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# The v-*erbB* Oncogene Confers Enhanced Cellular Susceptibility to Reovirus Infection

JAMES E. STRONG AND PATRICK W. K. LEE*

*Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, Calgary, Alberta, Canada T2N 4N1*

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**We have previously demonstrated that two mouse cell lines that are poorly infectible by reovirus become highly susceptible upon transfection with the gene encoding the epidermal growth factor receptor (EGFR) (J. E. Strong, D. Tang, and P. W. K. Lee, Virology 197:405–411, 1993). This enhancement of infection efficiency requires a functional EGFR, since such an enhancement is not observed in cells expressing a mutated (kinase-inactive) EGFR. The additional finding that reovirus is capable of directly binding to the N-terminal ectodomain of the EGFR (D. Tang, J. E. Strong, and P. W. K. Lee, Virology 197:412–414, 1993) has led us to question whether this interaction is required for the activation of a signalling cascade that somehow augments the ensuing infection process. In the present study, we address this question, using cells transfected with the v-*erbB* oncogene, which encodes a protein structurally related to the EGFR but lacking a large portion of the N-terminal ligand-binding domain. The v-erbB protein also possesses ligand-independent, constitutive tyrosine kinase activity. Control NIH 3T3 cells, which are poorly infectible by reovirus (serotype 3, strain Dearing), and NIH 3T3 cells transfected with the v-*erbB* oncogene (THC-11) were assayed for their susceptibilities to reovirus infection. Infectivity was determined by immunofluorescent detection of viral proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of radiolabeled cells, and plaque titration. All three assays demonstrated a drastically higher degree of susceptibility to infection in the THC-11 cell line. This enhanced susceptibility was found to be abrogated by treatment of the cells with genistein, an inhibitor of tyrosine protein kinases, but only partially by treatment with daidzein, an inactive analog of genistein. We propose that the mechanism of enhancement of infection efficiency conferred by EGFR and v-erbB is through the opportunistic utilization by the virus of an already activated signal transduction pathway.**

It is believed that the susceptibility of a cell to virus infection depends in large measure on the availability of cell surface receptors capable of interacting with the invading virus. Specific examples include the demonstration that rodent cells that are normally resistant to poliovirus or measles virus infection become susceptible upon transfection with cDNA clones encoding the respective receptor proteins of human origin (9, 23, 25). However, there is evidence that in some cases (e.g., poliovirus and human rhinoviruses), receptors are also present in tissues and cell types that are not sites of viral replication (12, 13, 19, 34). This evidence suggests that additional cellular factors are involved in controlling the outcome of infection. Little about the identities and functions of these factors is known.

For mammalian reoviruses, the cell surface recognition signal is sialic acid (17, 18, 28). Since many plasma membrane glycoproteins are sialylated, it follows that reovirus should, at least in theory, bind to multiple sialoglycoproteins rather than to a single homogeneous species. This was indeed found to be the case (5). However, the mere presence of sialic acid on the cell surface is insufficient to ensure a productive reovirus infection, which in turn implies the involvement of additional host cell factors in this process. Earlier studies have provided a few interesting clues. Hashiro et al. (20) reported that certain virally and spontaneously transformed cell lines of murine origin were susceptible to cytotoxic induction by reovirus, whereas normal human and subhuman primate cells, primary mouse cells, normal rat kidney cells, and baby hamster kidney cells were not. Duncan et al. (11) found that normal and simian virus 40-transformed WI-38 cells exhibited different sensitivities to reovirus infection, with cytopathology observed only in the transformed cells and not in normal cells, which nonetheless produced virus for a sustained period. Collectively, these observations suggest that reovirus infection efficiency is somehow linked to the transformed state of the cell. However, the molecular basis of this correlation remains obscure.

Recently, we reported that two mouse cell lines (NR6 and B82) expressing no epidermal growth factor receptors (EGFR) were relatively resistant to reovirus infection, whereas the same cell lines transfected with the gene encoding EGFR manifested significantly higher susceptibility (35). This enhancement of infection efficiency requires a functional EGFR, since it was not observed in cells expressing a mutated (kinase-inactive) EGFR. Thus, the reovirus infection process is closely coupled to the EGFR-mediated cell signal transduction pathway. Furthermore, we found that reovirus is capable of directly binding to the N-terminal extracellular domain of EGFR (36). Taken together, these observations suggest two alternative explanations for the augmentation of reovirus infection by functional EGFR. The first possibility is that reovirus plays an active role by first binding to EGFR, thereby activating the tyrosine kinase activity of the latter and triggering a cell signalling cascade which is somehow required for subsequent steps of the infection process. This mechanism would be similar to that proposed for *Salmonella typhimurium* invasion of mammalian cells (15, 26). The second possibility is that reovirus takes advantage of an already activated signal transduction pathway conferred by the presence of functional EGFR on the host cell. In this case, the binding of the virus to EGFR would

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, 3330 Hospital Dr., N.W., Calgary, Alberta, Canada T2N 4N1. Phone: (403) 220-7548. Fax: (403) 270-8520.

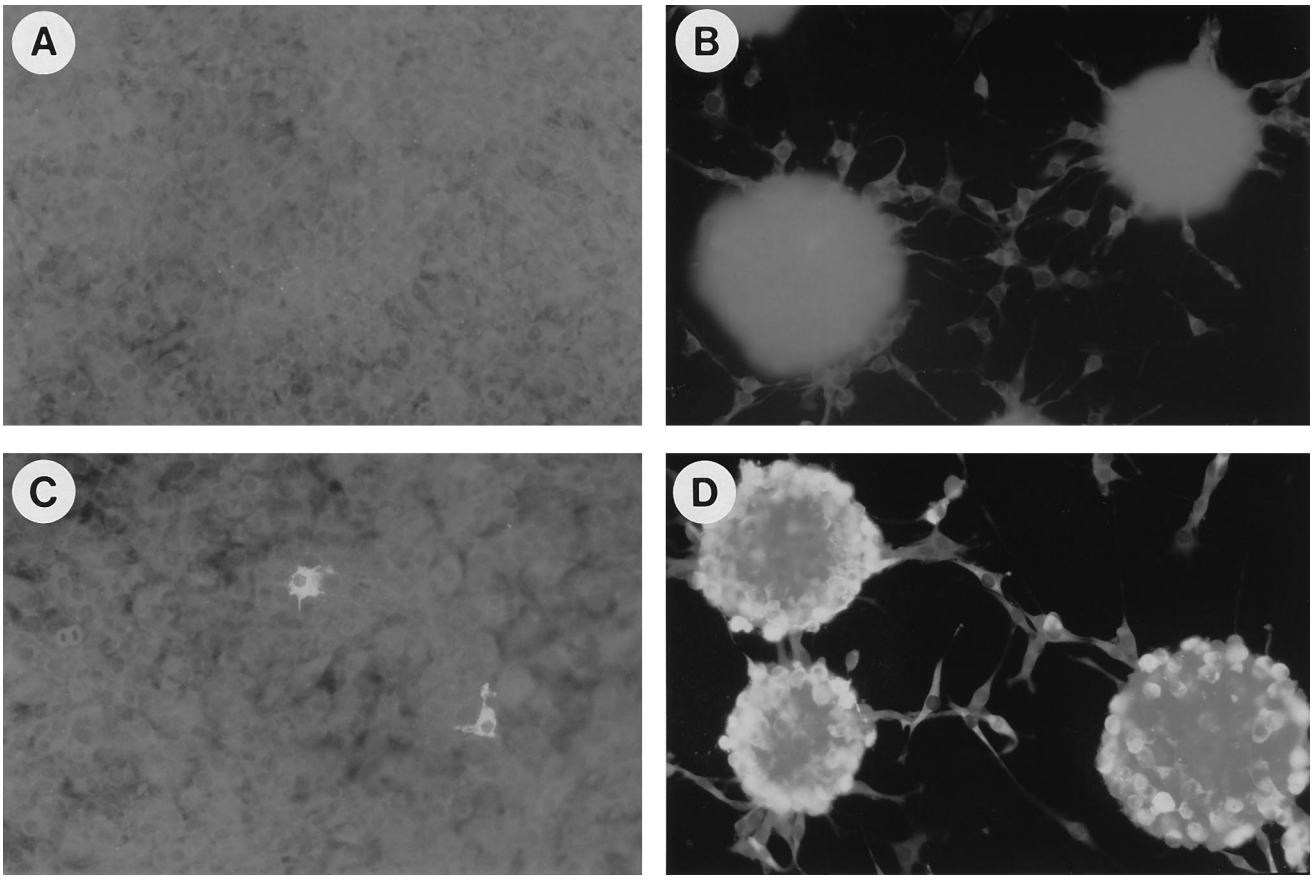


FIG. 1. Immunofluorescence assay of viral proteins expressed in reovirus-infected NIH 3T3 and THC-11 cells. Cells were infected with reovirus at an estimated MOI of 10 PFU per cell. At 48 h postinfection, cells were fixed, processed, and reacted with rabbit anti-reovirus type 3 antibody and then with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. The magnification for all pictures is ×132. (A) Uninfected NIH 3T3 cells. (B) Uninfected THC-11 cells. (C) Infected NIH 3T3 cells. (D) Infected THC-11 cells.

represent a fortuitous event that is unrelated to the ensuing infection.

To determine which of these two possibilities is more likely, we used the v-*erbB* oncogene to see whether it can confer infectibility to cells that are normally resistant to reovirus infection. The v-*erbB* oncogene, originally discovered as the transforming gene of avian erythroblastosis virus, is believed to have arisen through retroviral transduction of the cellular homolog, c-*erbB*, which encodes a growth factor receptor homologous to EGFR involved in the signalling of growth of erythroblasts (10, 27). During this transduction event, the avian erythroblastosis virus provirus genome is presumably inserted within a specific stretch of the c-*erbB* intron, effectively separating the portion encoding the ligand-binding domain from that encoding the transmembrane and the cytoplasmic regulatory domain (14, 24, 29). The resultant gene therefore encodes a receptor with a truncated extracellular binding domain (lacking amino acids 1 to 555 compared with human EGFR) possessing ligand-independent, constitutive tyrosine kinase activity (14, 22, 24, 29, 37). The v-*erbB* oncogene also contains point mutations and deletions within the cytoplasmic domain (6, 39) which have been shown to confer further transforming capabilities (2, 16, 32, 39), including the ability to transform fibroblasts (33).

The parental cell line used in the previous study, NR6, is a derivative of the Swiss 3T3 mouse fibroblast and manifests little susceptibility to reovirus (type 3, strain Dearing) infection (35). Since the similar NIH 3T3 cell line has been used extensively to assess the transforming activities of oncogenes, we selected these cells for our study of the effects of transformation with v-*erbB* on reovirus infection. Like NR6 cells, NIH 3T3 cells express a low number of EGFR and do not respond mitogenically to epidermal growth factor (8, 38). For this study, NIH 3T3 parental cells and NIH 3T3 cells transfected with the v-*erbB* oncogene (designated THC-11) were obtained from H.-J. Kung (Case Western Reserve University) (3). Compared with the parental NIH 3T3 cells, the THC-11 cells are highly transformed, with individual cells assuming an exaggerated spindle shape and losing most of their contact inhibition and with the majority of the growth taking place in large clumps of aggregated cells (Fig. 1).

The initial assessment of viral infectivity was carried out by indirect immunofluorescent microscopy (Fig. 1). For these studies, the NIH 3T3 and THC-11 cells were grown on coverslips and infected with reovirus at a multiplicity of infection (MOI) of ~10 PFU per cell or were mock infected. At various times postinfection, the cells were fixed in an ethanol-acetic acid (20:1) mixture for 5 min and then rehydrated by sequential washes in 75, 50, and 25% ethanol, followed by four washes with phosphate-buffered saline (PBS). The fixed and rehydrated cells were then exposed to the primary antibody (rabbit polyclonal anti-reovirus type 3 serum diluted 1/100 in PBS) for 2 h at room temperature. Following three washes with PBS, the cells were exposed to the secondary antibody (goat anti-rabbit

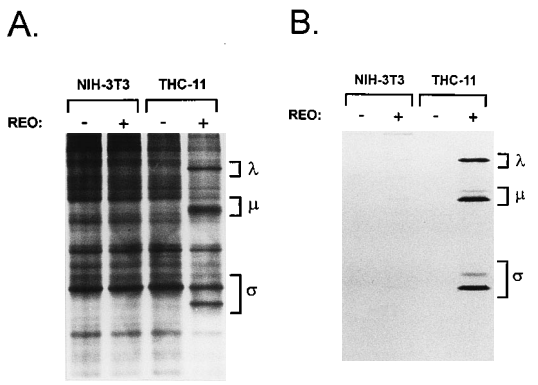


FIG. 2. Reovirus protein synthesis in mock-infected and reovirus-infected NIH 3T3 and THC-11 cells. Cells infected with reovirus (MOI ≈ 10 PFU per cell) and mock-infected cells were labeled with [$^{35}$S]methionine from 12 to 48 h postinfection. Lysates were then prepared and either analyzed directly by SDS-PAGE (A) or immunoprecipitated with the polyclonal anti-reovirus type 3 serum and then analyzed by SDS-PAGE (B). The positions of reovirus proteins are indicated on the right.

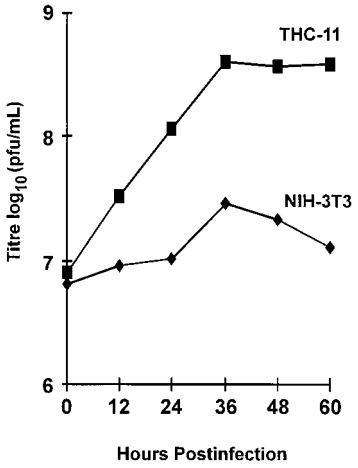


FIG. 3. Titers of reovirus produced by infected NIH 3T3 and THC-11 cells at various times postinfection. NIH 3T3 and THC-11 cells grown on 24-well plates were infected with reovirus at an estimated MOI of ~10 PFU per cell. At various times indicated, cells were harvested and lysed in PBS containing 1% Nonidet P-40 and 0.5% sodium deoxycholate. Virus yields were determined by plaque titration on L-929 cells. Data shown are the averages of three separate titrations ($10^8$ PFU/ml ≈ 200 PFU per cell).

immunoglobulin G [whole molecule]-fluorescein isothiocyanate conjugate diluted 1/100 in PBS containing 10% goat serum and 0.005% Evan's blue counterstain) for 1 h at room temperature. Finally, the fixed and treated cells were washed three more times with PBS and then once with double-distilled water, dried and mounted on slides in 90% glycerol containing 0.1% phenylenediamine, and viewed with a Zeiss Axiophot microscope on which a Carl Zeiss camera was mounted (the magnification for all pictures was ×132). The results (Fig. 1) indicate that only a very small percentage (2 to 5%) of parental NIH 3T3 cells expressed viral proteins at 48 h postinfection. In contrast, over 60% of THC-11 cells expressed viral proteins at this time point. These results are consistent with those of previous studies which showed that reovirus causes preferential cytotoxicity in transformed cells (11, 20), although the precise nature of transformation in these earlier studies was not characterized.

To further demonstrate that viral protein synthesis is more efficient in the v-*erbB*-transformed cells, cells were continuously labeled with [$^{35}$S]methionine from 12 to 48 h postinfection and the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results (Fig. 2A) show clearly that the level of viral protein synthesis was significantly higher in THC-11 cells than in the parental NIH 3T3 cells. The identities of the viral bands were confirmed by immunoprecipitation of the labeled proteins with polyclonal anti-reovirus type 3 antibodies (Fig. 2B). Since uninfected NIH 3T3 and THC-11 cells displayed comparable levels of cellular protein synthesis (Fig. 2A) and doubling times (data not shown), the observed difference in the level of viral protein synthesis could not be due to intrinsic differences in growth rates or translation efficiencies for the two cell lines.

To determine whether the increase in viral protein synthesis in the v-*erbB*-transformed cells corresponded to an increased output of infectious virus, lysates prepared from the infected cells harvested at various times postinfection were subjected to plaque titration. As can be seen from Fig. 3, the output of virus from THC-11 cells was significantly higher (~13-fold at the 36-h time point) than that obtained from NIH 3T3 cells.

These data collectively demonstrate that the introduction of the v-*erbB* oncogene into NIH 3T3 cells confers a significantly higher reovirus infection efficiency. This observation was similar to that previously made with NR6 cells transfected with human EGFR (35). This conference of infectibility occurs despite the truncation of the extracellular ligand-binding domain of the normal c-*erbB* or the homologous EGFR. It is therefore probable that reovirus does not require the binding to the extracellular domain of these receptors in order to infect. Rather, it is more likely that it requires the activation of the intrinsic tyrosine kinase activities that these receptors possess. We have already demonstrated that the expression of mutant EGFR that are fully capable of binding epidermal growth factor (and that have similar $K_d$s for epidermal growth factor binding) but that lack the kinase activity, and therefore the signal transducing function, are not capable of augmenting the viral infection efficiency (35). It was therefore suspected that the v-*erbB* oncogene also augments viral infectivity through the constitutive activation of tyrosine kinase activity.

To test this idea, we utilized the tyrosine kinase inhibitor genistein to determine if the chemical inhibition of tyrosine kinase activity could interfere with the enhancement of viral infectivity conferred by the v-*erbB* oncogene. Genistein, an isoflavone with specific tyrosine kinase inhibitory activity, has been used to study the physiologic role of tyrosine phosphorylation in a number of different cellular systems (1, 21, 31). For these studies, the NIH 3T3 and THC-11 lines were treated with 20 μg of genistein per ml or mock treated 6 h prior to infection with reovirus. The cells were then continuously labeled with [$^{35}$S]methionine for 36 h beginning at 12 h postinfection, and labeled proteins were either directly analyzed by SDS-PAGE (Fig. 4A) or immunoprecipitated with the antireovirus serum prior to SDS-PAGE analysis (Fig. 4B). The results demonstrate that genistein treatment prevented the enhancement of viral protein synthesis conferred by the v-*erbB* oncogene. The effect of genistein on the parental NIH 3T3 cells was minimal in terms of the overall level of protein synthesis in both infected and mock-infected cultures (Fig. 4A). Furthermore, genistein had only a very slight adverse effect on the low-level synthesis of viral proteins in this parental cell line (Fig. 4B). However, this was not the case for the THC-11 cells. Expression of reovirus proteins in these cells was drastically reduced by genistein treatment. Interestingly, host cell protein synthesis also appeared to be similarly affected. It thus appears that cells

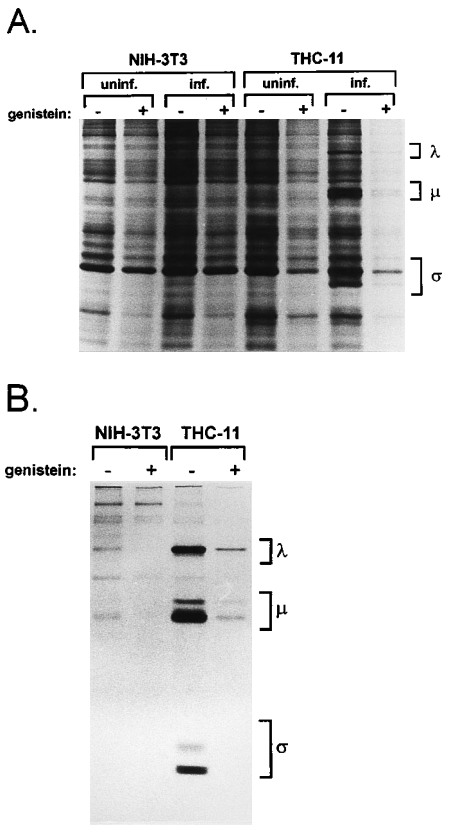


FIG. 4. The effects of genistein on host and viral protein synthesis in NIH 3T3 and THC-11 cells. Genistein (dissolved in dimethyl sulfoxide) or dimethyl sulfoxide alone was added to monolayers of NIH 3T3 and THC-11 cells (final concentration of genistein, 20 μg/ml) 6 h prior to infection with reovirus (MOI ≈ 10 PFU per cell). The cells were labeled with [$^{35}$S]methionine from 12 to 48 h postinfection in the presence or absence of genistein. Lysates were then prepared from harvested cells, and the equivalents of $\sim 3 \times 10^4$ cells were either analyzed directly by SDS-PAGE (A) or immunoprecipitated with the polyclonal anti-reovirus type 3 serum and then analyzed by SDS-PAGE (B). The positions of reovirus proteins are indicated on the right.

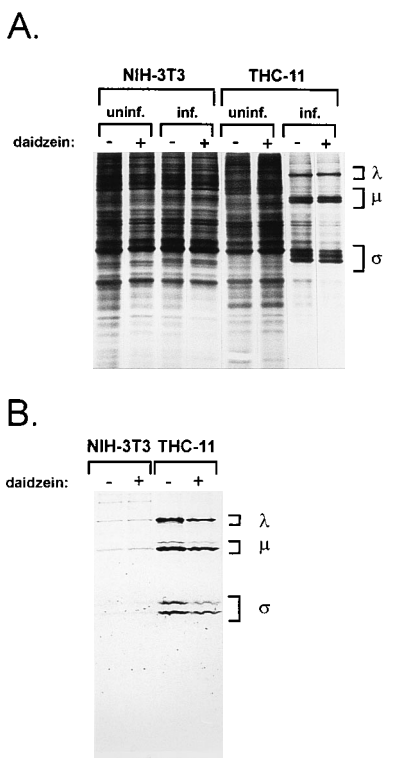


FIG. 5. The effects of daidzein on host and viral protein synthesis in NIH 3T3 and THC-11 cells. Daidzein treatment (final concentration, 20 μg/ml), infection with reovirus, and labeling of cells were carried out as described in the legend to Fig. 4. Cell lysates were either analyzed directly by SDS-PAGE (A) or immunoprecipitated with the polyclonal anti-reovirus type 3 serum and then analyzed by SDS-PAGE (B). The positions of reovirus proteins are indicated on the right.

containing EGFR or its homologs become dependent on tyrosine kinase activation for efficient translation of their mRNAs, and any acute disruption of this early activation may adversely affect subsequent protein synthesis. Indeed, a similar decrease in protein synthesis in response to genistein has also been observed in primary neuronal cultures (21). That the observed effect of genistein on infected THC-11 cells was due to an inhibition of tyrosine kinase activity was further confirmed by the demonstration that daidzein, a genistein analog with significantly less inhibitory activity on tyrosine kinase (1), manifested only partial inhibition of viral protein synthesis (Fig. 5A and B) and viral output (data not shown) from THC-11 cells. We cannot exclude the possibility that the disruption by genistein of other (i.e., non-EGFR-mediated) tyrosine kinase activities is responsible for the observed effects on viral growth in THC-11 cells. However, with the expression of v-erbB being the only difference between NIH 3T3 and THC-11 cells and considering the fact that tyrosine kinase activation is the first step in the EGFR (erbB) signalling pathway, our present results suggest that the enhanced infectibility of THC-11 is probably due to the EGFR-mediated tyrosine kinase activity. The previous observation that tyrosine kinase-defective EGFR were not capable of conferring enhanced viral infectibility to host cells (35) is compatible with this view. It is interesting to note in this regard that acute disruption of tyrosine kinase activity by genistein also results in the inhibition of replication of herpes simplex virus type 1 (40).

The present evidence that the v-*erbB* product, lacking a large portion of the extracellular ligand-binding domain and possessing constitutive tyrosine kinase activity, confers infectibility to cells suggests that the capability of reovirus to bind directly to EGFR, as was observed previously, is likely fortuitous and inconsequential. This notion is further supported by the additional observation that the binding of even high MOIs of reovirus to human epidermoid carcinoma cell line A431, which possesses a large number of EGFR (7) and which is susceptible to reovirus infection (4), fails to trigger tyrosine phosphorylation of the EGFR itself or other cellular substrates to any significant extent (unpublished data). It appears, therefore, that the mechanism of enhancement of infection efficiency conferred by EGFR or v-*erbB* is through the opportunistic utilization of an already activated signal transduction pathway. Since it has been well established that the activation of EGFR signalling triggers many downstream events (including changes in protein phosphorylation patterns, changes in intracellular ion levels, as well as changes in transcription and translation regulation), identification of the factor(s) directly involved in augmenting reovirus infectibility represents a formidable task. In this regard, the availability of a number of NIH 3T3-derived cell lines transformed with constitutively active oncogenes downstream of EGFR should prove to be invaluable for this task. Using this approach, we have obtained preliminary data which suggest that activated ras alone also confers enhanced reovirus infectibility. Current efforts are therefore focused on

the EGFR-ras pathway. The identification of an intracellular component(s) whose activation plays a direct role in promoting viral replication would represent a major step towards understanding the tissue tropism of reovirus as well as that of other viruses, such as the parvoviruses which also seem to target transformed cells (30).

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