Spiroplasma citri Virus SpV1-78, A Non-Lytic Rod-Shaped Virus with Single-Stranded, Circular DNA: Presence of Viral Sequences in the Spiroplasma Genome

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ABSTRACT. *Spiroplasma citri* virus SpV1 is a rod-shaped virus with a 8.4 kilobase single-stranded, circular DNA. SpV1 is non-lytic, and morphogenesis of the virion occurs while the viral DNA passes through the spiroplasma cell membrane. The virus seems to be intimately associated with *S. citri*, since many of the *S. citri* strains isolated over the years produce SpV1-type virus.

SpV1 type virus, SpV1-78, from a Turkish isolate of *S. citri* has been studied. The double-stranded replicative form (RF) of the virus has been purified. Using nick-translated SpV1 RF as a probe, we have detected the presence of viral sequences in the chromosomal DNA of *Spiroplasma citri* strain R8A2 HP, a strain which does not spontaneously produce SpV1 virions. Hybridization of SpV1 RF DNA restriction fragments with chromosomal DNA of *S. citri* used as the probe showed that most of the viral sequences are present in the host cell chromosome. SpV1 seems to be the first single-stranded DNA virus for which integration of viral sequences into the host cell chromosome occurs.
Index words. Spiroplasma virus, SpV1, replicative form, Southern hybridization.

SpV1-type viruses of spiroplasmas are naked, rod-shaped or filamentous viruses. They were first discovered in *Spiroplasma citri* cultures (3). Release of virions by the spiroplasma cell is non-lytic and seems to follow the pattern characteristic of filamentous coliphage f1-type viruses in which assembly of the viral DNA with the capsid proteins occurs while the DNA moves and is extruded through the cell envelope. Growth of SpV1-infected spiroplasmas is reduced and this results in the production of turbid plaques (1, 7), in contrast to the clear plaques which are obtained with lytic viruses such as spiroplasma virus SpV4 (10). SpV1 type virions are frequently found in primary cultures of *S. citri*. However SpV1 viruses are not restricted to the citrus stubborn agent. They infect many other *Spiroplasma* species, such as *S. melliferum* (6), *S. kunkelii*, *Spiroplasma* sp. strain 277F, *S. mirum* and the Drosophila spiroplasma (2).

S. citri virus SpV1-aa contains single stranded, circular DNA; the double-stranded DNA replicative form (RF) of the virus occurs as covalently closed circular (ccc) supercoiled molecules and open circular (oc) relaxed molecules of 8.5 kbp (4).

We have obtained, as shown here, similar results with another SpV1 virus of *S. citri*. The virus, SpV1-78, was present in large amounts in the primary culture and early passages of a *S. citri* strain isolated from stubborn-affected sweet orange leaves (sample 78) in the Adana region of Turkey in 1981. The main purpose of this paper is to report the presence of SpV1-78 DNA sequences in the genome of all *S. citri* isolates tested as well as in the genome of *S. phoeniceum*.

MATERIALS AND METHODS

Virus SpV1-78 was propagated in *S. citri* strain R8A2HP, an advanced passage of strain R8A2 (ATCC 27,556). Culture of the spiroplasma was in BSR medium (15). The virions were purified by polyethyleneglycol precipitation, Triton X-100 treatment, sedimentation through a sucrose cushion, CsCl equilibrium centrifugation, and dialysis of the CsCl fraction against 10 mM Tris-HCl, pH 7.8. Proteins from purified SpV1-78 virions were prepared and analyzed by polyacrylamide gel electrophoresis as described in Renaudin *et al.* (10). Total DNAs, respectively, from

SpV1-78 infected and uninfected cells of *S. citri* strain R8A2HP, as well as SpV1-78 viral DNA, were extracted and analyzed by agarose gel electrophoresis according to Renaudin *et al.* (10). SpV1-78 RF was extracted by the cleared lysate technique using SDS-lysis followed by NaCl clarification (6), and was further purified by equilibrium centrifugation in ethidium bromide-CsCl gradients. Agarose gel electrophoresis of DNA, transfer to nitrocellulose sheets and hybridization, was according to Southern (13) as described in Pascarel-Devilder *et al.* (9). Treatment of DNA by nuclease S1 was according to Renaudin *et al.* (10). Hydrolysis of DNA by restriction endonucleases was as recommended by the supplier (Boehringer). The size of DNA fragments was determined by their electrophoretic mobility in comparison with *Hind*III or *Pst*I digested bacteriohage λ DNA used as a marker. The α ^{32}P-labelled DNA probes were prepared with the Amersham PB10205 nick translation kit (11) according to the supplier's protocole. Electron microscopy techniques for virions and DNA were as described in Renaudin *et al.* (10).

RESULTS

The *S. citri* clone used to multiply SpV1-78 is strain Morocco R8A2HP (HP standing for High Passage). Strain R8A2HP has been selected for this work because: 1) it does not produce spontaneous SpV1 virions, and 2) it is sensitive to infection by SpV1-78. Upon infection with SpV1-78, R8A2HP cells produced large amounts of rod-shaped virions 2 to 8 hrs postinfection. Nascent virions were seen extruding from the spiroplasma cells, but no virions were ever observed within the cells. Free virions were also present and could be recovered from the supernatant of a centrifuged culture. Infection of a R8A2HP spiroplasma lawn produced only turbid plaques. These various observations indicate that release of virions from infected cells is non-lytic and occurs probably by the mechanism described for f1 type coliphages (14).

Free SpV1-78 virions were rod-shaped and measured 220-250 nm x 15 nm. Polyacrylamide gel electrophoresis showed that the major capsid protein had a molecular weight of less than 10,000. The major capsid proteins of coliphage f1 has also a low molecular weight (5240). The viral DNA was fully hydrolyzed by single-stranded DNA-specific nuclease S1 and could only be spread in denaturating conditions (30% formamide) for electron microscopical observations. These showed the molecule to be circular. SpV1-78 DNA is therefore single-stranded and circular. It gave only one band on 0.9% agarose gels (fig. 1, track 1).

In the cleared lysate of SpV1-78-infected R8A2HP *S. citri* cells, three extrachromosomal double-stranded (ds) viral DNA molecules could be detected (fig. 1, track 6): the open circular (oc) relaxed RFII, the covalently closed circular (ccc) supercoiled RFI and the linear (l) RF molecule. The cleared lysate of uninfected R8A2HP *S. citri* cells only showed chromosomal (c) DNA (fig. 1, track 5), also present in the cleared lysate of infected cells. Comparison of tracks 1 and 6 of fig. 1 shows that the single-stranded (ss) viral DNA had approximately the same electrophoretic mobility as ccc RFI. Comparison of tracks 5 and 6 clearly illustrates that the extrachromosomal virus-specific DNAs (ssDNA, RFI (ccc), RFII (oc), linear RF (l)) had an electrophoretic mobility greater than the chromosomal (c) DNA of the spiroplasma host, in other words, that chromosomal host DNA and viral extrachromosomal DNA were clearly separated on the gels.

When purified RF DNA was treated with restriction enzyme EcoRI, a large and a small fragment were obtained (fig. 1, track 2). The same two fragments were produced when the cleared lysate (fig. 1, track

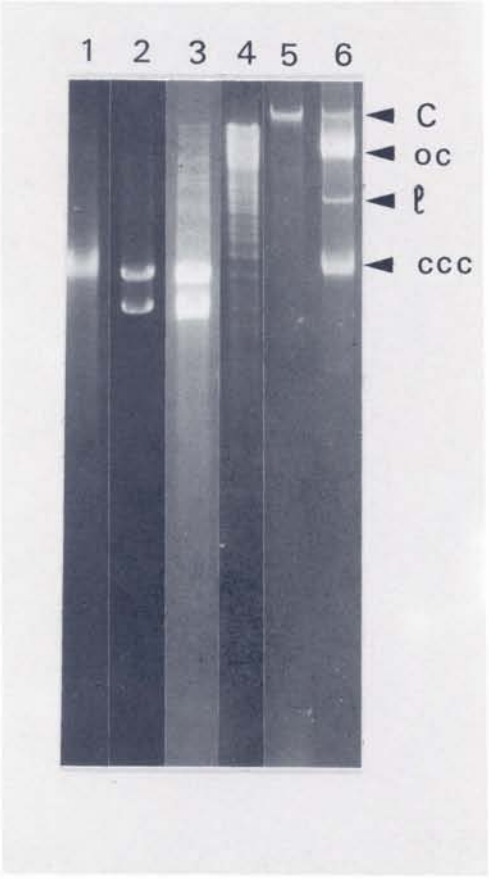

Fig. 1. Electrophoresis of SpV1-78 single stranded, circular DNA (track 1), *Eco*RI-treated SpV1-78 RF (track 2, *Eco*RI treated total DNA from SpV1-78-infected *S. citri* R8A2HP cells (track 3), *Eco*RI-treated total DNA from uninfected R8A2HP cells (track 4), DNA of cleared lysate from uninfected RBA2HP cells (track 5), DNA of cleared lysate from SpV1-78-infected R8A2HP cells (track 6) on 0.9 % agarose gel.

6) from infected R8A2 HP cells was submitted to *Eco*RI hydrolysis (fig. 1, track 3). The chromosomal DNA yielded a large number of *Eco*RI fragments (fig. 1, tracks 3 and 4).

Figure 2A (track 2) shows that the total DNA of uninfected cells of *S. citri* strain R8A2HP did not contain any extrachromosomal SpV1-specific DNA. Only the chromosomal host DNA band was detected. Had SpV1-specific DNA been present, it would have been revealed as the extra-chromosomal bands seen on fig. 1, track 6 (oc, l, ccc), the positions of which have also been indicated on fig. 2A. The absence of SpV1-specific DNA is also illustrated by track 3 of fig. 2A where the total DNA of *S. citri* was restricted by *Eco*RI before electrophoresis. Track 3 (like track 4 of fig. 1) shows only the *Eco*RI fragments given by the restricted host DNA; the two *Eco*RI fragments, characteristic of *Eco*RI restricted SpV1 RF are absent; these fragments are shown on fig. 2A, track 1.

On the basis of these experiments, it is clear that *S. citri* strain R8A2HP used in this work as host of virus SpV1-78 does not contain, prior to its infection by SpV1-78, any free SpV1 extra-chromosomal DNA, in agreement with the fact that this strain does not produce SpV1 virions spontaneously. Therefore, the strong Southern hybridization band observed on fig. 2B, track 2, between the total DNA of *S. citri* or strain R8A2 HP and the ^{32}P labelled SpV1-78 RF used as the probe, can only be due to the presence of SpV1-related DNA sequences in the chromosomal DNA of the uninfected *S. citri* strain R8A2HP.

This conclusion is strengthened by the hybridization pattern of fig. 2B, track 3, which reveals hybridization of the SpV1-78 RF probe with several of the *Eco*RI restriction fragments obtained from total R8A2HP DNA. One of these bands shows a particularly strong hybridization. It has the same electrophoretic mobility as the small *Eco*RI restriction fragment of SpV1-78 RF, shown by comparison of track 3 and track 1. The latter shows the hybridization pattern of the RF probe with *Eco*RI restricted SpV1-78 RF. The upper band of track 1 is contaminating chromosomal DNA of R8A2HP and, as expected, hybridizes with the probe.

Chromosomal DNA from 19 other *S. citri* strains from citrus, periwinkles or leafhoppers also hybridized with the SpV1-78 RF probe. Five of these strains were similar to strain R8A2HP in that they did not produce

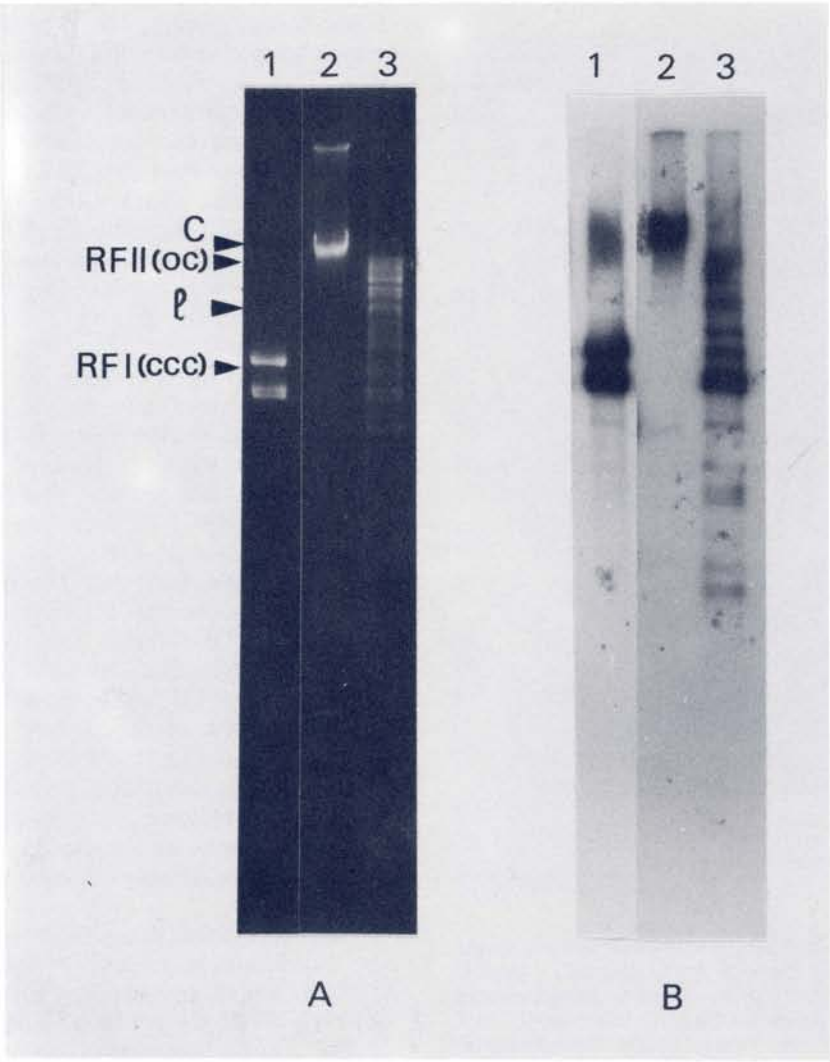

Fig. 2. A) Electrophoresis of *Eco*RI-treated SpV1-78 RF (track 1), total DNA from *S. citri* R8A2HP cells (track 2), *Eco*RI-treated total DNA from R8A2HP cells (track 3) on 1% agarose gel. B) Southern blot hybridization of the DNAs of A with ^{32}P-labelled SpV1-78 RF as the probe.

SpV1 virus spontaneously, but the other 14 strains gave SpV1 plaques on R8A2HP lawns.

The SpV1-78 RF probe hybridized also with chromosomal DNA of *S. phoeniceum* (12), a species having 60 per cent DNA homology with *S. citri* but not with that of *S. apis* (8) which has no relationships with *S. citri*.

Finally, the DNA of *S. citri* strain R8A2HP, free of extrachromosomal viral DNAs, was used as a probe to detect homologous sequences in SpV1-78 RF DNA (fig. 3). Such sequences were indeed detected by Southern blot hybridization not only in unrestricted RF DNA but also in the restriction fragments obtained when the RF was treated with *Eco*RI (fig. 3B, track 2) or *Taq*I (fig. 3B, track 3).

DISCUSSION

The experiments reported here clearly show the presence of SpV1-78-related DNA sequences in the chromosomal DNA of *S. citri* strain R8A2HP as well as in all 19 other

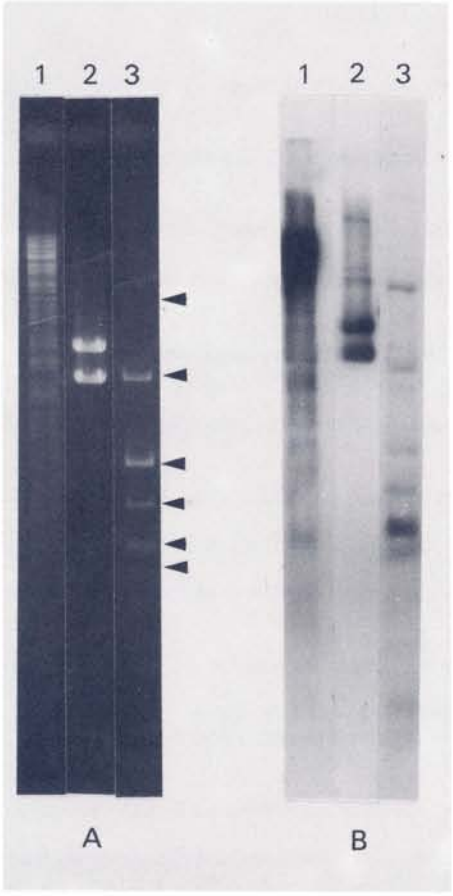

Fig. 3. A) Electrophoresis of *Eco*RI-treated total DNA of *S. citri* R8A2HP (track 1), *Eco*RI-treated SpV1I-78 RF (track 2), *Taq*I-treated SpV1-78RF (track 3) on 1.2% agarose. B) Southern blot hybridization of the DNAs of A with ^{32}P-labelled R8A2HP DNA as the probe. The arrows indicate *Taq*I restriction fragments.

strains tested. It is noteworthy that these viral sequences occur in *S. citri* strains, such as R8A2HP, which do not produce SpV1-type virus spontaneously, and prior to infection of such strains by SpV1-78 viruses. SpV1 seems to be the first single-stranded circular DNA virus for which integration of viral sequences into the host cell chromosome has been reported. At this stage it is not yet known if an entire SpV1 genome is integrated in the chromosomal DNA. The presence of an entire genome would explain the hybridization results, and also the spontaneous or sporadic production of SpV1 viruses by spiroplasmas. If only parts of the viral genome were integrated, no virions would be produced, but hybridization with viral DNA probes would be observed.

Dickinson and Townsend (5) have shown that *S. citri* colonies derived from cells that survived infection by SpV1-aa, a SpV1-type virus, were resistant to infection by that virus. Although the genome of *S. citri* strain R8A2HP contains sequences homologous to SpV1-78 sequences, this strain is not resistant to infection by SpV1-78, perhaps, because the viral sequences present in the genome of strain R8A2HP are similar, but not identical to those of SpV1-78. It is also possible that the viral sequences present in the genome of strain R8A2HP represent less than a full viral genome and lack what could be an immunity region. In the case of the short-tailed, double-stranded DNA virus SpV3-ai of *S. citri*, it has been shown that all strains of *S. citri* examined contained a deleted form of SpV3-ai DNA integrated as a cryptic prophage (5). The presence of these viral sequences did not prevent *S. citri* from becoming infected by SpV3-ai. Moreover, SpV3-ai was able to lysogenize the spiroplasma.

We have just cloned the SpV1-78 RF in *E. coli*. Restriction fragments of the cloned RF are now used as probes to investigate in more detail the precise nature of the SpV1 DNA sequences integrated in *S. citri* and other spiroplasmas.

Note added in proof. To exclude the possibility that the purified SpV1-RF could have been contaminated by chromosomal DNA, the Southern hybridization experiments have now been repeated with probes consisting of SpV1-RF-fragments cloned in *E. coli*. The hybridization results were essentially the same than those obtained with the uncloned RF. Also sequencing work has revealed more than 95 per cent homology between cloned SpV1-DNA fragments and cloned chromosomal DNA fragments.

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