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Wound Tumor Virus Polypeptide Synthesis in Productive Noncytopathic Infection of Cultured Insect Vector Cells

ANDREW J. PETERSON AND DONALD L. NUSS†*

Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201

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Inoculation of the leafhopper cell line AC-20 with wound tumor virus resulted in a productive noncytopathic infection with no detectable alteration of cellular protein synthesis. Virus-specific polypeptide synthesis, detectable by 8 h postinoculation, increased in a linear fashion, reaching a peak (approximately 10 to 15% of total protein synthesis) by 48 h postinoculation. The rate of viral protein synthesis continued at this level for several days but declined, relative to cellular protein synthesis, as infected cells were passaged. By passage 10, the synthesis of viral polypeptides was reduced to a level approximately 5% of that observed at 48 h postinoculation. Viral protein synthesis was not stimulated by superinfection. Viral antigens and infectious virus persisted in the majority (>90%) of cells in an infected culture even after more than 100 passages. The synthesis of wound tumor virus polypeptides in infected insect vector cells appears to be regulated in a coordinated and selective manner.

Wound tumor virus (WTV), a plant virus which is a member of the family *Reoviridae*, also replicates in its insect vector (5, 7). WTV is transmitted efficiently to plant hosts by only two species of leafhoppers (2, 3, 5). Although the virus multiplies to a high titer in the vector, the infection is inapparent and persistent (9, 14). Evidently, mechanisms which allow the efficient multiplication of the virus in the vector without the production of any significant pathology have evolved. Consequently, once infected, leafhoppers transmit WTV for the remainder of an apparently normal life-span (9).

A continuous cell culture (line AC-20) has been established from the leafhopper vector *Agallia constricta* (6). The cultured vector cells and the insect vector exhibit similar responses to WTV infection. Both support the multiplication of standard WTV to a high titer, and in both, an asymptomatic, persistent infection develops (3, 4). In addition, transmission-defective isolates of WTV that replicate in vegetatively propagated plant hosts fail to replicate in both the insect vector and cultured vector cells (18, 20). Therefore, cultured vector cells provide a suitable system with which to investigate the molecular details underlying virus-vector interactions and virus transmission.

We previously identified 12 WTV-encoded structural and nonstructural polypeptides in extracts of infected vector cells and in cell-free translation reactions programmed by WTV mRNA (17). The observations that the inoculation of leafhoppers or cultured leafhopper cells with WTV results in an asymptomatic, persistent infection prompted us to examine the rates of viral and host protein synthesis in WTV-infected vector cells during an extended time period after inoculation.

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AC-20 cells were grown in monolayer cultures at 28°C in growth medium prepared as described by Liu and Black (12). Synchronous infections were initiated as previously described (17) with 10 to 20 cell-infecting units (10) of WTV per AC-20 cell in 8-cm^2 culture dishes seeded with 3×10^5 cells 24 h before inoculation. At appropriate times after inoculation, growth medium was removed, and cell monolayers were incubated with 50 μCi of [^{35}S]methionine in 250 μl of growth medium lacking methionine and serum at 28°C for 30 min. Monolayers were washed twice with phosphate-buffered saline, drained, and lysed in 100 μl of electrophoresis sample buffer (11). The level of [^{35}S]methionine incorporation into protein was determined by the method of Mans and Novelli (13), and the labeled lysates were analyzed by electrophoresis in 12.5% polyacrylamide gels and autoradiographed (17). Each gel well received an equal amount of radiolabeled lysate. WTV-specific polypeptides were readily detectable by 8 h postinoculation (p.i.) (Fig. 1A). The rate of synthesis of WTV polypeptides continued to increase for the next 40 h and remained constant from 48 to 120 h p.i. The rate of synthesis of most cellular polypeptides remained constant throughout this time period. At the peak of virus-specific polypeptide synthesis (48 to 120 h p.i.), viral polypeptides represented approximately 10 to 15% of the total protein synthesized in a 30-min labeling period, as determined by the direct measurement of radioactivity in gel bands or by densitometric analysis of gel autoradiographs.

Examination of lysates prepared from pulse-labeled, subcultured infected cells revealed that the rate of viral polypeptide synthesis declined relative to the rate of cellular protein synthesis as the cells were passaged (Fig. 1B). By passage 5, viral polypeptide synthesis declined to approximately 35% of the rate observed at 48 h p.i. and was barely detectable with a 30-min pulse by passage 10. Viral polypeptide synthesis continued at this lower rate even after more than 100 passages. WTV antigens were present, as determined by a fluorescent-antibody assay, in more than 95% of cells in persistently infected cultures. This indicates that the decline in the rate of viral polypeptide synthesis observed after cell passage was not due to the selection of an uninfected cell population but rather reflects changes occurring within the majority of cells in the culture.

The relative changes in the accumulation of viral antigens were quantitated by Western blot analysis (Fig. 2). Viral polypeptide P_6, the 57,000-molecular-weight core compo-

* Corresponding author.
† Present address: Department of Cell Biology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.

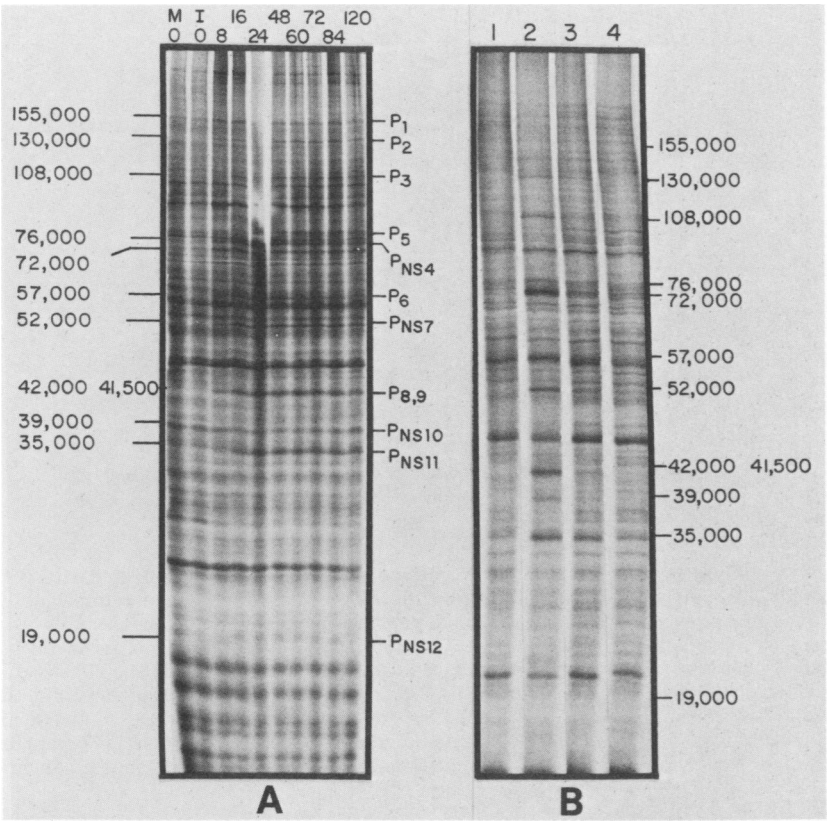

FIG. 1. Autoradiographs of polyacrylamide gels analyzing [^{35}S]methionine-labeled lysates of WTV-infected AC-20 vector cells. (A) Lysates of acutely infected AC-20 cells. The number at the top of each lane indicates the time (in hours p.i.) at which the pulse-labeling was performed. Lanes M and I contain lysates of mock-infected and infected cells, respectively, both prepared at zero time. The migration positions of WTV structural (P) and nonstructural (P_{NS}) polypeptides are indicated on the right, and the estimated molecular weights of the WTV gene products are indicated on the left. The nomenclature and estimated molecular weights of the WTV polypeptides are from Nuss and Peterson (17) and Nuss (16). (B) Lysates of persistently infected, subcultured AC-20 cells. Lane 1, Lysate of mock-infected cells; lane 2, lysate of infected cells pulse-labeled 48 h p.i.; lanes 3 and 4, lysates of infected cells pulse-labeled at passages 5 and 10, respectively. Estimated molecular weights are indicated on the right. For both panels A and B, each lane received an equal amount of radioactivity. The amount of radioactivity incorporated into individual polypeptides was determined directly by excising appropriate bands from dried gels and dissolving them in 0.3 ml of 15% hydrogen peroxide, followed by counting in Aquasol liquid scintillation fluid. Alternatively, the relative radioactivity incorporated was quantitated by densitometry of autoradiographs.

nent (17, 21), was most reactive with anti-WTV sera, whereas viral polypeptide P_5, the 76,000-molecular-weight product of genome segment 5 (15) and a component of the outer protein shell (21), reacted with the antisera to a lesser degree. A host protein, designated HP, was also detected, but it is unclear whether staining of this polypeptide was due to a reaction with anti-WTV sera or to intrinsic peroxidase activity. HP served as a convenient internal standard to indicate whether polypeptides in the different cell lysate preparations were equally transferred to the nitrocellulose membrane. The amount of cell-associated viral antigen decreased by 95% between 120 h p.i. (Fig. 2A) and passage 10 (Fig. 2B), consistent with the decrease in the rate of virus-specific polypeptide synthesis observed during this period (Fig. 1B). It should be noted that, although much diminished in amount, viral antigens did persist at a readily detectable level in subcultured infected cells.

Persistently infected cells were superinfected with WTV in an effort to stimulate virus-specific protein synthesis. No significant increase in the level of virus-specific polypeptide synthesis was detected in superinfected, persistently infected cells even though high levels of viral polypeptides were synthesized after inoculation of uninfected cells with the same virus preparation (Fig. 3). However, cells persistently infected with WTV were susceptible to infection with a heterologous virus, potato yellow dwarf virus (data not shown). It is not known whether the inability of superinfection with WTV to stimulate viral polypeptide synthesis in persistently infected cells is related to the decline in virus-specific polypeptide synthesis observed upon passage of infected cells.

Electron-microscopic analysis of persistently infected cells revealed that the majority (>90%) of cells in a persistently infected culture contained virus particles (Fig. 4). Mature virions were seen associated with membranous vesicles, as well as free within the cytoplasm and in electron-dense inclusions. Incomplete particles were also observed in significant numbers. The distribution of virus particles within the cell sections was consistent with the fluorescent-antibody patterns observed when infected cells were stained with fluorecein-conjugated anti-WTV sera (17). There appeared to be between 10^3 and 10^4 particles per cell based upon the number of particles observed per ultrathin section.

Infection of vector cells with WTV can be divided into an acute phase and a persistent phase. During the acute phase, viral polypeptide synthesis increases at a linear rate and peaks between 48 and 120 h p.i. During the persistent phase, viral polypeptide synthesis declines to a level approximately

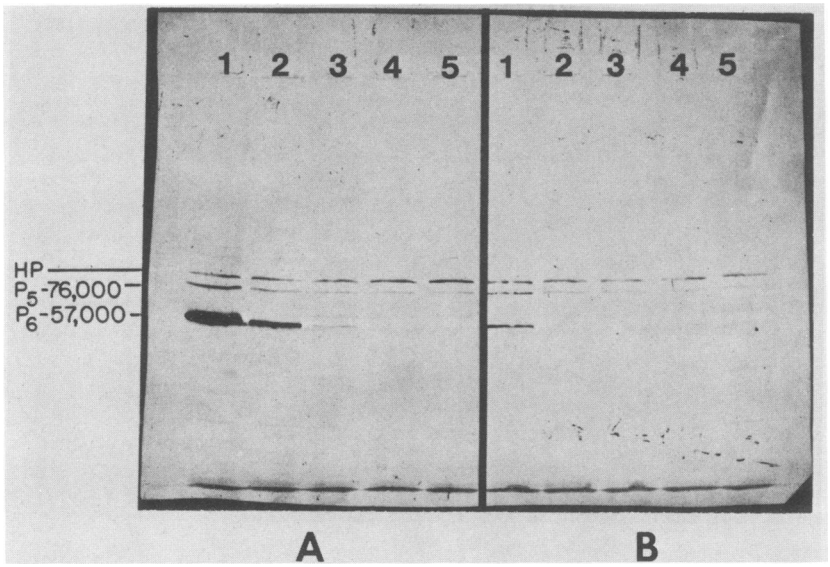

FIG. 2. Western blot analysis of lysates prepared from AC-20 cells acutely and persistently infected with WTV. After electrophoresis of lysates in 12.5% polyacrylamide gels, polypeptides were electrophoretically transferred to 0.45-μm nitrocellulose membranes by the method of Towbin et al. (22). WTV antigens were detected by the indirect immunoperoxidase technique (8) with the Bio-Rad Laboratories Immun-Blot kit and rabbit anti-WTV sera raised against virus particles purified from root tumors of infected sweet clover. Results obtained for lysates of acutely infected (120 h p.i.) and persistently infected (passage 10) cells are shown in panels A and B, respectively. In both cases, the infected cell lysates were serially diluted 1:10 with uninfected AC-20 cell lysate so that equal amounts of protein were applied to each gel well. Lane 1, Undiluted lysate; lanes 2 through 4, 10^{-1}, 10^{-2}, and 10^{-3} dilutions, respectively; lane 5, undiluted lysate of uninfected cells. Reactive polypeptides, including host polypeptide HP, are indicated on the left. A reactive nonviral polypeptide that comigrated with viral polypeptide P_5 was also detected. Relative signal strengths were determined by densitometry of photographic positives of the blot.

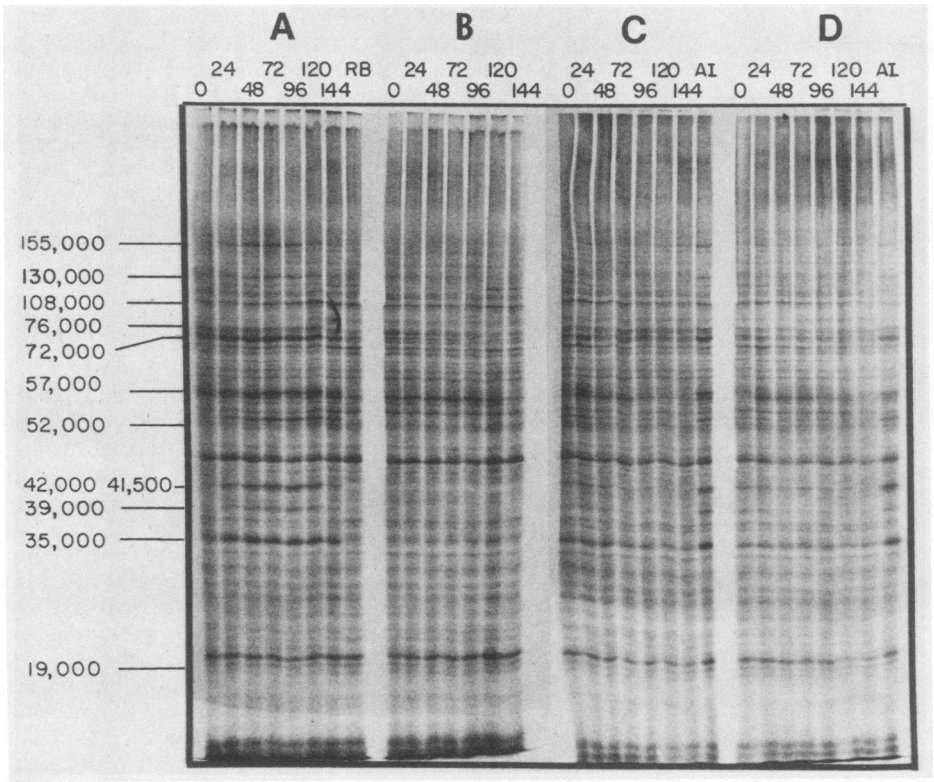

FIG. 3. Autoradiographs of polyacrylamide gels analyzing [^{35}S]methionine-labeled cell lysates prepared from persistently infected AC-20 cells after superinfection with WTV. Uninfected (AI) and persistently infected (RB) (infected for 1 year with standard WTV inoculum RB [19]) AC-20 monolayers were inoculated with the same virus preparation at a multiplicity of 20 cell-infecting units per cell. (A) Lysates of inoculated AC-20 cells. (B) Lysates of mock-inoculated AC-20 cells. (C) Lysates of inoculated, persistently infected AC-20 cells. (D) Lysates of mock-inoculated, persistently infected AC-20 cells. The number at the top of each lane indicates the time (in hours p.i.) at which the pulse-labeling was performed. The migration positions of WTV-specific polypeptides are indicated on the left. Each gel lane received an equal amount of radioactivity.

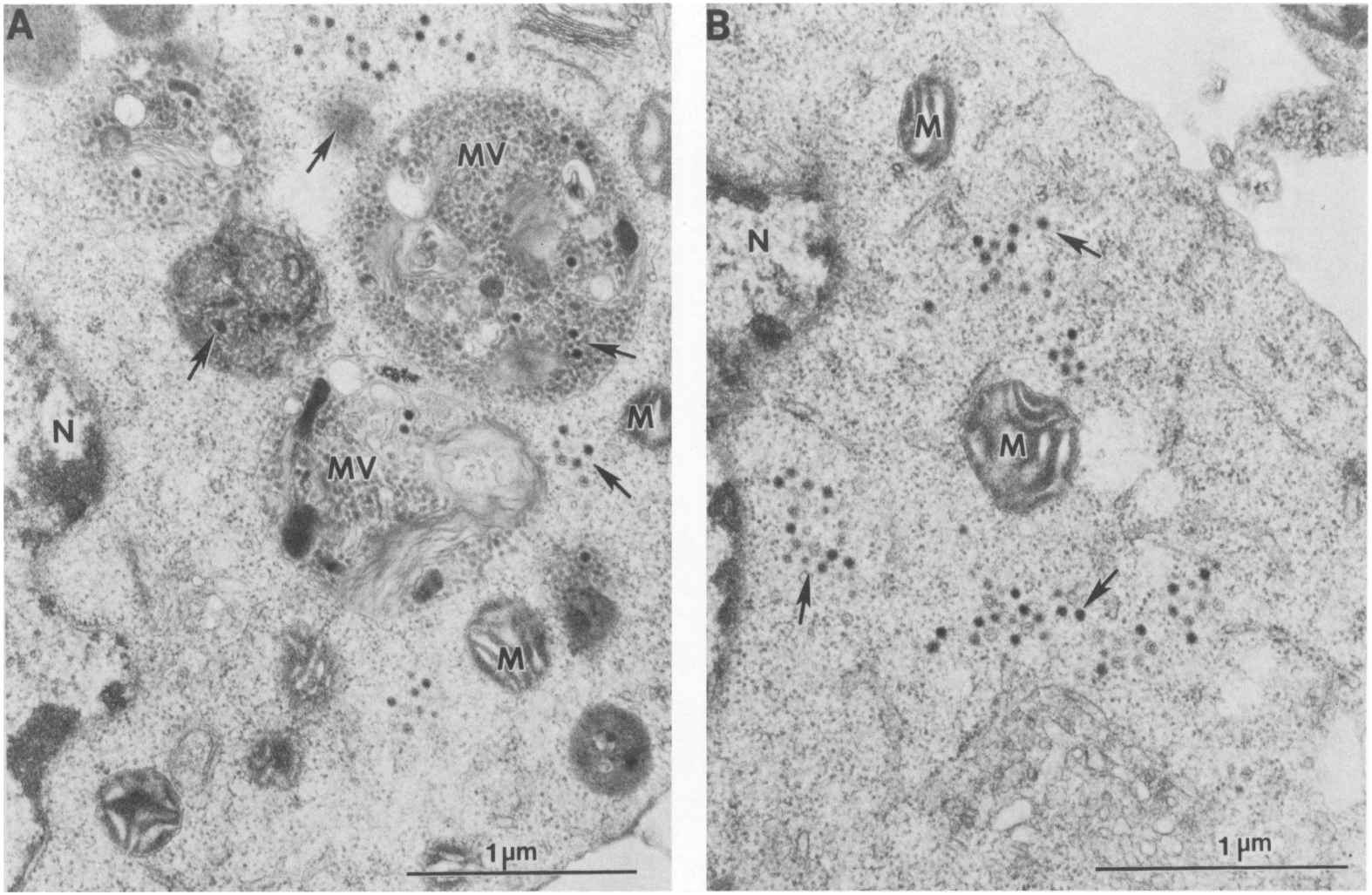

FIG. 4. Electron micrographs of ultrathin sections of AC-20 cells persistently infected with WTV. Persistently infected cells (passage 43) were fixed with 2.5% glutaraldehyde, postfixed with osmium tetroxide, dehydrated, and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate and photographed. Examples of intact virus particles in various structural environments are indicated by the arrows. N, Nucleus; M, mitochondria; MV, membranous vesicles.

5% of that observed at the peak of the acute phase and is maintained at this level for years of subculturing. The regulation of viral polypeptide synthesis is independent of cellular protein synthesis, which remains essentially constant during both the acute and persistent phases. The synthesis of each of the 12 viral polypeptides appears to increase and decline during the two phases in a coordinated manner. Each of the 12 WTV genome segments contains an identical hexanucleotide sequence at the terminus corresponding to the 5′ end of the plus strand and an identical tetranucleotide sequence (unrelated to the hexanucleotide) at the other terminus (1). These terminal sequences may be important in the coordinated regulation of viral gene expression.

A productive noncytopathic persistent infection results when cultured vector cells are inoculated with WTV prepared from infected plants, infected vector cells early (48 to 120 h p.i.) after inoculation, or persistently infected vector cells and can be summarized as follows. (i) No cytopathology develops in infected cultures at any point during the acute or persistent phase of infection. Persistently infected cells exhibit the same morphology, pattern of cellular protein synthesis, and growth rate as uninfected cells and do not suffer from a periodic crisis. (ii) Essentially all cells in the persistently infected culture remain infected for extended periods of subculturing. (iii) Infectious virus can be recovered from persistently infected cells. The inoculation of uninfected AC-20 cells with virus prepared from persistently infected cells results in another round of acute infection, which then becomes persistent. (iv) Viral polypeptide synthesis is not stimulated by superinfection of persistently infected cells with homologous virus. However, these cells are susceptible to infection with a heterologous virus.

The persistent infection exhibited by WTV most closely resembles that described as a "regulated infection" by Walker (23). It is not known whether the regulation of viral gene expression and viral replication involves virus-specific or host-specific factors or a combination of both. The regulation of WTV infection in cells of its natural insect vector favors the efficient transmission of the virus to plant hosts. The virus uses vector cell machinery to reproduce itself but does so in a manner that does not interfere with the vitality of the organism on which it depends for its own subsequent survival. Studies in progress are directed at determining the level at which WTV polypeptide synthesis is controlled and whether this regulation is mediated by host-specific or virus-specific factors.

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