in plants.

To investigate a biological role for DNA β in *A. conyzoides*, seedlings were co-agroinoculated with DNA A and DNA β by using clones pHNBin419 and pBin-AYVVβ. Of five inoculated plants, one produced yellow vein symptoms typical of the disease 12 days p.i. When agroinoculation was repeated on 25 plants by using pBin-AYVVAβ, containing both DNAs on a single binary vector, six plants developed typical symptoms 10 days p.i. (one example is shown in Fig. 3), and five additional plants produced symptoms by 18 days p.i. The presence of both DNA A and DNA β in systemically infected tissues was confirmed by blot hybridization (Fig. 4, lanes 1 and 2). In two separate experiments, individual infected plants were used to transmit the disease to

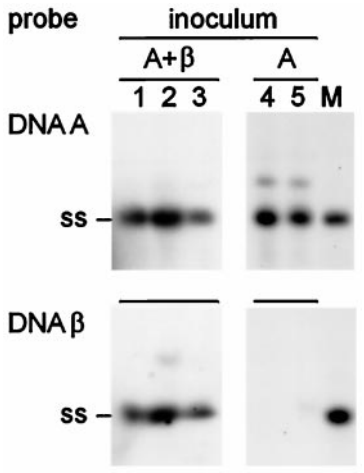

Fig. 4. Detection of viral DNAs in *A. conyzoides* plants. Samples (5 μg of nucleic acids) were extracted from individual plants infected either by agroinoculation with DNA A and DNA β (lanes 1 and 2; sampled 26 days p.i.) and DNA A alone (lane 4; sampled 42 days p.i.) or by whitefly transmission of the cloned progeny of DNA A and DNA β (lane 3; sampled 13 days after insect transmission) and DNA A alone (lane 5; sampled 23 days after insect transmission). A sample from a symptomatic plant (1/10 of the sample in lane 1) has been included as a marker (lane M) to allow comparison of the relative levels of DNA A in symptomatic and asymptomatic plants. Samples were fractionated by using agarose gels in TNE buffer, and blots were screened by using probes specific for DNA A (*Upper*) and DNA β (*Lower*). The position of single-stranded DNA (ss) is shown.

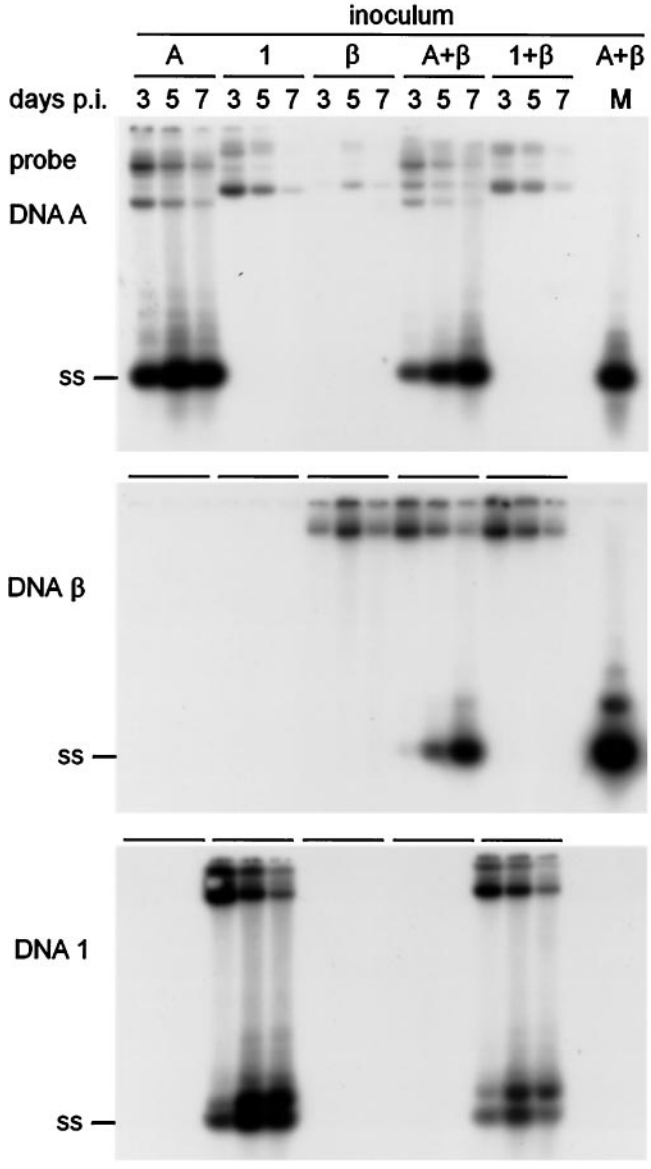

Fig. 5. Detection of viral DNAs in agroinoculated *N. benthamiana* leaf disks. Samples were extracted 3, 5, and 7 days p.i. from leaf disks agroinoculated with combinations of DNA A, DNA β, and DNA 1 as indicated. A nucleic acid sample from a symptomatic *N. benthamiana* plant infected by agroinoculation with DNA A and DNA β is in lane M. Equivalent amounts (5 μg) of nucleic acids were loaded in each lane. Samples were fractionated by using agarose gels in TNE buffer, and blots were screened by using probes specific for DNA A (*Top*), DNA β (*Middle*), and DNA 1 (*Bottom*). The position of single-stranded DNA (ss) is shown, and residual inoculum DNA is associated with the multiple bands at the top of each blot.

healthy *A. conyzoides* seedlings using *B. tabaci*. Severe yellow vein symptoms, indistinguishable from those associated with naturally infected plants from which the viral DNA was originally isolated, developed 13 days after recipient plants were exposed to viruliferous insects. The presence of both components in a symptomatic recipient plant was again verified by blot hybridization (Fig. 4, lane 3). Our data provide unequivocal proof that yellow vein disease of *A. conyzoides* is the result of co-infection by DNA A and DNA β.

Structural Features of DNA β. DNA A and DNA B of bipartite begomoviruses share a common region of $\approx$200 nucleotides, primarily located within the intergenic region, that contains cis-acting elements for replication and the control of gene expression. The nonanucleotide TAATATTAC, containing the nick site for the initiation of rolling circle replication (21, 22), occurs at the apex of a potential stem-loop structure located within the intergenic region of all geminiviruses. DNA β also contains the TAATATTAC motif and a stem-loop sequence between nucleotides 1,327–1,347/1–13. Interestingly, one recombination point in def17 corresponds to the Rep nick site in the DNA β motif, which has been shown for DNA A to be a recombinational hot-spot (21). Hence, it is likely that the recombinant was produced by a ligation error during DNA β rolling-circle replication. Sequences within begomovirus common regions, upstream of the stem-loop, contain the Rep binding site (23, 24). DNA A and DNA β do not share a common region, although DNA β clearly must possess sequences that are recognized by Rep to allow DNA A-mediated replication. The reiterated DNA A sequence GGTACTCA, which may participate in Rep binding (5), occurs between virion-sense nucleotides 2,617–2,624 and 2,652–2,659 and complementary-sense nucleotides 2,669–2,676. This sequence does not occur in DNA β, although the similar sequences GCTACGCA and GGTACACA, which are located upstream of the stem loop between virion-sense nucleotides 1,294–1,301 and complementary-sense nucleotides 1,263–1,270, respectively, may participate in replication. A region of DNA β between virion-sense nucleotides 704–972 is particularly A-rich (64%). Its function remains to be established but it may have originated from sequence duplications to satisfy size requirements for encapsidation and/or virus movement (discussed below). The coding capacity of DNA β is unknown, but two candidates for functional open reading frames (ORFs), encoding putative proteins of 13.8 kDa and 5.3 kDa, were predicted from the sequence by using the program TESTCODE (13) (Fig. 1*B*). Mutagenesis studies and transcript mapping are in progress to investigate gene expression from this component.

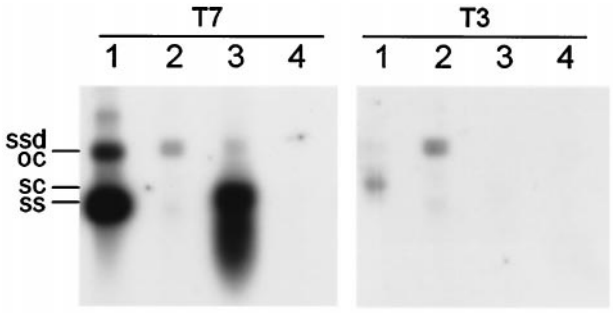

Fig. 6. Immunoprecipitation of DNA β. Nucleic acids were extracted either from symptomatic *N. benthamiana* plants that had been agroinoculated with DNA A and DNA β (lanes 1 and 2) or from virions that had been immunoprecipitated by using African cassava mosaic virus polyclonal antiserum (lanes 3 and 4). Duplicate samples were either untreated (lanes 1 and 3) or treated with mungbean nuclease (lanes 2 and 4) before fractionation on an agarose gel in TNE buffer. Blots were screened by using strand-specific probes synthesized *in vitro* from pBS-AYVVβ using the T7 or T3 promoter as indicated. The positions of single-stranded (ss), supercoiled (sc), open-circular (oc), and dimeric single-stranded (ssd) DNAs are shown.

DNA A and DNA β Are Mutually Dependent. Both DNA A and DNA 1 encode a Rep and can autonomously replicate (5, 10). We have investigated DNA β replication in the presence of these DNAs by using a *N. benthamiana* leaf disk assay (Fig. 5). Single-stranded forms of both DNA A and DNA 1, diagnostic of *de novo* replication, are readily detectable at 3 days p.i. DNA β was unable to autonomously replicate, but was replicated *in trans* by DNA A, although not by DNA 1. This result confirms that DNA 1 plays no role in the amplification of DNA β in plants.

To investigate DNA β encapsidation, *N. benthamiana* plants were infected with cloned DNA A and DNA β, and virions were immunoprecipitated from protein extracts by using a polyclonal antiserum known to cross-react with AYVV coat protein (5). Viral DNA forms associated with the immunoprecipitated complexes were characterized by blot hybridization (Fig. 6). A strand-specific probe synthesized from the T7 promoter of pBS-AYVVβ readily detected monomeric single-stranded DNA (ssDNA) as well as a less abundant form that is probably dimeric ssDNA in infected *N. benthamiana* extracts (Fig. 6, lane 1). The level of double-stranded DNA in both *N. benthamiana* and *A. conyzoides* tissues is variable and is often extremely low in comparison with ssDNA (for example, see Figs. 2 and 4). The scDNA is obscured by the ssDNA in this lane but is visible when using a probe synthesized from the T3 promoter that is unable to detect ssDNA. Treatment with mungbean nuclease removed the ssDNA forms and nicked the scDNA to produce open-circular DNA that comigrates with the dimeric ssDNA (Fig. 6, lane 2). The probe synthesized from the T7 promoter detected both monomeric and dimeric ssDNA forms in immunoprecipitated complexes (Fig. 6, lane 3). The single-stranded nature of these DNAs was confirmed by their susceptibility to mungbean nuclease digestion (Fig. 6, lane 4) and by the inability to detect them by using the probe synthesized from the T3 promoter (Fig. 6, lanes 3 and 4). An identical result was obtained by using an infected *A. conyzoides* extract (data not shown). The virion-sense (encapsidated) strand of DNA β, shown in Fig. 1, was deduced from the ability to detect ssDNA using only the probe synthesized from the T7 promoter together with a knowledge of the orientation of the DNA β insert in pBS-AYVVβ. The data demonstrate that the single-stranded form of DNA β is associated with AYVV DNA A-encoded coat protein, most likely encapsidated in AYVV virions. Encapsidation is required for whitefly transmission and may be necessary for systemic movement of the viral DNA, as has been suggested for other monopartite begomoviruses (25, 26). The fact that DNA β is approximately half the size of DNA A is consistent with a stringent size requirement for encapsidation, as has been suggested for DNA 1 and DNA A recombinants such as def17 (8–10). Alternatively, size diversity may be restricted by constraints imposed during systemic movement, as has been observed for begomovirus genomic components (27, 28).

Dot-blot hybridization was used to identify asymptomatic *A. conyzoides* plants that were systemically infected by agroinoculation with DNA A alone. Two such plants were chosen, and samples from 10 individual leaves from each plant, together with the equivalent leaves from a symptomatic plant infected with DNA A and DNA β, were assayed for viral DNA levels by dot-blotting. DNA A detection in the asymptomatic plants was sporadic (positive in only 3/20 samples, compared with 8/8 samples from symptomatic leaves), and when present was estimated to be $<5\%$ of that in equivalent symptomatic leaves (Fig. 4, lane 4; data not shown), indicating that DNA β significantly enhances the accumulation of DNA A in *A. conyzoides*. Nonetheless, whitefly transmission of DNA A alone from asymptomatic plants was achieved (Fig. 4, lane 5), although once again its occurrence in recipient plants remained sporadic and low, and plants remained asymptomatic.

DNA β shows little homology with DNA A, it is replicated *in trans* by DNA A, and it is most probably encapsidated by DNA A-encoded coat protein. Furthermore, we have demonstrated that it is not essential for DNA A systemic movement and whitefly transmission. In many respects, therefore, DNA β exhibits features generally associated with satellite RNAs (reviewed in ref. 29). Although some RNA satellites are known to exacerbate mild symptoms caused by the helper virus—for example, cucumber mosaic virus satellite RNAs modulate symptom development in a host-specific manner—the majority attenuate symptoms, and many reduce the helper virus titer. We have shown that DNA β is biologically functional, being required for amplification of DNA A to high levels and for the efficient systemic spread of infection throughout *A. conyzoides*, and that it is essential for the induction of symptoms in this natural host. Therefore, yellow vein disease of *A. conyzoides* is caused by a previously unrecognized virus complex comprising DNA A and DNA β, and these properties, coupled with the fact that it is a circular DNA component, make DNA β a unique subviral agent. In contrast to DNA β, DNA 1 is not required for maintenance of the disease. It can autonomously replicate but depends on helper virus (DNA A) for encapsidation and systemic movement (9, 10). In these respects, DNA 1 resembles beet western yellows virus ST9a RNA (30). Therefore, in view of its characteristics, we propose that DNA 1 represents a unique satellite-like DNA that has evolved from a nanovirus DNA by size adaptation to allow encapsidation and whitefly transmission. So far, no other unique nanovirus-like DNA component has been isolated from infected *A. conyzoides*.

At the present time we can only speculate about the role of DNA β in maintenance of the disease. DNA β may have a direct effect on DNA A replication. Alternatively, it may play a more indirect role, either by providing a cellular environment that is appropriate for viral DNA replication, as has been proposed for other geminiviruses (31–34), or by facilitating viral DNA movement throughout the plant, thereby amplifying DNA A levels (35, 36). Another possibility is that DNA β may encode a pathogenicity determinant that serves to counter a host defense mechanism by suppression of gene silencing (37), thereby allowing efficient systemic infection of the plant. DNA β is unrelated to any other known geminivirus or nanovirus component, and its origins remain obscure. In view of its size, it is possible that it evolved from a nanovirus component, as proposed for DNA 1. Thus, DNA β may have become associated with DNA A during co-infection by a begomovirus and a

nanovirus, making DNA B (if present) functionally redundant. A more intriguing possibility that cannot yet be ruled out is that DNA A and DNA β co-existed before the emergence of bipartite begomoviruses, and that DNA B usurped DNA β by a similar mechanism.

A database search revealed that DNA β shows homology (60% identity over a 350-nt sequence) to a satellite DNA (European Molecular Biology Laboratory Nucleotide Sequence Database accession no. U74627) associated with TLCV (38). Comparison of their entire sequences produced the approximate alignment shown in Fig. 1*A*. PCR-amplification from scDNA (sample 1) using abutting primers V4539 and V4540, located within this region of similarity (Fig. 1*A*), produced a 1.3 -kbp fragment corresponding to full-length DNA β. The sequences of two clones derived from this fragment differed from each other by $<1\%$ and showed 95% identity with the pBS-AYVVβ insert cloned from scDNA sample 2. PCR amplification using these primers failed to produce a fragment corresponding in size to the TLCV satellite DNA (682 nt), suggesting that a similar DNA does not occur, or is present in extremely low amounts, in infected *A. conyzoides.* Interestingly, TLCV is a presumed monopartite begomovirus that is most closely related to AYVV (5). We suggest that the satellite DNA isolated from TLCV-infected tomato is in fact a defective DNA β that has been retained in the virus population. TLCV DNA A alone can infect tomato, *N. tabacum*, and *Datura stramonium* (4), indicating that a functional DNA β is not essential, at least in these solanaceous species. Nonetheless, our data strongly suggest that TLCV has at some time been associated with a DNA β homologue, and it may be that such a component is still required for maintenance of the virus in alternative reservoir hosts. That such virus complexes are widespread and diverse is supported by our observations that distinct DNA β and DNA 1 homologues are associated with monopartite begomovirus infections of *A. conyzoides* and cotton in Pakistan and hollyhock in Egypt (refs. 9 and 39; R.W.B., P.G.M., K.S., and J.S., unpublished work).

We thank Professor J. W. Davies for his encouragement throughout this work and for critical discussion of the manuscript. This work was funded by the Biotechnology and Biological Sciences Research Council. Viruses were held and manipulated with the authority of the Ministry of Agriculture, Fisheries and Food under the Plant Health (Great Britain) Order 1993, license PHL 11/2300 (6/1998).

1. Hanley-Bowdoin, L., Settlage, S. B., Orozco, B. M., Nagar, S. & Robertson, D. (1999) *Crit. Rev. Plant Sci.* **18,** 71–106.
2. Kheyr-Pour, A., Bendahmane, M., Matzeit, V., Accotto, G-P., Crespi, S. & Gronenborn, B. (1991) *Nucleic Acids Res.* **19,** 6763–6769.
3. Navot, N., Pichersky, E., Zeidan, M., Zamir, D. & Czosnek, H. (1991) *Virology* **185,** 151–161.
4. Dry, I. B., Rigden, J. E., Krake, L. R., Mullineaux, P. M. & Rezaian, M. A. (1993) *J. Gen. Virol.* **74,** 147–151.
5. Tan, P. H. N., Wong, S. M., Wu, M., Bedford, I. D., Saunders, K. & Stanley, J. (1995) *J. Gen. Virol.* **76,** 2915–2922.
6. Briddon, R. W., Mansoor, S., Bedford, I. D., Pinner, M. S. & Markham, P. G. (2000) *Virus Genes* **20,** 17–24.
7. Wong, S. M., Swanson, M. M. & Harrison, B. D. (1993) *Plant Pathol.* **42,** 137–139.
8. Stanley, J., Saunders, K., Pinner, M. S. & Wong, S. M. (1997) *Virology* **239,** 87–96.
9. Mansoor, S., Khan, S. H., Bashir, A., Saeed, M., Zafar, Y., Malik, K. A., Briddon, R., Stanley, J. & Markham, P. G. (1999) *Virology* **259,** 190–199.
10. Saunders, K. & Stanley, J. (1999) *Virology* **264,** 142–152.
11. Timchenko, T., de Kouchkovsky, F., Katul, L., David, C., Vetten, H. J. & Gronenborn, B. (1999) *J. Virol.* **73,** 10173–10182.
12. Bedford, I. D., Briddon, R. W., Brown, J. K., Rosell, R. C. & Markham, P. G. (1994) *Ann. Appl. Biol.* **125,** 311–325.
13. Devereaux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395.
14. Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M. & Schell, J. (1983) *EMBO J.* **2,** 2143–2150.
15. Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. & Schilperoort, R. A. (1983) *Nature (London)* **303,** 179–180.
16. Klinkenberg, F. A., Ellwood, S. & Stanley, J. (1989) *J. Gen. Virol.* **70,** 1837–1844.
17. Covey, S. N. & Hull. R. (1981) *Virology* **111,** 463–474.
18. Feinberg, A. P. & Vogelstein B. (1983) *Anal. Biochem.* **132,** 6–13.
19. Maule, A. J., Hull, R. & Donson, J. (1983) *J. Virol. Methods* **6,** 215–224.
20. Stanley, J., Townsend, R. & Curson, S. J. (1985) *J. Gen. Virol.* **66,** 1055–1061.
21. Stanley, J. (1995) *Virology* **206,** 707–712.
22. Laufs, J., Traut, W., Heyraud, F., Matzeit, V., Rogers, S. G., Schell, J. & Gronenborn, B. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3879–3883.
23. Fontes, E. P. B., Gladfelter, H. J., Schaffer, R. L., Petty, I. T. D. & Hanley-Bowdoin, L. (1994) *Plant Cell* **6,** 405–416.
24. Argüello-Astorga, G. R., Guevara-González, R. G., Herrera-Estrella, L. R. & Rivera-Bustamante, R. F. (1994) *Virology* **203,** 90–100.
25. Rigden, J. E., Dry, I. B., Mullineaux, P. M. & Rezaian, M. A. (1993) *Virology* **193,** 1001–1005.
26. Wartig, L., Kheyr-Pour, A., Noris, E., de Kouchkovsky, F., Jouanneau, F., Gronenborn, B. & Jupin, I. (1997) *Virology* **228,** 132–140.
27. Etessami, P., Watts, J. & Stanley, J. (1989) *J. Gen. Virol.* **70,** 277–289.
28. Rojas, M. R., Noueiry, A. O., Lucas, W. J. & Gilbertson, R. L. (1998) *Cell* **95,** 105–113.
29. Liu, Y. Y. & Cooper, J. I. (1994) *Rev. Plant Pathol.* **73,** 371–387.
30. Passmore, B. K., Sanger, M., Chin, L.-S., Falk, B. W. & Bruening, G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10168–10172.
31. Xie, Q., Suárez-López, P. & Gutiérrez, C. (1995) *EMBO J.* **14,** 4073–4082.
32. Nagar, S., Pedersen, T. J., Carrick, K. M., Hanley-Bowdoin, L. & Robertson, D. (1995) *Plant Cell* **7,** 705–719.
33. Latham, J. R., Saunders, K., Pinner, M. S. & Stanley, J. (1997) *Plant J.* **11,** 1273–1283.
34. Liu, L., Saunders, K., Thomas, C. L., Davies, J. W. & Stanley, J. (1999) *Virology* **256,** 270–279.
35. Noueiry, A. O., Lucas, W. J. & Gilbertson, R. L. (1994) *Cell* **76,** 925–932.
36. Sanderfoot, A. A. & Lazarowitz, S. G. (1995) *Plant Cell* **7,** 1185–1194.
37. Voinnet, O., Pinto, Y. M. & Baulcombe, D. C. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 14147–14152.
38. Dry, I. B., Krake, L. R., Rigden, J. E. & Rezaian, M. A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 7088–7093.
39. Mansoor, S., Khan, S. H., Hussain, M., Zafar, Y., Pinner, M. S., Briddon, R. W., Stanley, J. & Markham, P. G. (2000) *Plant Dis.* **84,** 101.