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Polyomavirus Middle T Protein Encoded by a Retrovirus Transforms Nonestablished Chicken Embryo Cells

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A murine retrovirus encoding the middle T protein of polyomavirus infected and transformed nonestablished chicken embryo cells. The infected cultures formed colonies in soft agar-containing medium and released infectious transforming virus. Middle T protein expressed in the transformed chicken cells associated with $p60^{c\text{-}src}$ and, in immunoprecipitates, enhanced the tyrosine protein kinase activity of $p60^{c\text{-}src}$.

Transformation of cultured cells by polyomavirus is generally restricted to several rodent species (17). The middle T (mT) protein of polyomavirus is both necessary and sufficient to transform many established mouse and rat cell lines (8, 11, 14, 16, 18). However, the large T and small T proteins appear to contribute to stable transformation of nonestablished rat cells and also to the formation of tumors (12, 16). The mT protein in transformed cells forms a complex with the cellular proto-oncogene product $p60^{c\text{-}src}$ (2, 6, 7) and enhances its protein kinase activity (2, 4). We previously characterized a recombinant retrovirus mT-murine leukemia virus (MLV) encoding the mT protein of polyomavirus which transforms mouse and rat cell lines (8). Here we report the ability of an amphotropic pseudotype of mT-MLV [mT-MLV(ampho)] to infect and transform nonestablished chicken embryo cells. Chicken embryo cells that were infected by the recombinant retrovirus expressed an mT protein which associated with chicken $p60^{c\text{-}src}$ and enhanced its protein kinase activity. The infected cells were transformed, as judged by growth in soft agar, and released infectious mT-MLV(ampho).

mT-MLV(ampho) was produced by infecting a nonproducer rat cell line, which was transformed by mT-MLV, with the amphotropic MLV strain 1504A (15). Supernatants of the infected cultures were used to infect chicken embryo cells. Parallel cultures were infected with the Schmidt-Ruppin A strain of Rous sarcoma virus [RSV(SR-A)]. After several days, the cultures infected with mT-MLV(ampho) or RSV(SR-A) had the appearance of transformed cells (more rounded, less adherent, and more rapid acidification of the culture medium than the uninfected cultures), with the effects being more pronounced in the RSV(SR-A)-infected cultures. We also suspended newly infected cells in agar-containing medium to test for anchorage-independent growth. Both viruses induced anchorage-independent growth of the infected cells. Typical cultures are shown in Fig. 1. Preparations of each virus containing equal numbers of focus-forming units induced equal numbers of colonies of anchorage-independent chicken cells. Approximately 10% of the infected cells gave rise to colonies in the agar-containing medium.

Several colonies transformed by each virus were isolated from agar and passaged in liquid culture at 4-day intervals. After about 20 population doublings, the cultures grew more slowly and showed other signs of senescence. Before senescence, karyotype analysis was done to verify that the cells were chicken cells (data not shown). We concluded that mT-MLV(ampho) can transform chicken embryo cells to anchorage-independent growth.

To study the expression of the mT protein, chicken embryo cells were infected with mT-MLV(ampho) and passaged every 3 to 4 days by seeding at 2×10^6 cells per 100-mm-diameter plate. At 1 day before harvesting, the cells were seeded at 3×10^6 cells per 100-mm-diameter plate. Polyomavirus-transformed F2408 (10) cells grown in Dulbecco-Vogt modified Eagle medium (DME) supplemented with 10% calf serum were seeded in parallel. After three washes with ice-cold Tris-buffered saline, the cells were lysed by harvesting into 1 ml of RIPA buffer (10 mM sodium phosphate [pH 7.0], 1% Nonidet P-40, 1% sodium deoxycholate, 1% sodium dodecyl sulfate, 150 mM sodium chloride, 1% trasylol [Mobay Chemical Corp., New York, N.Y.]) and by incubating at 4°C for 15 min. After the lysates were clarified by centrifugation at $13,000 \times g$ for 30 min, 150 μl of chicken lysates or 100 μl of transformed rat cell lysates were used for immunoprecipitation. Immunoprecipitation was done at 4°C (4, 9) by adding to the lysates either 10 μl of a polyomavirus rat antitumor serum for 1 h or by adding 2 μl of monoclonal antibody (MAb) 327 (a monoclonal antibody which was raised against $p60^{v\text{-}src}$ and which also recognizes $p60^{c\text{-}src}$ [13]) for 40 min and then by adding 2 μl of rabbit anti-mouse serum (Cappel Laboratories, Cochranville, Pa.) for 20 min. MAb 327 was kindly provided by Joan Brugge. Immunoprecipitates were collected by adding 40 μl of Formalin-fixed Cowan strain *Staphylococcus aureus* (Calbiochem-Behring, LaJolla, Calif.) to the anti-T precipitates or by adding 10 μl of fixed *S. aureus* to the anti-C precipitates and then by washing three times with RIPA buffer and once with kinase buffer (100 mM PIPES [piperazine-*N*, *N'*-bis(2-ethanesulfonic acid)] [pH 7.0], 10 mM $MnCl_2$, 10 mM dithiothreitol) containing 0.5% Nonidet P-40. The pellet of *S. aureus* with bound proteins was suspended in 20 μl of kinase buffer containing 20 μCi [^{32}P]ATP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and incubated at 30°C for 30 min. The reaction was terminated by adding 1 ml of RIPA buffer followed by centrifugation. All lysates were suspended in 15 μl of sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 50 mM Tris hydrochloride [pH 6.8], 20% 2-mercaptoethanol) and analyzed on sodium dodecyl sulfate-polyacrylamide gels. The results of this experiment are shown in Fig. 2.

As a control, the proteins phosphorylated in immunoprecipitates of polyomavirus-transformed F2408 rat cell lysates were analyzed. mT protein and $p60^{c\text{-}src}$ were seen with MAb

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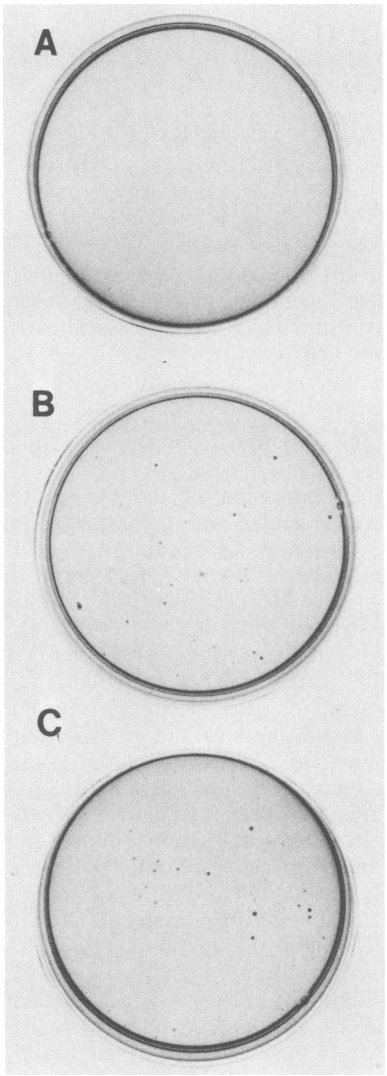

FIG. 1. Anchorage-independent growth of transformed chicken embryo cells. After explantation (3 days), chicken embryo cells (SPAFAS, Inc., Norwich, Conn.) were seeded at 10^7 cells per 100-mm-diameter plate. The next morning they were infected with serial 10-fold dilutions of mT-MLV(ampho) or RSV(SR-A) in 1 ml of DME containing 2 μg of polybrene per ml to facilitate virus adsorption. After 2 h at 37°C, the medium was changed to DME supplemented with 2% tryptose-phosphate (Difco Laboratories, Detroit, Mich.), 1% calf serum (GIBCO Diagnostics, Madison, Wis.), and 1% heat-inactivated (55°C for 1 h) chicken serum (GIBCO). At 6 h after infection, the cells were trypsinized and suspended at a density of 1.5×10^6 cells in 5 ml of DME supplemented with 10% tryptose phosphate, 4% calf serum, 1% heat-inactivated chicken serum, and 0.35% Bacto-Agar (Difco). The cell suspension was added to chilled 60-mm-diameter plates containing a 2-ml base of the same medium but supplemented with 0.6% agar. After the agar had solidified, triplicate plates at each dilution were returned to a 37°C incubator for 21 days. Shown are uninfected chicken embryo cells (A), cells infected with 10^4 focus-forming units of mT-MLV(ampho) per ml (on the F2408 rat cell line) (B), and cells infected with 10^4 focus-forming units of RSV(SR-A) per ml (on chicken embryo cells) (C). The number of colonies decreased linearly with the dilution of each virus.

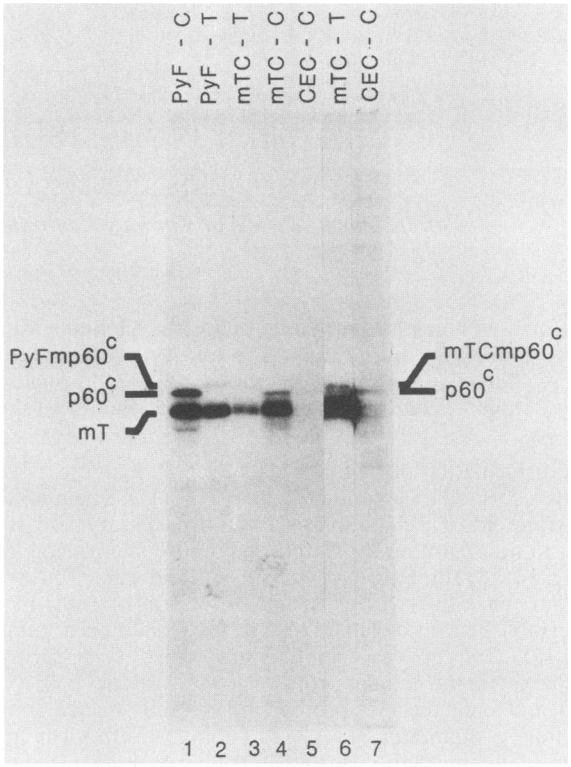

FIG. 2. Detection of transforming proteins in transformed chicken embryo cells. Freshly explanted chicken embryo cells (10^7 cells per 100-mm-diameter plate) were infected as described in the legend to Fig. 1 and were passaged for 2 weeks. Cell lysates were immunoprecipitated with MAb 327 or polyomavirus rat antitumor serum and phosphorylated in vitro as described in the text. The products of the protein kinase reaction were fractionated on an 8.5% polyacrylamide gel (28.8% acrylamide:0.735% bis-acrylamide). The dried gel was exposed to Kodak XAR-5 film for 2 h without (lanes 1 to 5) or with (lanes 6 and 7) an intensifying screen at −70°C. Lanes 6 and 7 are the same as lanes 3 and 5, respectively, and are included to show the more slowly migrating form of $p60^{c\text{-}src}$ from the transformed chicken cells and $p60^{c\text{-}src}$ from uninfected chicken cells. Abbreviations: CEC, uninfected chicken embryo cells; mTC, mT-MLV(ampho)-transformed CEC; PyF, polyomavirus-transformed F2408 rat cells; $p60^c$, $p60^{c\text{-}src}$; $mp60^c$, slower-migrating form of $p60^{c\text{-}src}$ as described in the text; T, rat polyomavirus antitumor serum immunoprecipitates; C, MAb 327 immunoprecipitates.

327 (Fig. 1, lane 1), whereas mT protein and a more slowly migrating form of $p60^{c\text{-}src}$ (4, 19) were seen with anti-T serum (lane 2). When the proteins phosphorylated in immunoprecipitates of mT-MLV(ampho)-transformed chicken embryo cell lysates were analyzed, the results were similar. mT protein and $p60^{c\text{-}src}$ were seen with MAb 327 (Fig. 1, lane 4), whereas mT protein, $p60^{c\text{-}src}$, and a more slowly migrating form of $p60^{c\text{-}src}$ were seen with anti-T serum (lanes 3 and 6). Lanes 5 and 7 show the position of $p60^{c\text{-}src}$ from uninfected chicken cells. The identity of the various phosphorylated proteins was confirmed by partial proteolytic mapping with *S. aureus* V8 protease (data not shown). We concluded that the mT protein is expressed in chicken embryo cells transformed by mT-MLV(ampho). The amount of mT protein in the chicken cells varied between different batches of cells but always fell within the range observed for polyomavirus or mT-MLV-transformed rodent cells (data not shown). The mT protein associated with chicken $p60^{c\text{-}src}$ and appeared to

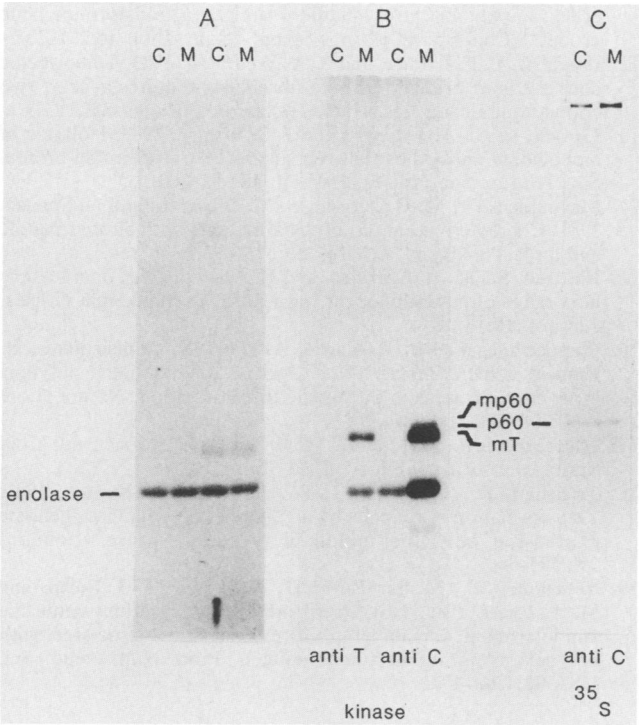

FIG. 3. Activation of p60$^{c\text{-}src}$ activity in mT-protein-transformed chicken cells. Unlabeled or [^{35}S]methionine-labeled lysates of uninfected or mT-MLV(ampho)-infected chicken embryo cells were immunoprecipitated as described in the text. Cells were labeled with 200 μCi of [^{35}S]methionine (Amersham) for 16 h in 2 ml of methionine-free DME supplemented with 5% undialyzed calf serum. For the in vitro kinase assay, 1 μl of activated enolase was included in the kinase buffer. Rabbit muscle enolase (5 mg/ml) was stored at −70°C in 50% glycerol and was activated by adding an equal volume of 100 mM glacial acetic acid and by incubating at 30°C for 5 min (5). The reaction was terminated by adding 25 μl of 2× sample buffer, and one-third of the sample was loaded on the gel. The gel conditions were the same as described in the legend to Fig. 2. (A) Photograph of the dried, stained gel showing the stained enolase band. (B) In vitro kinase reaction. The enolase band comigrates exactly with the stained band shown in panel A. (C) Metabolic label with [^{35}S]methionine, showing the p60$^{c\text{-}src}$ band. The gel was impregnated with PPO (2,5-diphenyloxazole) (3) and exposed to Kodak XAR-5 film for 96 h (methionine) or 2 h (kinase) at −70°C. Panels B and C are from adjacent lanes of the same gel. Symbols are as described in the legend to Fig. 2. C, Uninfected chicken embryo cells; M, mT-MLV(ampho)-infected chicken embryo cells.

be phosphorylated by p60$^{c\text{-}src}$ in immunoprecipitates made with MAb 327 or anti-T serum.

Association of the mT protein with p60$^{c\text{-}src}$ resulted in enhancement of the protein kinase activity of p60$^{c\text{-}src}$ as measured by autophosphorylation of p60$^{c\text{-}src}$ and by phosphorylation of exogenous substrates (2, 4). We measured the phosphorylation of enolase added to MAb 327 immunoprecipitates of uninfected and mT-MLV(ampho)-infected chicken embryo cell lysates (Fig. 3). The phosphorylation of enolase was 7.5- to 10-fold higher in immunoprecipitates made from mT-MLV(ampho)-infected cells compared with uninfected cells (Fig. 3B) as measured by scanning densitometry of the autoradiograms or by direct scintillation counting of the excised bands. Phosphorylation of enolase occurred predominantly on tyrosine residues (data not shown). The increase in phosphorylation was not caused by an increase in the amount of p60$^{c\text{-}src}$ in the extracts of cells infected by mT-MLV(ampho) because the amounts of [^{35}S]methionine-labeled p60$^{c\text{-}src}$ were similar in extracts of uninfected and infected cells (Fig. 3C).

We tested medium from mT-MLV(ampho)-infected chicken embryo cultures for the presence of virus capable of transforming rat F2408 cells (10). Medium harvested after overnight incubation contained infectious virus with a titer of about 10^4 focus-forming units per ml. This titer was comparable to the titer of the original virus stocks grown on rat cells. Rat cells transformed by the virus released from mT-MLV(ampho)-transformed chicken cells contained mT protein (data not shown).

Several conclusions emerged from these experiments. (i) Amphotropic pseudotypes of mammalian retrovirus vectors can be used to introduce new genes into chicken cells with high efficiency. In addition to mT-MLV(ampho), we have used a recombinant murine retrovirus which encodes the transforming gene of RSV(SR-A) (1) to transform chicken embryo cells. This virus expresses p60$^{v\text{-}src}$ and is released by the transformed cells. The use of mammalian retrovirus vectors may provide a useful alternative to DNA transfection and microinjection of chicken embryo cells. (ii) The polyomavirus mT protein can transform nonestablished chicken embryo cells to anchorage-independent growth. Land et al. (12) reported that mT-protein-encoding plasmids failed to induce foci on monolayers of nonestablished rat embryo fibroblasts. However, the mT-protein-encoding plasmids could induce foci of transformed cells if the plasmid was introduced into the cells along with a plasmid encoding resistance to mycophenolic acid and if a selection was applied. In addition, the plasmid could induce some growth of the infected cells in agar, although the colonies failed to grow to visible size under the conditions used. The results described here are compatible with the above observations and show that the mT protein can transform chicken cells as well as rodent cells. (iii) The mT protein associates with and enhances the protein kinase activity of chicken p60$^{c\text{-}src}$, as is true for mouse and rat p60$^{c\text{-}src}$ (2, 4). These observations support the idea that the association of mT protein and p60$^{c\text{-}src}$ may be important for cell transformation by polyomavirus. It will be interesting to see whether the ability of mT protein to associate with p60$^{c\text{-}src}$ extends to more distantly related p60$^{c\text{-}src}$ molecules in other species.

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