DNA Synthesis in Tobacco Mosaic Virus-Infected *Nicotiana tabacum* Protoplasts and Regeneration of Persistently Infected Calli

YEHUDA STRAM† AND ARTHUR WEISSBACH*

Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110

Received 17 March 1986/Accepted 18 July 1986

Tobacco mosaic virus infection of *Nicotiana tabacum* mesophyll protoplasts did not affect the pattern of chloroplast or total cellular DNA synthesis for at least 120 h when compared with that of mock-infected cells. Calli derived from infected protoplasts often showed large amounts of tobacco mosaic virus RNA and coat protein.

Our knowledge of the events which take place at the molecular level in tobacco mosaic virus (TMV)-infected cells is sparse due in part to the asynchronous nature of the infection. The use of protoplasts *in vivo* has overcome the problem of asynchronous infection and has become a potentially powerful tool in studying the replication mechanism of TMV and other viruses. However, profound changes occur in cells during the enzymatic preparation of protoplasts, including the cessation of DNA synthesis for a few days after protoplast formation (3, 12). Our objective in this study was to see if there is any effect of TMV infection on DNA synthesis in tobacco protoplasts.

Replication of TMV in protoplasts. Protoplasts were prepared from the leaves of sterile shoot cultures of *Nicotiana tabacum* L. cv. xanthi which had been grown under 10,000 lx of light in a light-dark cycle of 16 and 8 h, respectively. The protocol for preparation and isolation of the protoplasts was essentially as previously described (6, 11), except that the enzyme solution contained 0.4% of partially purified cellulase RS (Yakult Biochemical, Japan) and 0.04% of partially purified pectolyase Y23 (Seichin Pharmaceutical Co., Ltd., Japan) in 0.4 M mannitol (pH 5.7). The incubations were carried out for 1 h. The partially purified enzymes were 33 to 55% ammonium sulfate fractions of the commercial crude powder.

The inoculation of protoplasts with TMV (2×10^4 virions per cell) was as described by Kikkawa et al. (6). In each experiment, one half of the protoplasts were infected with TMV and the other half were mock infected; TMV replication in protoplasts was monitored by dot blot hybridization (9). At the time of initial infection, the input viral RNA was 12 ng/ 10^6 protoplasts (time zero). By 24 h after initial infection, the amount of TMV RNA had increased to 775 ng/ 10^6 protoplasts and by 120 h had reached a level of 10 µg of TMV RNA per 10^6 cells (Table 1). The percentage of protoplasts that were infected was found to be at least 70%, as estimated from the TMV distribution in calli derived from the infected protoplasts (Table 2).

DNA synthesis in mock-infected and TMV-infected protoplasts. To determine if DNA synthesis in protoplasts is affected by TMV infection, we measured the incorporation of [^{3} H]thymidine in the infected and mock-infected

protoplasts. Cells were pulse-labeled with [^{3} H]thymidine for 3 h from the time the infection was established (time zero) and every 24 h thereafter. In both the infected and uninfected protoplasts, the kinetics of total cellular DNA synthesis were similar after protoplast formation (Fig. 1). Both types of protoplasts started to synthesize DNA between 2 and 3 days after protoplast formation, and the rate of DNA synthesis increased in a similar fashion in both infected and mock-infected cells for 48 to 96 h.

There is evidence suggesting an involvement of the chloroplast compartment in TMV infection (8, 10). To study the effect of TMV infection on chloroplast DNA synthesis, we used molecular hybridization techniques which have been previously described (2, 4, 5). Total radiolabeled DNA from 6-h pulse-labeled infected and uninfected protoplasts were hybridized to cloned *N. tabacum* chloroplast DNA immobilized on nylon membranes, and the percentage of labeled chloroplast DNA was measured (Fig. 2). At 48 h after infection, when DNA synthesis recommenced, about 30% of the DNA synthesized in both infected and uninfected protoplasts was chloroplast DNA (Fig. 2). This percentage dropped to 11 to 14% by 72 h in both cultures and remained constant for at least another 48 h in the two cultures.

Our results demonstrate that DNA synthesis in the chloroplasts of the mesophyll protoplasts is not altered by TMV infection and that the DNA of the chloroplast replicates after infection of the protoplasts. Furthermore, chloroplast replication seems to recover 24 h before that of the nuclear DNA (Fig. 2). This phenomenon is consistent

TABLE 1. Replication of TMV in *N. tabacum* protoplasts^a

Time postinfection (h)	TMV RNA (ng/ 10^6 protoplasts)
0	12
24	775
48	1,500
72	2,750
96	3,000
120	10,000

^a Nucleic acid-containing extracts obtained at each time point were put on a nitrocellulose membrane in 3 successive 10-fold dilutions each. Different amounts of TMV RNA (15 ng, 1.5 ng, 150 pg, and 15 pg) were spotted to serve as standards. After hybridization with [^{32} P]cDNA prepared from TMV RNA (9), each spot was cut out and counted. The amount of TMV RNA at each time point was calculated by comparing the counts in each sample with those of the standards.

* Corresponding author.

† Present address: Department of Plant Pathology, University of Kentucky, Lexington, KY 40546.

TABLE 2. TMV RNA and coat protein in calli originated from infected protoplasts^a

Samples	Mo after infection	TMV RNA (ng/10 ⁶ cells)	TMV coat protein (ng/10 ⁶ cells)	TMV RNA as % of RNA + coat protein ^b
B ^c	2	48	828	5.5
C ^c	2	26	726	3.4
D ^c	2	19.2	785	2.3
4	2	710	15,423	4.4
9	2	1146	16,639	6.5
12	2	ND ^d	ND	— ^e
20	2	ND	—	—
44	2.5	0.75	<12	—
47	2.5	1.3	<12	—
5	3.5	ND	—	—
53	3.5	1577	38,326	3.9
54	3.5	1	ND	—
55	3.5	1176	40,019	2.8
57	3.5	262	14,425	1.7
63	3.5	ND	—	—
64	3.5	17.85	355	4.8
53	5	290	4,170	6.7
56	5	300	6,517	4.4
72	5	ND	—	—
82	5	315	9,916	3
83	5	ND	—	—
92 ^f	5	ND	ND	—

^a Calli were regenerated from protoplasts as described by Aviv et al. (1). The amount of the coat protein was determined by enzyme-linked immunosorbent assay as described by Orchansky et al. (7).

^b TMV RNA is 5% of the virion weight.

^c Mix of calli from liquid medium.

^d ND, Not detectable.

^e —, Analysis not done.

^f Calli originated from uninfected protoplast culture.

FIG. 1. [³H]thymidine incorporation into protoplast DNA. Time points indicate the middle of the 3-h labeling period. ●, Infected protoplasts; ▲, uninfected protoplasts.

FIG. 2. [³H]thymidine incorporation into chloroplast DNA. The times indicated between points are the times after the isolation of the protoplasts and the inoculation with TMV. Each time point represents the middle of the 6-h labeling period. ●, TMV-infected protoplasts; ▲, control uninfected protoplasts.

with the observation that chloroplast DNA synthesis is independent of nuclear DNA synthesis. The autonomous nature of the chloroplast DNA synthesis has been shown in tobacco cell suspension culture by Heinhorst et al., who have shown that inhibition of the nuclear DNA synthesis does not affect the chloroplast DNA synthesis (4, 5).

Persistence of TMV in cells. We determined whether regeneration of the infected protoplasts in calli and plants would be influenced by TMV infection and whether the virus would persist in calli and regenerated plants. Of the calli examined 2 to 5 months after infection, 70% contained the viral genome in the range from 1×10^0 to 1.5×10^3 ng/10⁶ cells (Table 2). The percentage of calli containing TMV RNA did not change in the 5 months after infection. To see whether the viral RNA in the calli was being expressed, the calli that were used for the detection of viral RNA were checked for the TMV coat protein. These experiments showed a direct correlation between the amount of RNA and the amount of coat protein in the calli (Table 2): the calli that showed that high amounts of TMV RNA also contained high amounts of coat protein. The results suggested that calli derives from infected protoplasts containing TMV virions, since the percentage of TMV RNA relative to the coat protein is about 5% similar to that of the mature virion (9). That calli regenerated from the infected protoplasts contain, in many cases, high levels of TMV is consistent with the observation that TMV infection does not seem to affect cell function or cellular DNA synthesis. The variability in the amount of the virus in different calli may suggest that they can show variable resistance to TMV infection.

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