Partial Reversion of Conditional Transformation Correlates with a Decrease in the Sensitivity of Rat Cells to Killing by the Parvovirus Minute Virus of Mice but Not in Their Capacity for Virus Production: Effect of a Temperature-Sensitive *v-src* Oncogene

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Received 7 March 1989/Accepted 18 July 1989

The cytolytic effect of the autonomous parvovirus minute virus of mice, prototype strain (MVMP), was studied in cultures of *ts* 339/NRK rat cells that display a temperature-sensitive transformed phenotype as a result of their transformation with a Rous sarcoma virus strain mutated in the *v-src* oncogene. A shift from restrictive (39.5°C) to permissive (34.5°C) temperature was associated with a marked sensitization of these cells to killing by MVMP. In contrast, *ts* 339/NRK cell derivatives supertransformed with a wild-type *src* oncogene were sensitive to MVMP at both temperatures, suggesting that the expression of a functional oncogene product may determine, at least in part, the extent of the parvoviral cytopathic effect. Although *ts* 339/NRK cells were quite resistant to parvoviral attack at 39.5°C, they were similarly proficient in MVMP uptake, viral DNA and protein synthesis, and infectious particle production at both permissive and restrictive temperatures. Consistently, electron microscopic examination of infected *ts* 339/NRK cultures incubated at 39.5°C revealed the presence, in the majority of the cells, of numerous full and empty virions that were predominantly located in autophagic-type vacuoles. Thus, in this system, the reversion of transformed and MVMP-sensitive phenotypes appears to correlate with the setting up of a noncytotoxic mode of parvovirus production. These results raise the possibility that the physiological state of host cells may affect their susceptibility to parvoviruses by modulating not only their capacity for virus replication but also cellular processes controlling the cytopathic effect of viral products.

Parvoviridae constitute a family of single-stranded DNA-containing viruses that infect a wide variety of animal species, including humans (1, 35). Members of this viral family can be classified according to the degree of dependence of their replication on coinfection with a helper virus (2, 47). Thus, the so-called autonomous parvoviruses are able to replicate efficiently without the aid of a helper virus in a range of permissive cells (9, 47). Interest in these viruses arises from their small size and low genetic complexity that render them especially dependent on their host cells. In particular, parvoviral replication appears to rely on cellular functions, some of which are restricted to proliferating cells (S-phase factors) in appropriate differentiation states (20, 37, 38, 46). Such strong requirements are interesting from a fundamental point of view because they reflect interactions between highly regulated cellular functions and viral determinants (9). Moreover, the requirements of parvoviruses towards host cells also are major factors influencing the tissue tropism and the pathogenicity of these viruses (39).

Another intriguing property of parvoviruses that may be a reflection of the foregoing is the potent ability of these viruses to suppress spontaneous, virally or chemically induced, and implanted tumors in laboratory animals (10, 15, 35, 42). This oncosuppressive activity is still poorly understood. Interestingly, recent observations showed that a number of *in vitro* cultures of transformed cells constitute a preferential target for lytic replication of autonomous parvoviruses. Thus, a series of human and murine fibroblasts and

epithelial cells transformed by various oncogenic treatments, including radiation, a chemical carcinogen, and viral or cellular oncogenes, are found to be more sensitive to the killing action of parvovirus H-1 or minute virus of mice (prototype strain, MVMP) than corresponding normal cells (3, 7, 8, 21, 22, 23). However, no absolute correlation between hypersensitivity to parvoviruses and transformation can be drawn, since transiently and a few stably transformed cells tested prove to resist the killing action of these viruses (23). Previous work indicated that the enhanced killing of transformed cells coincides with their greater capacity for supporting the parvoviral life cycle, although infection is not necessarily productive (7). Viral DNA is amplified to a greater extent in some but not all transformed cells, compared with their normal parents (7, 8, 21, 23). Moreover, in all cases studied so far, parvovirus-sensitive transformed cells accumulate greater amounts of viral nonstructural proteins than do corresponding normal cultures (8, 44a). Since parvoviral nonstructural proteins are suspected of being cytotoxic (25, 30), the up-modulation of their expression may constitute one factor that enhances the viral cytopathic effect (44a). However, no perfect correlation has been found between the relative sensitivities of various transformed cell lines and their proficiency in viral DNA or protein synthesis (7, 8, 21, 23), suggesting that cellular factors other than those involved in virus replication may contribute to the overall cellular susceptibility to parvoviral attack.

The dependence of parvovirus replication on cell proliferation and differentiation (see above) suggests that the mod-

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ulation of cellular functions involved in these processes, as induced by malignant transformation (24), may account for the frequent cosegregation of parvovirus-sensitive and transformed phenotypes. However, it cannot be ruled out that transformation preferentially affects a minor fraction of preexisting parvovirus-sensitive cells. In order to distinguish between causal and consequential interrelations of transformation and susceptibility to parvoviral attack, there is a need for a conditional transformation system allowing the modulation of the transformed phenotype of a given cell strain. This rationale prompted us to analyze the susceptibility of normal rat kidney (NRK) cells that contain an integrated Rous sarcoma virus (RSV) mutant conferring a temperature-sensitive transformed phenotype to MVMP.

The transforming potential of RSV can be ascribed to the *v-src* oncogene that encodes a 60-kilodalton phosphoprotein, pp60^{v-src} (16, 34, 50). This protein is associated with the internal surface of the plasmic membrane as well as with the cytoskeleton and possesses a tyrosine-specific protein kinase activity (5, 16, 17, 27). Although a number of potential cellular substrates of this enzyme have been identified and a series of RSV mutants has contributed to delineate *v-src* functions (6, 16, 48, 50), the exact mode of transformation by pp60^{v-src} still remains elusive. In this study, we used the *ts* 339/NRK cell line that is transformed with a temperature-sensitive RSV mutant, allowing the reversion of several transformation parameters (e.g., morphological changes, efficient growth in agar suspension, high hexose uptake, and lectin agglutinability) when cultures are shifted from permissive to restrictive temperature (14).

Results presented in this report show that the expression of a functional *v-src* protein correlates with an increase in the susceptibility of rat cells to the killing effect of MVMP, suggesting that changes induced by the *src* oncogene may affect determinants of parvoviral cytopathogenicity. Surprisingly, the greater MVMP resistance of cells grown at restrictive temperature is not accompanied by a detectable decrease of their ability to sustain a productive parvovirus infection and may rather be due to a modulation of their responsiveness to viral cytotoxic factors.

MATERIALS AND METHODS

Cells and virus. *ts* 339/NRK was derived from the NRK cell line (11) by transformation with a temperature-sensitive (*ts*) mutant of RSV strain B77 (14) and was kindly provided by T. Graf (Heidelberg, Federal Republic of Germany). NRK and *ts* 339/NRK cells, as well as the *ts* 339-*wt* SRA/NRK derivative described in this report, were grown as monolayers in Dulbecco modified Eagle minimal essential medium supplemented with 10% calf serum (GIBCO Ltd., Renfrewshire, Scotland) and 1% sodium pyruvate. A9, a line of mouse fibroblasts, was grown in Eagle minimum essential medium supplemented with 5% fetal calf serum (GIBCO).

MVMP parvovirus was propagated in A9 cells and purified as described by Tattersall et al. (40). ³H-labeled virus was produced by incubating infected cells with [³H]thymidine (25 Ci/mmol; C.E.A.-Saclay, Saclay, France) as described by Rhode (29). Conditions for cell infection and virus harvesting have been described previously (21), except that incubation of virus-infected cells took place at 34.5 or 39.5°C. MVMP was titrated by plaque assay on A9 cells as described by Rommelaere et al. (33). Virus titers were expressed as PFU per ml. The multiplicity of infection (MOI) defines the number of PFU inoculated per cell.

Transformation with a wild-type *v-src* oncogene. M-*src* is a murine retrovirus constructed by inserting the *v-src* onco-

gene from the Rous sarcoma virus strain Schmidt-Ruppin subgroup A (SRA) into the retroviral vector pMV-4, thus facilitating integration and expression of the *v-src* gene in mammalian cells (45). A cell line producing this recombinant virus was kindly provided by B. Vennström (Stockholm, Sweden). *ts* 339/NRK cells, grown at 39.5°C, were infected with M-*src* in the presence of 0.8 µg of polybrene per ml and were seeded at low densities into 100-mm culture dishes. After 1 to 2 weeks of incubation at 39.5°C, cell clones exhibiting a morphologically transformed appearance were isolated with cloning cylinders and further amplified. These transformed clones were denoted *ts* 339-*wt* SRA/NRK.

pp60^{src} immunoprecipitation, kinase assay, and gel electrophoresis. Cells (2.5 × 10⁶ per 100-mm dish) were metabolically labeled for 3.5 h in 2 ml of methionine-free Dulbecco modified Eagle minimal essential medium supplemented with 5% dialyzed calf serum and 200 µCi of [³⁵S]methionine (800 Ci/mmol; Amersham Corp., Amersham, England). Cells were lysed in 20 mM Tris (pH 7.5)–150 mM NaCl–1 mM EDTA–1% Nonidet P-40–1% Aprotinin, in the presence of 0.2 mM Na₃VO₄ to prevent tyrosine-specific phosphatases (12). After clarification by centrifugation at 120,000 × *g* for 1 h, lysate fractions containing equal amounts of trichloroacetic acid-precipitable radioactivity (2.5 × 10⁷ cpm) were supplemented with 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS), immunoprecipitated by using an excess of a specific anti-*src* monoclonal antibody (MAb 327) (Oncogene Science Inc., Manhasset, N.Y.), fractionated on 10% SDS-polyacrylamide gels, and processed for fluorography as described by Martin et al. (18).

Kinase activity was determined by using enolase as an exogenous substrate for phosphorylation. Equal amounts of pp60^{src}, as determined by densitometric scanning of autoradiograms, were immunoprecipitated as described above, except that sodium deoxycholate and SDS were both omitted to prevent denaturation of kinase activity (13). Immunoprecipitates were suspended in kinase buffer (20 mM Tris, pH 7.5, 5 mM MnCl₂) containing 4 µg of acid-denatured (26) rabbit muscle enolase (Sigma Chemical Co., St. Louis, Mo.) and 5 µCi of [γ-³²P]ATP (3,000 Ci/mmol, Amersham Corp.). The mixtures were incubated at either 34.5 or 39.5°C for 10 min, fractionated by 10% SDS-polyacrylamide gel electrophoresis, and examined by autoradiography by using a Lightning-plus enhancing screen with two sheets of aluminum foil between the dried gel and the X-ray film to block ³⁵S radiation.

Measurement of cell growth and cell cloning ability on plastic. For the measurement of cell growth, cultures (10⁵ cells) were infected with MVMP at various MOIs. At intervals postinfection (p.i.), living cells in the cultures were identified by the trypan blue exclusion method and counted with a hemacytometer.

To determine the ability of cells to clone on plastic, cultures were trypsinized 4 h p.i. and replated at low densities onto 60-mm dishes. After 10 to 15 days of incubation, colonies were fixed in ethanol-acetic acid (3:1 [vol/vol]), stained with Giemsa blue, and counted. Plating efficiencies of the uninfected cells studied were greater than 80%.

Parvoviral life cycle parameters. (i) Virus uptake. Cells (2 × 10⁵ cells per 60-mm dish) were infected by ³H-labeled MVMP (MOI ≈ 2.5 PFU per cell, 4,000 to 8,000 cpm per dish) and incubated for 1 h at 34.5 or 39.5°C. The inoculum was removed, and the cultures were further incubated for 1 h in complete medium. After removal of the medium, the cultures were rinsed with phosphate-buffered saline, incu-

bated for 5 min in 10^{-3} M EDTA, and harvested by trypsinization. The radioactivity recovered at each step was measured in a liquid scintillation counter. Cell-associated radioactivity prior to and after EDTA rinsing was taken as a measurement of virus binding and uptake, respectively.

(ii) **Total viral DNA production.** Total intracellular viral DNA was measured in whole-cell lysates by dispersed cell assays (49). Briefly, cultures (5×10^5 cells per 60-mm dish) were infected by MVMP at various MOIs, incubated for 2 or 30 h, harvested by trypsinization, and trapped by filtration onto nitrocellulose membranes. Filters were denatured with alkali and neutralized, and viral DNA was quantitated by hybridization to ^{32}P -labeled MVM DNA. The probe was the recombinant plasmid pMM984A containing the entire MVMP genome (19) and was kindly given by D. C. Ward (Yale University, New Haven, Conn.). The probe was labeled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ by nick translation (32). Hybridized radioactivity was quantitated by liquid scintillation spectrometry.

(iii) **Viral capsid protein synthesis.** Cells grown on cover slips were fixed as described above. Viral capsid proteins were stained by indirect fluorescence (27) by using first a 1:50 dilution of rabbit serum raised against empty MVMP capsids and then a 1:400 dilution of fluorescein isothiocyanate-conjugated sheep serum raised against rabbit immunoglobulins (Biosys, Compiègne, France). The anti-viral capsid protein serum was a generous gift from P. Tattersall (Yale University).

(iv) **Production of infectious virus and infectious centers.** Cultures (10^5 cells) were infected at MOIs ranging from 10^{-2} to 10 PFU per cell. For the determination of infectious virus production, cultures were frozen at intervals p.i. and subjected to three freeze-thaw cycles. The suspensions were diluted in minimal essential medium supplemented with 5% fetal calf serum, and the released particles were titrated by plaque assay by the method of Rommelaere et al. (33).

For the determination of infectious centers, appropriate numbers of virus-infected cells were harvested at 3 to 4 h p.i. and seeded on top of 3×10^5 A9 indicator cells in 60-mm dishes. At 3 to 4 h after seeding, the cultures were processed as for plaque assays. The proportion of productively infected cells was determined from the fraction of seeded cells giving rise to plaques.

Electron microscopy. Cells were accumulated at the beginning of the S phase by incubation with an excess of thymidine (7.5 mM; Calbiochem-Behring, La Jolla, Calif.) during 18 to 20 h. Immediately after release from this thymidine block, cells were infected with MVMP (MOI = 10) or mock treated and transferred to Dulbecco modified Eagle minimal essential medium supplemented with 10% calf serum and 1% sodium pyruvate. At various times after MVM or mock infection, with or without subcultures, cells were washed and fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2.5 h at room temperature and processed for electron microscopy by the method of Richards et al. (31), with slight modifications. Briefly, after extensive washes in cacodylate buffer, cells were postfixed for 1 h with 2% OsO_4 in the same buffer at room temperature, dehydrated in ethanol, and embedded in situ in Epon. Ultrathin sections of the cell monolayers, cut parallel to the growth plane, were stained with uranyl acetate and then with lead citrate. Electron micrographs were taken in an AE1 EM6B electron microscope at a 60-kV acceleration voltage.

RESULTS

Transformed phenotypes of *ts* 339/NRK and *ts* 339-*wt* SRA/NRK cells. *ts* 339/NRK cells were transformed by a temperature-sensitive avian sarcoma virus mutant and, as originally described by Graf and Friis (14), they reverted to a morphologically normal phenotype after a temperature shift from 34.5 to 39.5°C for 2 to 3 days. In contrast, the *ts* 339-*wt* SRA/NRK clonal derivative, which was isolated in this study after infection of *ts* 339/NRK cells with a wild-type *src*-containing murine retrovirus, was selected on the basis of the maintenance of a transformed morphology at 39.5°C (data not shown).

The expression of *src* oncogenes was determined in the NRK cell line and its *v-src*-containing derivatives by immunoprecipitation with a specific antiserum after in vivo labeling with ^{35}S methionine. As shown in Fig. 1A, untransformed NRK cells sustained only a low extent of pp60^{src} production that may be due to the activity of the endogenous cellular oncogene. On the other hand, *ts* 339/NRK and *ts* 339-*wt* SRA/NRK cells showed high levels of pp60^{src} synthesis at both 34.5 and 39.5°C. It is noteworthy that *ts* 339/NRK cultures accumulated a three- to fivefold greater amount of pp60^{src} at 34.5°C compared with the same cell line at 39.5°C or with the *ts* 339-*wt* SRA/NRK clone at either temperature.

pp60^{src} from the NRK cell line and its transformed derivatives was tested for its ability to phosphorylate enolase. In vitro kinase assays were performed on *src* proteins immunoprecipitated from cell lysates with MAb 327. No significant kinase activity was observed from parental NRK cells. For equivalent amounts of *src* protein, pp60^{src} from *ts* 339/NRK cells had a threefold higher kinase activity at 34.5 than at 39.5°C. On the other hand, the protein kinase activity of *ts* 339-*wt* SRA/NRK cells was 10- to 20-fold greater than that of *ts* 339/NRK cells at 34.5°C (Fig. 1B).

It follows that the related cell lines used in this study display graded *src* protein kinase activities, ranging from insignificant to low, intermediate, and high levels in NRK cells, *ts* 339/NRK cells at 39.5°C, *ts* 339/NRK cells at 34.5°C, and *ts* 339-*wt* SRA/NRK cells at either temperature, respectively. This result may be put together with the facts that (i) *ts* 339/NRK cells grown at 39.5°C were not completely normalized and showed, in particular, a greater saturation density and anchorage independence than did parental NRK cells, and (ii) the phenotype of *ts* 339-*wt* SRA/NRK cells was hypertransformed compared with that of *ts* 339/NRK cells at 34.5°C (data not shown).

Cell sensitivity to the killing effect of MVMP. Previous studies showed that untransformed NRK cells resist infection with parvovirus MVMP (44a). Therefore it was of interest to determine whether temperature modulation of the transformed phenotype of the *ts* 339/NRK derivative would affect its susceptibility to the parvoviral killing action. The cytopathic effect of MVMP was assessed by measuring either the growth of infected cultures at intervals p.i. or the residual ability of infected cells to form colonies on plastic.

As shown in Fig. 2A, the growth of MVMP-infected *ts* 339/NRK cultures (MOI = 1 PFU per cell) was only slightly inhibited at 39.5°C but was virtually suppressed at 34.5°C. Similar results were obtained after cell infection with MVMP at an MOI of 10 PFU per cell (data not shown). The reduced or arrested growth of MVM-infected *ts* 339/NRK cultures may result from the ability of MVMP to kill a fraction of the cells, the size of which would depend on culture conditions. This possibility was tested by measuring the efficiency of

FIG. 1. Immunoprecipitation and protein kinase assay of pp60^{src} from transformed and untransformed NRK cells. (A) Autoradiograph of a 10% SDS-polyacrylamide gel showing proteins immunoprecipitated with anti-pp60^{src} monoclonal antibodies from cell lysates labeled with [³⁵S]methionine. Equal amounts of trichloroacetic acid-insoluble radioactivity were used for all immunoprecipitations. Exposure time, 6 days. (B) Autoradiograph of a 10% SDS-polyacrylamide gel showing pp60^{src} and enolase phosphorylations from cell lysates incubated with acid-treated enolase and [³²P]ATP. Equivalent amounts of labeled pp60^{src} were used for *ts* 339 and *ts* 339-wt SRA immunoprecipitations. Exposure time, 6 h. Cultures were maintained at 34.5 or 39.5°C, as mentioned on top of lanes. Size markers (in kilodaltons), are on the left.

infected cells in forming colonies on plastic. As shown in Fig. 3, MVMP caused only a slight reduction in the survival of *ts* 339/NRK cells at 39.5°C, consistent with their almost unaffected growth (Fig. 2A). In contrast, MVMP infection proved to be lethal for a large fraction of *ts* 339/NRK cells grown at 34°C. The cytotoxic effect of MVMP, as measured by this assay, results only from the primary infection; indeed, similar cell survivals were obtained in the absence or presence of antiserum directed against MVM capsid proteins, i.e., under conditions allowing or not allowing secondary infections (data not shown). Thus, both cell growth and survival curves show that *ts* 339/NRK cultures are more susceptible to the lytic effect of MVMP at 34.5 than at 39.5°C, i.e., under conditions allowing full expression of the transformed phenotype.

These results raise the question of whether the temperature sensitivity of the *v-src* oncogene product is responsible for such a modulation of the susceptibility of *ts* 339/NRK cells to MVMP as a function of temperature. The role of the oncogene was determined by testing whether the introduction of a wild-type *src* oncogene in these cells would render their sensitivity to MVMP less dependent on temperature. Indeed, the supertransformed *ts* 339-wt SRA/NRK strain was prevented from growing in vitro at both 34.5 and 39.5°C upon infection with MVMP at an MOI of 1 (Fig. 2B) or 10 (data not shown) PFU per cell. Moreover, MVMP drastically reduced the colony-forming ability of *ts* 339-wt SRA/NRK cells grown at either temperature (Fig. 3). The toxic effect of MVMP on supertransformed cells was still somewhat greater at 34.5 than at 39.5°C, a fact that may be the result of a low

FIG. 2. Effect of parvovirus MVMP on the growth of *ts* 339/NRK (A) and *ts* 339-wt SRA/NRK (B) cultures. Mock- or MVMP-treated cells (MOI = 1 PFU per cell) were incubated at 34.5 or 39.5°C and counted at intervals p.i.

FIG. 3. Susceptibility of *ts* 339/NRK and *ts* 339-*wt* SRA/NRK cells to the killing effect of MVMP. Cells were infected with MVMP (MOI = 1 or 10 PFU per cell), and their survival was determined by measuring their residual colony-forming ability on plastic compared with that of mock-treated cells.

extent of intrinsic temperature sensitivity of the parvoviral life cycle (44), the contribution of the *ts* 339 *src* oncogene, or both. However, the resistance accompanying the transition from 34.5 to 39.5°C was less for *ts* 339-*wt* SRA/NRK than for *ts* 339/NRK cells, suggesting that the temperature sensitivity on the *v-src* oncogene in the latter cells plays a major role in the modulation of their susceptibility to MVMP by temperature.

Taken together, these data strongly suggest that the expression of a functional *v-src* oncogene correlates with the sensitivity of the rat cells studied to the cytopathic effect of MVMP. Thus, pp60^{src} may induce qualitative or quantitative modifications of cellular or viral determinants of MVMP cytotoxicity. Whether such modifications are directly involved in the transformed phenotype or are merely coincidental remains to be determined. In any case, the differential MVMP sensitivity of *ts* 339/NRK cells grown at 34.5 versus 39.5°C can be related more particularly to a subset of *src*-induced transformation parameters, namely, those which are reversed at 39.5°C and include the cytoskeletal organization and the ensuing cell morphology (see above).

Cell susceptibility to the MVMP replicative cycle. We have tested whether the differential MVMP sensitivity of *ts* 339/NRK cells grown at 34.5 or 39.5°C is related to their capacity for supporting parvovirus replication at either temperature.

(i) **Ongoing parvoviral life cycle.** MVMP was adsorbed and internalized to similar extents by *ts* 339/NRK cells, irrespective of whether cultures were incubated at 34.5 or 39.5°C (Table 1). Therefore, the hypersensitivity of these cells to the killing effect of MVMP at 34.5°C is unlikely to result from their greater proficiency in viral uptake at this temperature. This conclusion was confirmed by showing that cells grown at either temperature had similar intracellular MVMP DNA contents at 2 h p.i., as measured by hybridization against dispersed cell lysates (data not shown). By using this technique, it was also found that *ts* 339/NRK cells sustained high levels of MVMP DNA amplification at both 34.5 and 39.5°C

TABLE 1. Effect of temperature on the ability of *ts* 339/NRK cells to replicate MVMP^a

Temperature (°C)	Virus uptake ^b (%)	Viral DNA amplification ^c	Capsid protein synthesis ^d (%)	Infectious virus production ^e (PFU/culture)	Infectious center efficiency ^f (%)
34.5	19	470	48	5 × 10 ⁵	54
39.5	17	1200	73	41 × 10 ⁵	52

^a Data shown are from a typical experiment. Similar relative values were obtained in two to three independent experiments.

^b Percentage of inoculated virus which becomes internalized.

^c Ratio of viral DNA contents per culture at 30 h versus 2 h p.i. (MOI = 1).

^d Percentage of immunofluorescence-positive cells 36 h p.i. (MOI = 1).

^e Infectious virus recovered from cultures 120 h p.i. of 10⁵ cells (MOI = 1).

^f Percentage of infected cells (MOI = 1) initiating plaque formation.

(Table 1). Moreover, a large fraction of infected cells produced viral structural proteins at both temperatures, as detected by immunofluorescence staining (Table 1).

Taken together, these data indicate that the resistance to MVMP, as acquired by *ts* 339/NRK cultures upon incubation at 39.5°C, does not coincide with a decrease in their ability to support the above-mentioned viral replicative steps. This feature contrasts with the reported failure of parental untransformed NRK cells to amplify and express MVMP DNA (44a). Thus, *ts* 339/NRK cultures maintained at 39.5°C, although quite resistant to MVMP (Fig. 3), are not equivalent to untransformed NRK cells with respect to their permissiveness to virus replication.

(ii) **Production of infectious MVMP particles.** MVMP was inoculated in the *ts* 339/NRK cultures maintained at 34.5 or 39.5°C, and virus titers were measured at intervals p.i. As shown in Fig. 4, a production of infectious virus took place at both temperatures. For each time point considered, greater amounts of progeny virions were recovered from cultures incubated at 39.5 than from those kept at 34.5°C (Fig. 4), consistent with the higher extent of parvovirus replication achieved at the former temperature for a given interval p.i. (Table 1). The fact that yields of virions and viral products were greater at 39.5 than at 34.5°C, when measured at the same time after infection, can probably be ascribed to the faster progression of the parvoviral life cycle (28) and also to the continued growth of the cultures at the former temperature (Fig. 2A). On the other hand, it came as a surprise that such an efficient production of infectious MVMP virions at 39.5°C was compatible with the proliferation of infected cultures, as analyzed concomitantly (Fig. 2A). The proportion of productively infected cells was determined by measuring the ability of inoculated cells to initiate plaque formation in indicator cultures (infectious center assay). As shown in Table 1, about half of the primarily infected cells (MOI = 1 PFU per cell) gave rise to infectious centers, irrespective of temperature. This proportion is similar to the fraction of cells that were killed by MVMP at 34.5°C (about 50% for an MOI of 1; see Fig. 3). In contrast, at 39.5°C, the fraction of killed cells was significantly lower than that of infectious centers (10 and 52%, respectively, for an MOI of 1; see Fig. 3 and Table 1). This discrepancy raises the unexpected possibility that at least part of the MVMP resistance acquired by *ts* 339/NRK cultures at 39.5°C may result from the ability of a majority of infected cells to release the virus without being killed.

(iii) **Ultrastructural analysis of *ts* 339/NRK cells grown at 39.5°C.** It is expected that virions, including full particles, will become detectable in the majority of MVMP-infected *ts* 339/NRK cells grown at 39.5°C, in spite of the fact that these

FIG. 4. MVMP production in *ts* 339/NRK cells at 34.5 or 39.5°C. Cultures were infected with MVMP (MOI = 1 PFU per cell) and, in parallel to the determination of their growth (see Fig. 3), were analyzed at intervals p.i. for the production of infectious virus.

cells survive and proliferate. This prediction was tested by electron microscopic examination of MVMP- or mock-infected *ts* 339/NRK cultures incubated at 39.5°C.

Mock-treated *ts* 339/NRK cells often displayed micronuclei that may result from lobulation, followed by fragmentation of interphase nuclei. Numerous vesicles containing several individual masses of flocculent and membranous materials were present in the cytoplasm of these cells and became included inside large autophagic vacuoles. In fact, greater autophagic activity distinguished *ts* 339/NRK cells grown at 39.5°C (Fig. 5A) from those maintained at 34.5°C (data not shown).

ts 339/NRK cells, incubated at 39.5°C, were also analyzed at intervals after infection with MVMP, in order to detect viral particles and virus-induced alterations of cellular ultrastructures. Numerous spherical particles having the size of parvoviruses (about 25 nm in diameter) became clearly visible in numerous cytoplasmic vesicles present in all the cells observed (from about 100 to 500) from 24 h p.i. onwards and appeared as a lining on the inner membrane surface of these vesicles (Fig. 5B). As expected of parvoviral bursts, only a fraction of the viral particles appeared to be full (Fig. 5B and E). Infected cells maintained at 39.5°C continued to proliferate (Fig. 2A). Consistently, occasional mitotic figures were observed, showing a more or less even distribution of

virus-containing vesicles between daughter cells (Fig. 5C). Virion-containing vesicles progressively became encumbered with membrane whorls and coalesced into larger vesicles comprising heterogeneous internal material, especially myelinlike structures (Fig. 5D and E). Such vesicles were often trapped within larger autophagic vacuoles (Fig. 5D, E, and F), which may also contain other cellular material, such as mitochondria, ergastoplasmic sacs, and lipid droplets. Some of these vacuoles appeared to be in the process of excreting their content outside the cell (Fig. 5D). Moreover, such vesicles were present in similar amounts in subcultured cells analyzed at 9 days p.i. (Fig. 5F) or as late as 13 days p.i., suggesting that infection may become persistent at 39.5°C.

Taken together, these observations indicate that the proliferation of infected *ts* 339/NRK cultures at 39.5°C is compatible with the accumulation of large amounts of MVMP virions, including full particles, in practically all the cells, consistent with the putative noncytotoxic mode of virus production inferred above.

DISCUSSION

Modulation of the cytolytic action of MVMP by host cell transformation. In the present work, functional expression of a *v-src* oncogene was found to correlate with an increase in the sensitivity of rat cells to the killing effect of MVMP. On the one hand, *ts* 339/NRK cells that are conditionally transformed by a temperature-sensitive mutant of RSV resisted MVMP infection to a much greater extent under conditions reducing the protein kinase activity of pp60^{src} and causing a reversion of the morphological transformation (incubation at 39.5 versus 34.5°C). On the other hand, supertransformation of the *ts* 339/NRK cells with a wild-type *v-src* oncogene led to a concomitant increase in pp60^{src} activity and in the levels of both cell transformation and sensitivity to MVMP, irrespective of whether cells were grown at 34.5 or 39.5°C. These results are in agreement with previous reports (3, 7, 8, 21, 22, 23, 41) showing that a series of in vitro-transformed human and murine cells are more susceptible to the attack of MVMP or H-1 parvovirus than are corresponding normal cells. Such a hypersensitivity is demonstrated for cells transformed by a variety of agents, including several viral or cellular oncogenes (8, 21, 23). In addition to extending these observations to another oncogene, *v-src*, the present study points to the parallel variation of the parvoviral cytopathic effect and the expression of the transformed phenotype in one and the same cell strain, strongly suggesting that the former may be modulated by the latter.

Similar to other mutants of the *v-src* oncogene (16, 48, 50),

FIG. 5. Ultrastructure of mock- and MVMP-infected *ts* 339/NRK cells grown at 39.5°C. Electron micrographs of mock-treated cells (A) or MVMP-infected cells (MOI = 10 PFU per cell) (B to F) incubated at 39.5°C. Parasynchronous cultures were examined at 24 h (B), 36 h (C), 72 h (D), 150 h (A and E), and 9 days (after one subculture [F]) after mock or MVMP infection. N, Nucleus; e, dilated rough endoplasmic reticulum cisternae; m, mitochondria. (A) Two large vacuoles containing cellular debris are observed in the cytoplasm. Magnification, $\times 22,500$. (B) Vesicles containing empty and full viral particles, as indicated by small and large arrowheads, respectively, are observed in the cytoplasm. Magnification, $\times 55,000$. (C) Mitotic cell division. Virus-containing vesicles (arrowheads) are distributed between the two daughter cells connected by the midbody. Magnification, $\times 20,000$. (D) Cell in the process of excreting undigested material into the medium, including virus-containing vesicles (arrowheads) coming from a large vacuole (large arrow) whose limiting membrane has fused with the plasma membrane. On the right side, two vacuoles (autolysosomes) (small arrows) contain virus at their periphery and myelinic figures. Magnification, $\times 13,000$. (E) Cytoplasm on the left side, near the cell surface, contains a large vacuole filled with heterogeneous material and many virions, including empty and full particles (small and large arrowheads, respectively). On the right side, more vacuoles, whose limiting membrane appears interrupted in places, contain myelinic figures and viral particles. Magnification, $\times 39,000$. (F) Lysosomal vacuoles containing myelinic figures and viral material (arrowheads) are observed in the cytoplasm near dilated rough endoplasmic reticulum cisternae (e). Magnification, $\times 39,000$. Figure 5D through F continues on the following page.

FIG. 5—Continued

the *ts* 339 strain involved in this study is characterized by a reduced tyrosine kinase activity *in vitro* and a defect in the induction of morphological transformation at 39.5 versus 34.5°C. The mechanism by which the pp60^{v-src} protein may potentiate the killing action of MVMp is not known. The pp60^{v-src} protein is mainly located at the inner face of the plasma membrane (particularly at regions of cell-to-cell contact and adhesion plaques) but is also associated with intracellular membranes and with the cytoskeleton (6, 16, 27). It remains to be elucidated whether such an intracellular location of pp60^{v-src} accounts for the greater autophagic activity occurring at 39.5 than at 34.5°C. Although several substrates of the tyrosine kinase activity of pp60^{v-src} have been identified (6, 16, 50), the targets whose phosphorylation is temperature sensitive and which may contribute to morphological transformation and susceptibility to MVMp remain to be determined.

Dissociation of MVMp production from cell killing. The reason for the greater resistance of *ts* 339/NRK cells to MVMp upon temperature shift to 39.5°C is a matter of speculation. Previous work showed that transformed cells which are sensitized to parvoviral infection have a greater capacity for replicating (3, 7, 8, 21) and expressing (7, 8, 21) viral DNA, although progeny virions are not necessarily produced. This feature strongly suggests that the enhancement of the parvoviral lethal action on transformed cells may be due, at least in part, to their ability to synthesize higher amounts of specific viral products, such as the nonstructural protein(s) suspected of being cytotoxic (25, 30). Results presented in this report suggest that the up-modulation of the viral life cycle may not be the only reason for the sensitization of transformed cells to parvoviral attack. Indeed, the greater resistance of *ts* 339/NRK cells to MVMp at 39.5°C does not coincide with a reduction in the efficiency of any of the steps of virus replication measured, including the production of infectious particles. Therefore, the reversion of transformation in this system uncovered an apparent dissociation of permissiveness from cytopathogenicity. If this dissociation is confirmed by further analyses of parvovirus replication, the possibility would arise that transformation-modulated cellular determinants of the killing effect of MVMp may be involved not only in virus replication but also in cell responsiveness to viral cytotoxic products.

It is worth noting that the resistance of *ts* 339/NRK cultures to MVMp at 39.5°C is compatible not only with virus replication but also with the release of a burst of infectious progeny virions. Different hypotheses may be put forward to reconcile the much reduced cytopathic effect of MVMp at 39.5 versus 34.5°C with the maintenance of a level of virus production that is similar or even higher at the former temperature. On the one hand, productively infected cells may only represent a very minor fraction of the cultures. This possibility is not supported by the fact that the proportion of infectious centers is similar at 39.5 and 34.5°C and much higher than the fraction of cells killed at the former temperature. On the other hand, a noncytotoxic mode of virus production may take place in *ts* 339/NRK cells grown at 39.5°C. Such an unconventional release mechanism may normally be masked or suppressed by a cytotoxic process that is no longer active in the partial revertants. This hypothesis is consistent with our electron microscopic observations showing that at 39.5°C, all *ts* 339/NRK cells observed become filled with cytoplasmic inclusions comprising empty and full particles, while cultures continue to proliferate. It is worth noting that virions accumulated into cytoplasmic vesicles are often enclosed in larger autophagic

vacuoles and are occasionally seen to be released from the cells. It should also be stated that such a subcellular confinement of the virus may account for the characteristic punctuated immunofluorescence staining of the cytoplasm for viral structural proteins in *ts* 339/NRK cells grown at 39.5°C, in contrast with the diffuse staining observed for cultures incubated at 34.5°C (data not shown). Parvovirus trapping in cellular vesicles has been described previously in relation to the uptake of parental particles, although its role in virus transfer to the nucleus is unclear (9, 31). Virus-containing organelles in *ts* 339/NRK cells grown at 39.5°C are much more complex than the endocytotic vesicles thought to be involved in virus uptake. However, entry of secondarily infecting virions has to be considered as a possible contribution to the intracellular accumulation of viral particles seen by electron microscopy, at least for late times after inoculation. Alternatively, virus transit in a subcellular secretory autophagic compartment may constitute a potential step of a noncytotoxic mechanism for the production of progeny particles. This issue is particularly intriguing, since parvovirus release is usually considered to result from generalized cell degeneration leading to the disruption of the plasmic membrane (9, 31, 36). On the other hand, noncytotoxic virus shedding takes place in cells infected with certain enveloped viruses, such as retroviruses that bud at the cell surface (43). However, as expected for nonenveloped viruses, no budding of parvovirus particles has been observed in infected *ts* 339/NRK cells grown at 39.5°C. Taken together, our results raise the possibility of an alternative noncytotoxic release mechanism related to virus sequestration and exocytosis. Work to test the latter possibility is presently in progress in our laboratory.

The permissiveness of normalized *ts* 339/NRK cultures at 39.5°C contrasts with the failure of the original NRK cell line to support the MVMp life cycle (44a). The present work does not allow one to identify the distinctive feature(s) of the former cells that accounts for their permissiveness in the absence of cytopathic effect at 39.5°C. The proficiency of *ts* 339/NRK cells in parvovirus replication at 39.5°C may be related, at least in part, to the residual pp60^{v-src} activity that is shown to exist under these conditions. An alternative, nonexclusive possibility would be that the *ts* 339/NRK clonal derivative undergoes an additional unspecified change stimulating the parvoviral life cycle, irrespective of temperature. Consistently, a BALB/c 3T3 variant cell line has been isolated which appears to have a similar MVM-resistant phenotype, in that resistance is modulated by transformation and that the untransformed cells produce and export progeny virus without concomitant cell lysis (R. Moir and P. Tattersall, personal communication). It follows that the concurrence of full permissiveness to MVMp and resistance to virus-induced killing is not unique to the *ts* transformant used in this study, even though parvovirus replication proves to be lytic in most permissive cellular systems analyzed so far. Similarly, release of simian virus 40 virions from epithelial cells has been recently reported to occur without cell lysis (4). Taken together, these observations indicate that cell disruption is not the obligatory outcome of a productive infection by nonenveloped viruses.

ACKNOWLEDGMENTS

We are greatly indebted to T. Graf for his generous gift of the *ts* 339/NRK cell line. We thank P. Tattersall for anti-MVM capsid protein serum and B. Vennström for M-src producing cells. We also thank J. J. Cornelis, P. Tattersall, and S. Cotmore for stimulating discussions; K. E. Boulukos for her critical reading of the manu-

script; and N. Duponchel for technical assistance. We are grateful of N. Devassine, M. C. Bouchez, and S. Redjem for taking care of the manuscript.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Association pour la Recherche sur le Cancer, and Institut Pasteur de Lille (France), the Caisse Générale d'Epargne et de Retraite (Belgium), and the Commission of the European Communities. N.S. and B.v.H. were supported by grants from the Association pour la Recherche sur le Cancer and Centre National de la Recherche Scientifique, respectively. M.G. is a senior research associate of the Belgian National Fund for Scientific Research.

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