Molecular Analysis of *Campoletis sonorensis* Virus DNA in the Lepidopteran Host *Heliothis virescens*

By **DAVID A. THEILMANN** and **MAX D. SUMMERS**

*Department of Entomology, Texas A & M University and Texas Agricultural Experimental Station, College Station, Texas 77843-2475, U.S.A.*

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**SUMMARY**

Nucleic acid hybridization techniques were used to analyse the fate of *Campoletis sonorensis* virus (CsV) DNAs in naturally parasitized and virus-injected *Heliothis virescens* larvae. Viral DNA persisted in injected *H. virescens* larvae from 0 to 10 days post-injection but no increase in the amount of viral DNA could be detected. Similarly, no increase in the amount of viral DNA was detected in naturally parasitized *H. virescens* larvae before the development of the *C. sonorensis* pupae. However, a dramatic increase of viral DNA was detected in pharate and newly emerged (0 to 48 h) adult *C. sonorensis* wasps. The results from these *in vivo* molecular analyses suggest that CsV replication does not occur in naturally parasitized or virus-injected *H. virescens* and that viral replication may be restricted to tissues of *C. sonorensis* wasps.

**INTRODUCTION**

Polydnaviruses are observed in the reproductive tracts of many species of parasitic wasps in the families Ichneumonidae and Braconidae (Stoltz & Vinson, 1979a). The most thoroughly studied polydnavirus is the *Campoletis sonorensis* virus (CsV) which replicates in the calyx epithelial cells of the female wasp, *C. sonorensis* (Ichneumonidae). CsV buds from the surface of the calyx cells into the oviduct lumen where it is the major component of the 'calyx fluid' (Norton et al., 1975). During oviposition by the female wasp the calyx fluid is injected along with the wasp egg into the haemocoel of its lepidopteran host, *Heliothis virescens*. Injection of calyx fluid or purified virus into *H. virescens* larvae has been shown to prevent encapsulation of the *C. sonorensis* eggs by the host's haemocytes and will also cause a marked retardation of larval growth and development (Edson et al., 1981; Vinson et al., 1979). A recent study has also shown that injection of CsV will also cause a reduction in the plasmatocyte population in *H. virescens* (Davies et al., 1986). The results of all experiments to date suggest that CsV not only plays an important role in the protection of the developing *C. sonorensis* endoparasite from the cellular defences of the host *H. virescens* but can also have a profound effect on the overall metabolism and growth of the parasitized larvae.

Electron microscopic observation of CsV after oviposition in *H. virescens* detected the virus in the nuclei of haemocytes, tracheal epithelium, muscle and fat body cells, but no replication of virus was observed in any of these tissues (Stoltz & Vinson, 1979b). Although replication is not observed, transcription from a number of the 28 CsV superhelical (SH) DNAs occurs in naturally parasitized *H. virescens* and in larvae injected with purified virus (Blissard et al., 1986a, b; Fleming et al., 1983). These previous studies have not identified the mechanism by which CsV is transmitted from one generation of wasps to the next. A possible route of virus transmission is by the developing endoparasite acquiring CsV *per os* from virus-containing haemolymph or tissues of the lepidopteran host. Fleming & Summers (1986) demonstrated that both male and female wasps contain DNA sequences homologous to viral DNA and that some of the viral sequences may be integrated into the wasp genome. This suggests that CsV could be transmitted vertically through the germline to each successive generation.
The purpose of this study was to analyse CsV replication in *H. virescens* tissues using sensitive nucleic acid hybridization techniques. Since no CsV permissive tissue culture system exists, all experiments were performed *in vivo*. Our results show that in *H. virescens* larvae injected with calyx fluid (from which *C. sonorensis* eggs had been removed), CsV DNA remained relatively constant from 0 to 10 days post-injection. Similar results were obtained in naturally parasitized *H. virescens* larvae. However, a dramatic increase in the amount of CsV DNA was detected in pharate and newly emerged *C. sonorensis* adults. These results suggest that CsV does not replicate in *H. virescens* larvae and that CsV replication may be restricted to the tissues of *C. sonorensis*.

**METHODS**

*Insect cultures.* *C. sonorensis* and its host *H. virescens* were reared at 25 °C on a 14:10 h (light:dark) photoperiod as described by Krell et al. (1982). Third instar *H. virescens* larvae were parasitized by mated *C. sonorensis* females (Krell et al., 1982).

*Virus purification and viral DNA isolation.* CsV was purified from the oviducts of *C. sonorensis* females by the method of Krell et al. (1982). DNA was isolated from the purified virions as described by Fleming et al. (1983).

*Nucleic acid extraction.* For those experiments involving injection of *H. virescens* larvae with calyx fluid, *Autographa californica* nuclear polyhedrosis virus (AcNPV) or phosphate-buffered saline (PBS), three groups of four larvae were selected at the designated times post-injection (except at 10 days post-injection when only a single group of four larvae was analysed) and each group was separately homogenized using a sterile Dounce homogenizer. Total DNA was extracted by the procedure of Fuchs & Green (1979) as modified by Fleming et al. (1983). RNA was removed from the DNA samples by RNase A digestion (10 μg/ml) at 37 °C for 1 h followed by one extraction with phenol:chloroform:isoamyl alcohol (25:24:1). The purified DNAs were resuspended in 400 μl H2O and stored at 4 °C.

For analysis of CsV DNA in naturally parasitized host larvae, 50 *H. virescens* larvae and/or *C. sonorensis* pupae or *C. sonorensis* adults were randomly selected at the designated times post-parasitization, homogenized with a Polytron (Brinkmann Instruments) and the DNA was extracted using the method described above. Since the *C. sonorensis* larvae do not all pupate at the same time after parasitization, some of the samples of 50 insects consisted of mixtures of parasitized *H. virescens* larvae and *C. sonorensis* pupae. A *C. sonorensis* pupa contains no identifiable *H. virescens* tissue. The proportion of parasitized *H. virescens* larvae and *C. sonorensis* pupae in each sample is indicated in the appropriate figures. All DNA samples from the groups of 50 larvae were resuspended in 5 ml H2O and stored at 4 °C. To monitor the efficiency of DNA recovery, pUC8 plasmid DNA was added to most homogenates as an internal standard. Efficient recovery of pUC8 DNA was analysed for each sample by dot blot hybridization. If total DNA recovery was below normal, digest samples were normalized relative to pUC8 recovery.

*Southern blot and dot blot hybridizations.* To determine whether specific viral sequences were present, purified DNAs from homogenized insects were initially digested with the restriction endonucleases EcoRI or PstI. For CsV DNA samples this prevents the occurrence of duplicate signals from the relaxed circular and superhelical DNAs (Krell et al., 1982). The digested DNAs (maximum 15 μg/lane) were separated by electrophoresis in 1% agarose gels (Maniatis et al., 1982). The gels were unidirectionally transferred to nitrocellulose (Smith & Summers, 1980). The digested DNAs (maximum 15 μg/lane) were separated by electrophoresis in 1% agarose gels (Maniatis et al., 1982). The gels were unidirectionally transferred to nitrocellulose (Smith & Summers, 1980) and hybridized to specific 32P-labelled nick-translated probes (108 to 2 x 108 d.p.m./gg; Rigby et al., 1977). All hybridizations were performed in 50% formamide at 43 °C using conditions described elsewhere (Fleming et al., 1983). Hybridized blots were exposed to Kodak X-Omat XAR-5 film using Cronex intensifying screens at -70 °C.

*Injection of H. virescens larvae.* To obtain wasp calyx fluid, ovarioles and oviducts were dissected from 1- to 3-week-old female wasps and minced in sterile PBS (1 mM-CaCl2, 2.5 mM-KCl, 1.5 mM-KH2PO4, 0.5 mM-MgCl2, 137 mM-NaCl). The cell debris was pelleted (15600 g for 10 s) and the supernatant (calyx fluid) collected. The volume of the supernatant was adjusted with PBS to a final concentration of one pair of dissected ovarioles per 10 μl.

Hybridization of 32P-labelled CsV DNA to dot blots of DNA isolated from naturally parasitized *H. virescens* larvae showed that from 1 to 20 ng per larva (average of 6.5 ± 4.6 ng) was transferred from the wasp to the host during oviposition. The maximum transferred (20 ng) represents ≤14% of the total viral DNA found in a single female wasp (Fleming & Summers, 1986). For injection experiments each third instar *H. virescens* larva was injected with 15% of the average amount of calyx fluid recovered from one pair of ovarioles in a total volume of 1.5 μl. Third instar *H. virescens* larvae were anaesthetized with CO2 before injection with 1.5 μl of calyx fluid solution, AcNPV or PBS. The calyx fluid was injected behind the head capsule directly into the haemocoel using a drawn capillary micropipette (Davies et al., 1986).
Plasmid DNA. CsV DNA was separated by electrophoresis in a 0.7% agarose gel, and the 6.6 kbp viral SH band identified as B (Krell et al., 1982) was excised from the gel and the DNA recovered by electroelution (Smith & Summers, 1979). The SH DNA B was digested with EcoRI or BamHI and the restriction fragment cloned into the plasmid vector pUC8. The recombinant plasmid designated as pBE-6600 used in this study contains the entire B superhelix as a single EcoRI fragment. Plasmid DNA was isolated by the method of Holmes & Quigley (1981) and purified on CsCl–ethidium bromide gradients.

RESULTS

To investigate CsV replication in the tissues of the lepidopteran host in vivo, third instar H. virescens larvae were injected with calyx fluid from which wasp eggs had been removed. This ensured that there was no viral DNA present due to CsV replication in the wasp egg or the developing wasp endoparasite. Changes in weight gain of naturally parasitized or injected H. virescens larvae were used to compare and assess some of the gross physiological effects of CsV on H. virescens larvae in the presence or absence of the endoparasite and to demonstrate that the quantity of injected CsV was sufficient to mimic the effects of natural parasitization. Fig. 1 compares the fresh weight of H. virescens larvae injected with calyx fluid, AcNPV, PBS, and larvae naturally parasitized by C. sonorensis females. The reduction in host weight gain observed in larvae injected with calyx fluid was similar to that of naturally parasitized larvae. Differences between these two curves did occur after day 7, which is presumably due to the feeding of the endoparasite (which is not present in calyx fluid injected larvae) upon the tissue of the H. virescens larvae. Larvae injected with AcNPV, a virus pathogenic to H. virescens, exhibited a similar growth rate as mock-infected larvae during the first 48 h but became moribund by 72 h and died shortly thereafter. The growth and development of PBS-injected larvae was the same as healthy larvae that had not been injected.

Southern blot analysis of H. virescens DNAs which were extracted at the designated times after injection of calyx fluid (Fig. 2a) showed no significant differences in the levels of viral

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Fig. 2. Southern blot and dot blot analysis of DNAs extracted from *H. virescens* larvae injected with CsV or AcNPV. *H. virescens* larvae were injected with 1-5 μl of calyx fluid or 1-5 μl AcNPV (8.3 × 10⁴ p.f.u.), and the DNAs were extracted from groups of four *H. virescens* larvae at the various times post-injection (days) indicated. For Southern blot analysis equal fractions (10%) of the total DNA sample from the CsV- or AcNPV-injected samples were digested with *EcoRI* and *PstI* respectively. The digested DNAs were separated in 1% agarose gels by electrophoresis, transferred to nitrocellulose (Smith & Summers, 1980), and hybridized to ³²P-labelled CsV DNA (a) and ³²P-AcNPV DNA (c). Replicate lanes in (a) represent duplicate samples from separate groups of four *H. virescens* larvae. Lane *R* is a reconstruction of DNA from mock-injected larvae to which 2 ng of CsV DNA was added. *HindIII* digest fragments of phage λ DNA were used as molecular size markers and sizes are indicated in kbp (lane M). The Southern blots (a) and (c) were exposed to Kodak X-Omat XAR-5 X-ray film using an intensifying screen, at −70 °C for 9 days and 12 h respectively. Dot blot analysis of CsV DNA (b) or AcNPV DNA (d) was performed to analyse the changes in total viral DNA. Equal fractions (10%) of each DNA sample were applied to the nitrocellulose using the method of Kafatos *et al.* (1979). Each data point in (b) represents samples from separate groups of four *H. virescens* larvae and is an average of two separate dots.
DNA at any of the time points examined. In addition, the relative intensities of viral \textit{EcoRI} bands appeared to be consistent for each time point tested with no single band separately increasing or decreasing in intensity at the different times post-injection. Analysis of the total amount of CsV DNA present at each time point by dot hybridization (Fig. 2b) also showed that there was no detectable change of the amount of viral DNA in the \textit{H. virescens} larvae until 10 days after injection. The single sample analysed at 10 days showed a decrease of approximately 35\% relative to the previous time points; the significance of this drop is unknown. An apparent decrease in viral DNA at 2 days post-injection was not reproducible in duplicate experiments. Hybridization to CsV probes was not detected in mock-injected samples at any time post-injection. After injection of the pathogenic baculovirus AcNPV into \textit{H. virescens} larvae a dramatic increase in the level of \textit{PstI}-digested AcNPV DNA could easily be detected (Fig. 2c, d) using these techniques.

Even though the injection of calyx fluid mimics the effects of natural parasitization, it is possible that viral replication in \textit{H. virescens} requires the presence of the developing endoparasitic wasp. To test for this possibility the levels of CsV DNA were analysed in naturally parasitized \textit{H. virescens} larvae (which would include DNA extracted from the developing \textit{C. sonorensis} endoparasitic larvae), \textit{C. sonorensis} pupae, and \textit{C. sonorensis} adults. For the analysis of CsV replication in naturally parasitized \textit{H. virescens} it would have been preferable to separate male and female \textit{C. sonorensis} larvae and pupae because of the large differences in viral DNA content that have been reported for adults. \textit{C. sonorensis} male and female adults contain 0.01 ng and 150 ng of CsV DNA, respectively (Fleming & Summers, 1986). Because it is not possible to determine the sex of the early endoparasitic instars of \textit{C. sonorensis} larvae, groups of 50 parasitized \textit{H. virescens} larvae and/or \textit{C. sonorensis} pupae or \textit{C. sonorensis} adults were randomly selected at the designated times post-parasitization and the DNAs extracted (Fig. 3). At each time point there was a range of developmental stages present with one form usually predominating. Each sample analysed by this method therefore represented a sex and developmental stage average (Fig. 3b). Southern blot analysis of \textit{EcoRI}-digested DNAs extracted from each of these groups of naturally parasitized \textit{H. virescens} larvae, \textit{C. sonorensis} pupae, or \textit{C. sonorensis} adults at the designated times post-parasitization is shown in Fig. 3(a). No major differences in the levels of any of the CsV DNAs could be detected up to 9 days after parasitization. The intensity of the CsV bands reproducibly decreased from 10 to 13 days which was followed by an increase in viral DNA at 15 days. In addition, the relative intensity of the separate viral bands at each time point examined appeared to remain constant. Dot hybridization analysis of the total amount of CsV DNA present at each time point (Fig. 3b) showed a fluctuation in the level of CsV DNA detected from 0 to 9 days which was probably due to the variations in the amount of virus that could be oviposited by female wasps during parasitization (i.e. 6.5 ± 4.6 ng). The decrease in the amount of viral DNA at 9 to 13 days correlated with the decrease in the number of parasitized \textit{H. virescens} larvae still living in each sample of 50 insects. For example, at 12 and 13 days after parasitization when viral DNA was lowest the samples contained no living parasitized \textit{H. virescens} larvae or identifiable \textit{H. virescens} tissue (i.e. 100\% \textit{C. sonorensis} pupae, Fig. 3a, b).

The genome of CsV is complex in its physical structure and organization. To conduct a less complicated analysis of CsV DNA replication a plasmid containing the entire CsV SH B, pBE-6600, was used as a hybridization probe. The CsV SH B DNA when digested with \textit{EcoRI} migrated as a single 6.6 kbp fragment (Fig. 4a). Hybridization of pBE-6600 to DNA samples from the naturally parasitized larvae that had been digested with \textit{EcoRI} showed that the 6.6 kbp viral band (CsV B DNA) was not detected at 12 or 13 days post-parasitization but could again be detected by 14 days, after which the amount of viral CsV B DNA increased (Fig. 4b). The plasmid pBE-6600 also hybridized to two bands of 5-1 kbp and 4-2 kbp that are not found in \textit{EcoRI}-digested viral DNA (lane R, Fig. 4b). These two bands have been suggested to represent integrated forms of the viral DNA in the wasp genome (Fleming & Summers, 1986). From 10 days after parasitization to adults the intensity of the 5-1 kbp and 4-2 kbp putative integrated genomic bands did not change whereas the level of 6-6 kbp viral band increased significantly.
Fig. 3. Southern blot and dot blot analysis of CsV DNA in naturally parasitized *H. virescens* larvae during *C. sonorensis* development. DNA extracted from 50 parasitized *H. virescens* larvae or *C. sonorensis* pupae and adults at the indicated times post-parasitization (days). (a) Equal amounts (0-4%) of each DNA sample were digested with *Eco*RI and the DNA fragments separated by electrophoresis in a 1% agarose gel, transferred to nitrocellulose (Smith & Summers, 1980) and hybridized to 32P-labelled...
Fig. 4. Southern blot analysis of the CsV SH B DNA in naturally parasitized *H. virescens* larvae and *C. sonorensis* pupae. (a) Native CsV DNA or EcoRI-digested CsV DNA electrophoresed in a 1% agarose gel and stained with ethidium bromide. Lane 1, 1-3 μg CsV genomic DNA; 2, 1-3 μg EcoRI digest of CsV genomic DNA. Arrows indicate the positions to which CsV superhelical B (SH-B) and EcoRI-digested linear B (L-B) migrate. (b) Equal fractions (0-3%) of the same DNAs as those described in Fig. 3 were digested with EcoRI and the fragments were separated by electrophoresis on a 1% agarose gel, transferred to nitrocellulose filters (Smith & Summers, 1980) and hybridized to 32P-labelled CsV B DNA that had been isolated from the plasmid pBE-6600 by electroelution. The 6.6 kbp band represents the linear form of the CsV SH-B. The 5.1 kbp and 4.2 kbp bands are the putative wasp genomic bands (Fleming & Summers, 1986). The plasmid pUC8 was added to the samples as an internal standard and hybridizes to residual vector sequences in the electroeluted probe. Lane NP contains DNA from non-parasitized *H. virescens* larvae and lane R is a reconstruction of non-parasitized insect DNA to which 2 ng of CsV viral DNA was added; the arrows indicate viral sequences other than SH-B that cross-hybridize to pBE-6600. The proportion of *C. sonorensis* pupae present in each group of 50 insects is given as a percentage below each lane. HindIII digest fragments of phage λ DNA were used as molecular size markers and sizes are indicated in kbp (lanes M).

CsV DNA. For adult samples, 30 male and 20 female wasps were collected and pooled (based on an average male to female sex ratio in our colony of 3:2). Wasps were 0 to 24 h (lane A1) or 24 to 48 h (lane A2) post-emergence. Lane NP represents DNA from 50 non-parasitized *H. virescens* larvae and lane R is a reconstruction of non-parasitized insect DNA to which 2 ng of CsV DNA was added. The proportion of *C. sonorensis* pupae present in each group of 50 insects is given as a percentage below each lane. HindIII digest fragments of phage λ DNA were used for molecular size markers and are indicated in kbp (lane M). (b) Dot hybridizations were performed with the DNA from the naturally parasitized larvae to compare the total amount of viral DNA present at each time point. Equal fractions (0.6%) of the total DNA sample were applied to nitrocellulose filters using the method of Kafatos et al. (1979) and were hybridized to 32P-labelled CsV DNA. The horizontal black bars represent the developmental stages of *C. sonorensis* present during the time course of this experiment; Hv + CsE, parasitized *H. virescens* larvae containing a *C. sonorensis* egg (Wilson & Ridgway, 1975); Hv + CsL, parasitized *H. virescens* larvae containing a *C. sonorensis* larva; CsP, *C. sonorensis* pupae; CsA, *C. sonorensis* adults.
DISCUSSION

CsV has been observed by electron microscopy to replicate only in the calyx cells of the female parasitic wasp, *C. sonorensis*, but not in the tissue of the lepidopteran host, *H. virescens* (Norton & Vinson, 1983; Norton et al., 1975; Stoltz & Vinson, 1979). The virus is transferred to the host *H. virescens* larvae during oviposition where it appears to be the causal agent of marked physiological changes as evidenced by a reduction in growth, and by some direct or indirect process to interfere with the mechanism by which the wasp egg is encapsulated (Edson et al., 1981; Vinson et al., 1979). It has been shown that CsV-specific mRNAs can be detected in parasitized *H. virescens* larvae. Some of these messages have been mapped to specific CsV DNAs (Blissard et al., 1986a, b; Fleming et al., 1983). Using Southern blot analysis of DNA extracted from *H. virescens* larvae injected with calyx fluid containing CsV, no increase in viral DNA was detected, suggesting that (i) viral DNA will persist at, or near, the original levels injected for up to 10 days after injection and (ii) a detectable level of viral or individual SH DNA replication does not occur. Similarly, no increase in viral DNA could be detected in naturally parasitized *H. virescens* larvae. However, our techniques did detect an increase of CsV DNA in *C. sonorensis* pupae and adults where viral replication is known to occur (Norton & Vinson, 1983).

Analysis of DNAs from naturally parasitized *H. virescens* larvae showed an apparent correlation between the amount of viral DNA and the ratio of *H. virescens* larvae to *C. sonorensis* pupae (Fig. 3), i.e. the amount of CsV-specific DNA decreased as the number of parasitized *H. virescens* larvae in the sample of 50 insects also decreased (9 to 12 days post-parasitization). In early *C. sonorensis* pupal samples which did not contain tissue from parasitized *H. virescens* larvae (12 and 13 days; Fig. 3 and 4), the amount of viral DNA was extremely low or undetectable. Therefore, these results suggest that all or most of the viral DNA detected from 0 to 13 days post-parasitization is that found in *H. virescens* tissues and not in the developing *C. sonorensis* endoparasite. The low amounts of viral DNA detected at 12 and 13 days may be from the ingested remains of *H. virescens* tissues. CsV replication is first detected at 14 days when the predominant pupal form had a pigmented head, thorax and compound eyes. This is the same developmental stage at which virus replication was first observed in calyx tissue by electron microscopy (Norton & Vinson, 1983). Our interpretation of this observation and our data is that CsV replication does not occur in *C. sonorensis* during the endoparasitic life stages or early pupae (i.e. before 14 days post-parasitization) but does replicate in pharate or newly emerged *C. sonorensis* adults. Hybridization experiments with the CsV SH B confirmed the above experiments. Analysis with CsV SH B also showed that only the 5-1 kbp and 4-2 kbp wasp genomic sequences remained constant for the time points examined. This is consistent with the hypothesis that they represent integrated sequences since approximately equal quantities of wasp genomic DNA (as estimated by ethidium bromide-stained agarose gels) were analysed at 12 to 15 days and in the adult DNA samples A1 and A2 (Fig. 4b).

CsV mRNA expression can be detected in *H. virescens* larvae by 2 h post-parasitization and continues for the entire developmental period of the *C. sonorensis* larval endoparasite (Blissard et al., 1986a, b; Fleming et al., 1983). Preliminary comparisons between CsV transcription in *C. sonorensis* pupae and parasitized *H. virescens* have shown that there are differences in the regions of the viral genome from which mRNAs are expressed (Fleming et al., 1983). This suggests that in female *C. sonorensis* wasps, where replication is known to occur, different viral genes may be expressed than in parasitized *H. virescens* larvae. An alternative explanation of our data is that CsV replicates in *H. virescens* at undetectable levels to produce virus for a *per os* mode of transmission. If this explanation is correct then structural mRNAs are also likely to be expressed and translated in the lepidopteran host. Therefore, the identification of genes coding for structural proteins and comparison of their expression in *H. virescens* and *C. sonorensis* may provide further evidence for the presence or absence of CsV replication in *H. virescens*. It is also possible that intact virions from the initial oviposition event survive to infect the developing endoparasite via a *per os* mechanism.

The discovery of putative integrated forms of viral DNA in the male and female wasp genome suggests that CsV is transmitted vertically through the germine (Fleming & Summers, 1986).
Since CsV appears to be essential for the successful development of the endoparasitic stages of *C. sonorensis* (Edson *et al.*, 1981), vertical transmission would ensure that all female wasps contain virus. Therefore, if CsV is transmitted vertically, it is possible that viral genes are expressed in *H. virescens* in order to protect the developing endoparasite from the host’s defence systems, but the virus does not replicate for the purposes of a *per os* transmission. If, as our data imply, CsV replicates in *C. sonorensis* but not in *H. virescens*, CsV will be unique in that this genetically very complex virus is expressed in its two natural hosts yet replicates only in one.

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