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Cellular *ras* Gene Activity Is Required for Full Neoplastic Transformation by Polyomavirus

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To investigate the role of *ras* gene activity in cellular transformation by polyomavirus, murine C3H10T1/2 cells were rendered ras deficient by transfection with an antisense *ras* gene construct. Ras deficiency resulted in a partial suppression of the polyomavirus-induced transformed phenotype. The production of viral middle T antigen and its association with pp60$^{c\text{-}src}$, increased membrane-associated protein kinase C activity, and morphological transformation were unaffected by the downregulation of c-*ras* gene expression. On the other hand, stimulated proliferation, focus formation on confluent monolayers of normal cells, and colony formation in soft agar were all greatly reduced in cells containing reduced p21^{ras} levels. It is concluded that *ras* gene activity is needed for full cell transformation by polyomavirus.

Among the three proteins encoded in the early region of polyomavirus, the middle tumor antigen (mT) is thought to be the principal protein involved in transformation (47). mT attaches onto the host cell plasma membrane, where it associates with the carboxy terminus of pp60$^{c\text{-}src}$, the cellular homolog of the transforming protein of Rous sarcoma virus, pp60$^{v\text{-}src}$ (8, 13), as well as other cellular oncogenes of the *src* family (7, 27–29). This interaction results in an increase in the activity of pp60$^{c\text{-}src}$ protein-tyrosine kinase (4), partly because mT locks the enzyme into a configuration in which it cannot be inactivated by phosphorylation of its tyrosine 527 residue (10–12, 21). In this membrane-associated complex with pp60$^{c\text{-}src}$, mT can induce parts of the membrane signaling apparatus to generate a stream of signals which transforms the cell and its progeny.

There are two reasons to believe that the 21-kDa Ras proteins might mediate the responses to the transforming signals from mT:pp60$^{c\text{-}src}$ complexes. First, microinjected anti-Ras antibodies block the cellular responses to normal and oncogenic protein-tyrosine kinases, including the viral pp60$^{v\text{-}src}$ (46). Second, the viral pp60$^{v\text{-}src}$ is one of several membrane protein-tyrosine kinases phosphorylating the GTPase-activating protein (GAP), which modulates the activity of Ras proteins (17, 22, 24, 34). It must be noted however, that the abundance of proteins phosphorylated on tyrosine in response to v-*src* gene expression makes the identification of substrates which are essential for transformation a very difficult task (44). Contrary to pp60$^{v\text{-}src}$, expression of the polyomavirus mT does not result in any increase in total cellular phosphotyrosine, indicating that this system might offer distinct advantages for the identification of the functionally significant substrates of membrane tyrosine kinases mediating signal transduction, transformation, and cell proliferation.

Suppression of the transformed phenotype of cells expressing the polyomavirus T antigens by inactivation of the *ras* gene product would provide strong evidence for the involvement of cellular Ras proteins in transformation by this virus. This can be achieved through the introduction of neutralizing anti-Ras antibodies into polyomavirus-transformed cells by microinjection (36, 45) or electroporation (41). Although microinjected antibodies persist for a sufficient amount of time to allow assessment of the role of the *ras* gene in various mitogenic responses (36), this approach cannot be used to determine the long-term consequences of Ras inactivation on transformation, such as agar growth or tumorigenicity. Therefore, we chose to study the effects of p21$^{c\text{-}ras}$ on the polyomavirus-induced transformation of murine C3H10T1/2 cells by downregulating its expression through the introduction of an antisense *ras* gene construct, the messages from which would produce a Ras deficiency by blocking c-*ras* mRNA translation (1).

In this report, we show that Ras deficiency suppressed parts of the transformed phenotype of C3H10T1/2 cells expressing the early region of polyomavirus, such as accelerated proliferation, focus formation on confluent monolayers of normal cells, and formation of colonies in soft agar, without affecting viral mT production or association with pp60$^{c\text{-}src}$, the stimulation of membrane-associated protein kinase C (PKC) activity by the mT:pp60$^{c\text{-}src}$ complex, or morphological transformation.

MATERIALS AND METHODS

Materials. Tissue culture media and sera were from Flow Laboratories, Inc. Geneticin (G418 sulfate) was from GIBCO Laboratories, Grand Island, N.Y., and hygromycin B was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. [γ-^{32}P]ATP (2,000 Ci/mmol) was purchased from ICN Pharmaceuticals Inc., Irvine, Calif.

Cell lines, culture techniques, and plasmid transfections. All cells were grown in 10% fetal calf serum–90% Dulbecco's modification of Eagle's medium in a humidified CO_2 incubator.

Plasmid transfections were performed as described previously (42). For expression of the *ras* antimessage, the pMt-I antisense *ras* construct containing the human c-H-*ras* gene

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downstream and in the opposite orientation from the heavy-metal-inducible mouse metallothionein (MT) promoter and the G418 resistance gene under control of the simian virus 40 promoter was transfected into C3H10T1/2 cells with selection for neomycin resistance (48). Stable transfectants were isolated and grown into clones.

For expression of the polyomavirus T antigens, plasmid pPB21, containing the entire polyomavirus genome cloned in the *Bam*HI site of plasmid pBR322 (19), was introduced by calcium phosphate transfection along with a plasmid conferring resistance to hygromycin, serving as a selectable marker (pY3 [3]). The late region of polyomavirus is interrupted in plasmid pPB21, so that only the T antigens can be expressed after transfection. One microgram of total DNA per 2×10^5 cells plated in Linbro trays was used, at a polyomavirus pPB21/pY3 ratio of 50:1.

Measurement of RNA expression. Total cellular RNA was extracted by the method of Chirgwin et al. (9) and electrophoresed on a formaldehyde-containing 1% agarose gel. The RNA was transferred to a nylon-based BIOTRANS membrane (ICN Pharmaceuticals) and hybridized with 10^7 cpm of nick-translated *ras* probe, using the conditions recommended by the manufacturer. For this probe, a 602-bp *Sma*I fragment containing all of the 5′ untranslated sequence as well as exons 1 and 2 and introns 1 and 2 of the transforming human c-H-*ras* gene (from the EJ bladder carcinoma cell line [48]) was introduced into the gemini pGEM-1 vector (Promega Biotec). As a control, the probe was stripped and the same membrane was hybridized with a nick-translated β-actin probe (a gift of B. Elliott [20]).

$p21^{ras}$ and polyomavirus mT analysis. C3H10T1/2 clones in which the *ras* antimessage plasmid had been introduced were tested for expression of the antimessage and levels of $p21^{c\text{-}ras}$ protein essentially as described previously (1). Briefly, the protein concentration of detergent cell lysates was carefully determined, using the technique described by McKnight (33). Total protein (100 μg) was separated by polyacrylamide gel electrophoresis and transferred to a Hybond ECL nitrocellulose sheet (Amersham, Arlington Heights, Ill.). The membrane was probed with the anti-Ras monoclonal antibody pan-ras Ab2 (Oncogene Science) at 10 μg/ml. The specifically bound immunoglobulin G was visualized by using a horseradish peroxidase-labeled F(ab′) sheep anti-mouse immunoglobulin G antibody and an enhanced chemiluminescence detection system as instructed by the manufacturer (Amersham). The amount of $p21^{ras}$ present was determined by densitometry of the film after multiple exposures of the gel for different lengths of time. To improve quantitation, the experiments were conducted in triplicate and the values obtained were averaged. As a control of blotting efficiency, the gel was stained by Coomassie blue after the electrophoretic transfer. As a further control of total protein concentration, the same protein samples were run on a parallel gel which was similarly stained.

Induction of the MT promoter was achieved by the addition of 50 μM $ZnSO_4$ to the growth medium for 24 h.

Polyomavirus mT analysis was performed essentially as described previously (42). The protein concentration of the detergent extracts was determined by the method of McKnight (33). These extracts were precipitated with ascites fluid from brown Norwegian rats containing antibodies to all three polyomavirus T antigens as previously described (42). In all in vitro phosphorylation experiments, the gels had been treated with alkali prior to autoradiography. The extent of phosphorylation was determined by densitometry of the autoradiogram.

Analysis of in situ membrane-associated PKC activity levels. The analysis was conducted essentially as described previously (6). Briefly, cells were hypotonically lysed, and nuclei were removed by centrifugation. Protein concentration in the supernatant was determined by the Bradford method (Bio-Rad, Richmond, Calif.). Native membranes with their associated PKC from 150 μg of the postnuclear supernatant were pelleted by ultracentrifugation; 50 μg of a specific 85-kDa PKC substrate from S49 T-lymphoma cells was added to the pellet together with calcium and [γ-^{32}P]ATP. After incubation, the proteins were separated by polyacrylamide gel electrophoresis, and the extent of phosphorylation of the 85-kDa PKC substrate protein was determined by densitometry of the autoradiogram after multiple exposures of the gels for different lengths of time. Addition of a fivefold excess of substrate to all membrane preparations did not result in any increase in phosphorylation, indicating that the substrate was not limiting in this assay. The specificity of this assay for PKC was demonstrated by the selective downregulation of phosphorylation of the 85-kDa band by a long treatment of the cells with tetradecanoyl phorbol acetate (5, 30a).

Construction and expression of an H-*ras* gene. A 2.3-kb fragment of the human c-H-*ras*-1 gene, coding for the four *ras* exons and three introns as well as a poly(A) signal, was introduced into the retroviral vector pDOL (26) at the unique *Bam*HI cloning site. The ψ2 packaging line (30) was transfected with this plasmid, and the culture supernatant from a mixture of G418-resistant stable transfectants was used to infect C3H10T1/2 cells and their derivatives. Because of the aberrant splicing of the message in the packaging line caused by the *ras* introns, more than 90% of the infected target cells contained various combinations of *ras* gene exons, while a very small proportion of them expressed a correctly spliced c-H-*ras* gene (11a).

For expression of a transforming *ras* gene, the Nψ-6 line, which produces recombinant retroviruses carrying a leucine 61-activated c-H-*ras*-1 cDNA (15) in the pDOL retroviral vector (26), was used (11a). C3H10T1/2 cells, infected with the vector-containing supernatant from Nψ-6 cells and selected for G418 resistance, yielded colonies which displayed a transformed morphology and were capable of anchorage-independent growth. The titer was approximately 2,000 G418-resistant colonies per ml of Nψ-6 culture supernatant.

RESULTS

Production of antisense *ras* C3H10T1/2 transfectants. The MT-antisense *ras* construct was introduced through transfection into C3H10T1/2 cells with selection for G418 resistance. Resistant clones were grown and tested for levels of *ras*-specific mRNA and $p21^{c\text{-}ras}$ protein in the presence or absence of 50 μM $ZnSO_4$ as described in Materials and Methods. To avoid problems stemming from toxicity of the heavy-metal inducers of the MT promoter, two C3H10T1/2 clones expressing significant levels of *ras* antimessage even in the absence of the $ZnSO_4$ inducer (lines R5 and R14) were selected for further transfection experiments. Very similar results were obtained with both lines. In this report, results with the R14 cells and their polyomavirus-transfected derivatives are presented. As described below (Fig. 1 to 5), the R14 cells had approximately one-third the Ras protein levels and displayed a very slow growth and a very flat morphology relative to the parent C3H10T1/2 line. As a control,

C3H10T1/2 cells were transfected with an identical plasmid but lacking the *ras* antimessage sequence. These cells behaved identically to the parent C3H10T1/2 line (not shown).

Production of R14 clones stably expressing the polyomavirus early region. The polyomavirus early region was expressed in the R14 clone or the parent C3H10T1/2 line through transfection of plasmid pPB21 and selection for hygromycin resistance. Growth of the drug-resistant colonies was substantially slower for the R14 than for the C3H10T1/2 cells. This was true even in the absence of the inducer of the antimessage RNA, in keeping with the high constitutive levels of *ras* antimessage expression in the R14 line. After selection in hygromycin-containing medium for 2 weeks for C3H10T1/2 cells or 6 weeks for R14 cells, individual colonies were picked without regard to morphology. These were propagated and screened for levels of mT-associated kinase activity in a standard immunoprecipitation assay, as described previously (42). To avoid problems stemming from double-stranded RNA unwinding (49), all tests were conducted with confluent cells. As in the case of transfected NIH 3T3 or F111 clones (39, 42), mT levels varied from clone to clone, while several clones displayed kinase levels comparable to those of the mouse NIH 3T3 line transformed by whole polyomavirus (py6 [2]). Of 400 R14 hygromycin-resistant, polyomavirus pPB21-transfected clones, 52 showed mT-associated kinase activity levels higher than 80% of the activity of the py6 line, compared with 8 of 58 C3H10T1/2 clones.

The transformation parameters and tumorigenicity of rat F111 cells expressing the early region of polyomavirus under control of the dexamethasone-regulatable mouse mammary tumor virus promoter correlated with the level of mT phosphorylated in immune complexes rather than the total amount of T antigens determined by metabolic labeling (42). Therefore, to ensure that the Ras-deficient R14 clones examined had levels of active mT similar to those of the C3H10T1/2 cells after transfection of the polyomavirus early-region genes, 40 independently produced representatives of R14 clones expressing high levels of in vitro kinase activity and five polyomavirus-transformed C3H10T1/2 cell lines were chosen for further study (42) and named Rpy-1 to Rpy-40 and 10py-1 to 10py-5, respectively. As shown in Fig. 1A, the in vitro phosphorylation of mT in five representative Rpy lines (Rpy-1 to Rpy-5) was as high as or higher than that of two representative 10py lines (10py-1 and 10py-2). Furthermore, a direct comparison of the in vitro mT:pp60$^{c\text{-}src}$ kinase activity levels between all of the R14 and C3H10T1/2 pPB21 transfectants obtained suggested that downregulation of c-*ras* gene expression did not appreciably suppress the production of mT protein or its association with pp60$^{c\text{-}src}$.

To exclude potential problems of clonal variability stemming from transfected genome instability, Northern (RNA) blotting and Western immunoblotting studies were conducted to determine whether the antisense construct was functioning in the R14 cells and their polyomavirus-transfected derivatives and was able to reduce the levels of the p21^{ras} protein. In agreement with previous results (1), an RNA was detected with the predicted size of an antisense transcript produced from the integrated MT-antisense *ras* vector (~4 kb). An RNA molecule corresponding to the size of the normal c-*ras* mRNA (~2 kb) was also detected in the C3H10T1/2, while this molecule was undetectable in the R14 or Rpy clones (Fig. 1C, lanes 1 to 6). At the same time, the levels of p21$^{c\text{-}ras}$ protein were verified in two 10py and seven Rpy clones through immunoblotting of total cell extracts as described in Materials and Methods. As shown in Fig. 1B,

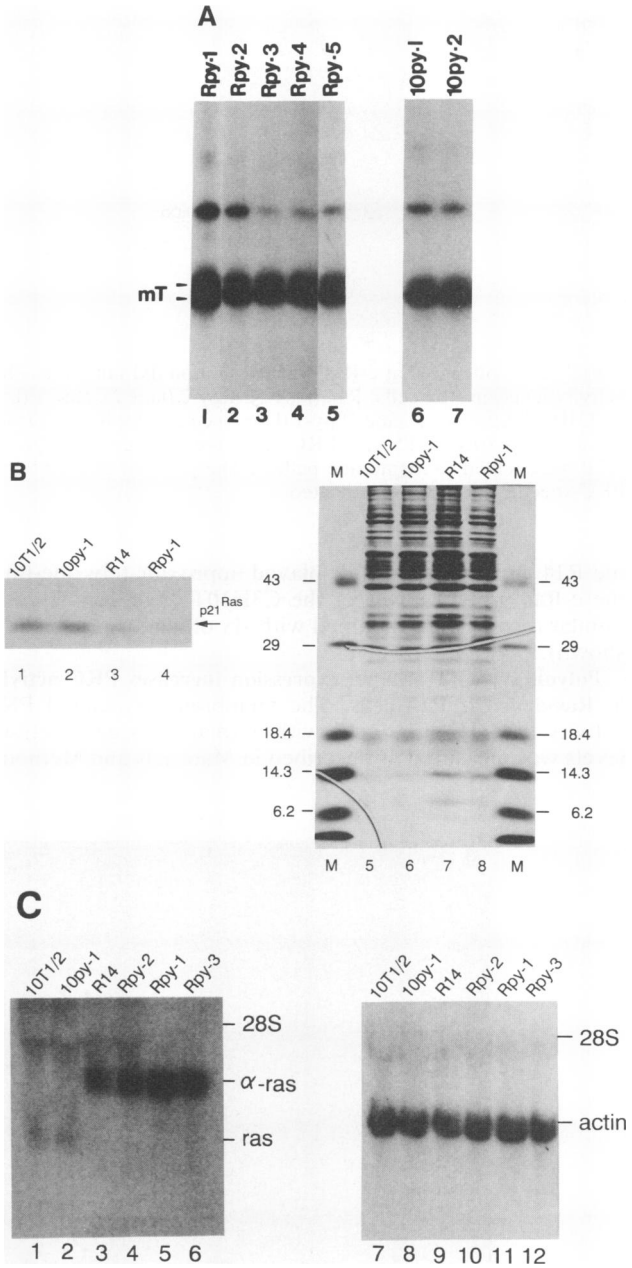

FIG. 1. (A) In vitro phosphorylation of mT produced in the indicated clones after transfection with plasmid pPB21 encoding the early region of polyomavirus. Washed immunoprecipitates were incubated with [γ-^{32}P]ATP and electrophoresed on a 10% acrylamide-sodium dodecyl sulfate gel. Gels were treated with NaOH, dried, and exposed to X-ray film. Position of the 56- and 58-kDa phosphorylated mT forms are indicated. (B) Quantitation of c-Ras protein levels in the indicated clones by immunoblotting of cell lysates and probing with the pan-ras Ab2 antibody. Lanes 5 to 8 show the total protein profile of an identical gel as revealed by Coomassie blue staining. The arrow points to the position of the 21-kDa Ras protein. Lanes M, molecular weight markers. Positions of migration are indicated in kilodaltons. (C) Measurement of *ras*-specific RNA levels in the indicated lines before (lanes 1 and 3) and after (lanes 2, 4, 5, and 6) expression of the early region of polyomavirus. Purified RNA was electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a nick-translated *ras*-specific probe (see Materials and Methods). As a control, the same membrane was stripped and rehybridized with a β-actin probe (lanes 7 to 12). Positions of the 18S and 28S rRNAs are indicated.

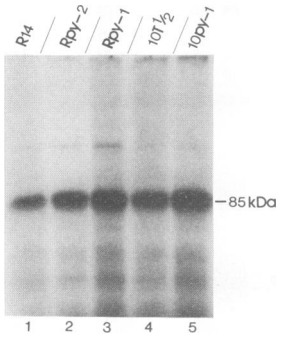

FIG. 2. Evidence that c-Ras downregulation did not affect PKC activity levels in Rpy cells. R14 (lane 1), Rpy-2 (lane 2), Rpy-1 (lane 3), C3H10T1/2 (lane 4), and 10py-1 (lane 5) cells were hypotonically lysed, and a specific 85-kDa PKC substrate, calcium, and [γ-^{32}P] ATP were added to membrane pellets. The position of the 85-kDa PKC-specific substrate is indicated.

the R14 and Rpy-1 lines displayed approximately one-third the c-Ras protein levels of the C3H10T1/2 or 10py-1 cells. Similar results were obtained with six other Rpy clones (not shown).

Polyomavirus T-antigen expression increases PKC activity in Ras-deficient R14 cells. The membrane-associated PKC activity in C3H10T1/2 cells with normal c-*ras* gene activity levels was measured as described in Materials and Methods. As shown in Fig. 2 (lanes 4 and 5), it was found to be increased slightly by polyomavirus transformation. Membrane-associated PKC activity was much lower in the Ras-deficient R14 cells (Fig. 2, lane 1), but it was stimulated four- and threefold in the polyomavirus-expressing Rpy-1 and Rpy-2 derivatives of R14 cells, respectively (Fig. 2, lanes 2 and 3). Similar results were obtained with five other Rpy and two 10py clones (not shown). Therefore, reducing c-*ras* expression did not prevent polyomavirus from stimulating membrane-associated PKC activity, which is a major component of mitogenic and other signals from a variety of protein-tyrosine kinase receptors, a viral K-Ras protein, and pp60$^{v\text{-}src}$ (16, 16a, 31, 50).

c-*ras* downregulation inhibits transformation by polyomavirus. Ras deficiency did not prevent the polyomavirus early-region genes from causing the morphological transformation of R14 cells (Fig. 3). As expected, the polyomavirus-transformed 10py-1 cells with normal c-*ras* expression levels displayed a transformed morphology compared with the parental C3H10T1/2 cells (Fig. 3A and C). R14 cells with reduced c-*ras* expression levels looked larger and flatter (Fig. 3B) than the parental C3H10T1/2 cells (Fig. 3A). However, R14 cells were dramatically transformed into overlapping, round cells after expression of the early region of polyomavirus (e.g., line Rpy-1; Fig. 3D).

Although c-*ras* downregulation did not reduce mT-antigen expression, the stimulation of membrane-associated PKC, or morphological transformation, it did suppress other parts

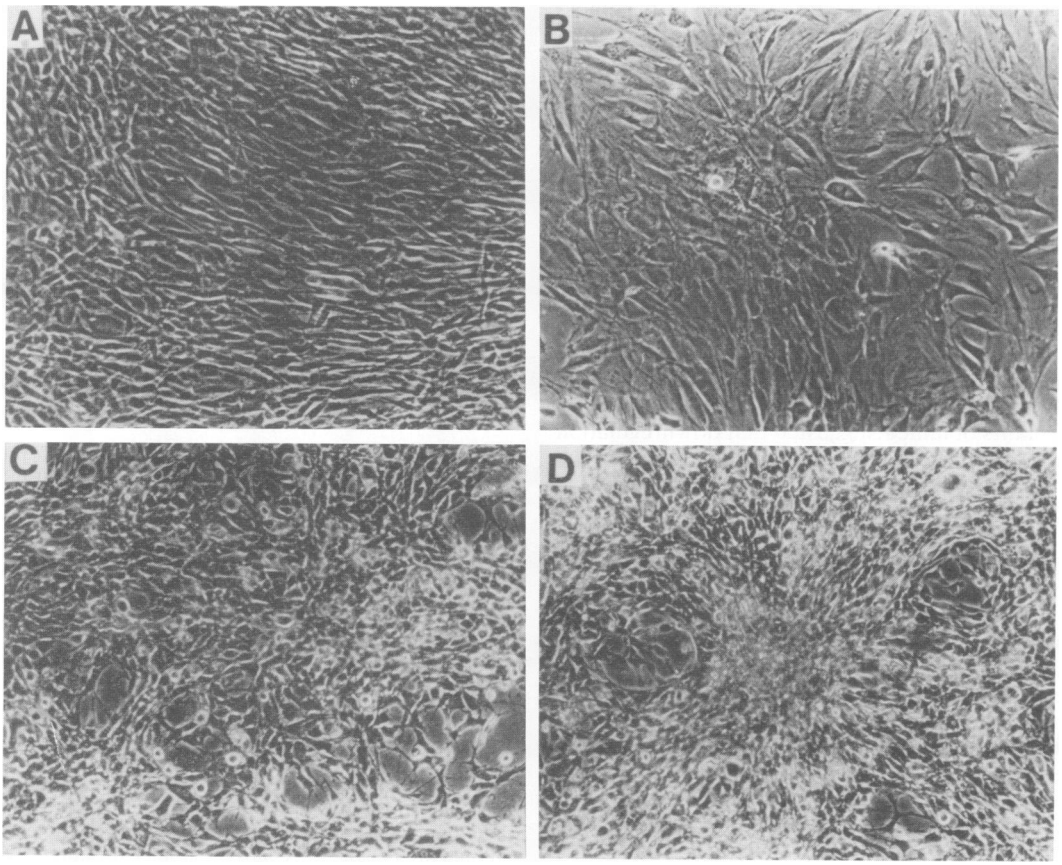

FIG. 3. Evidence that Ras deficiency did not affect morphological transformation of C3H10T1/2 cells by polyomavirus. C3H10T1/2 (A), R14 (B), 10py-1 (C), and Rpy-1 (D) cells were grown to 90% confluency and photographed under phase contrast.

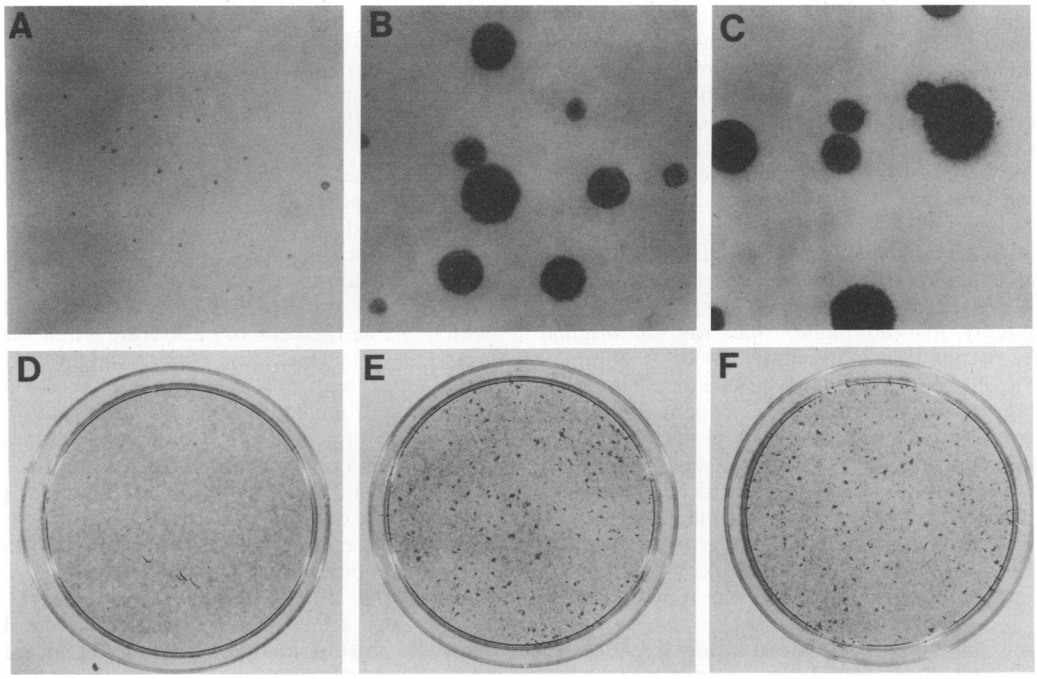

FIG. 4. Evidence that Ras deficiency prevented full transformation of C3H10T1/2 cells by polyomavirus, whereas the expression of an activated ras gene reversed this effect. Rpy-1 (A and D), 10py-1 (B and E), or Rpy-ras-1 (C and F) cells were suspended in agar (A to C) or plated on top of a confluent monolayer of R14 cells (D to F). Ten days later for 10py-1 and Rpy-ras-1 and 45 days for Rpy-1, cells were photographed under low power (A to C) or fixed and stained with Coomassie blue (D to F).

of the transformed phenotype. Polyomavirus-transformed 10py-1 cells with normal c-*ras* expression levels produced colonies when suspended in soft agar (Fig. 4B) and formed foci on confluent R14 cell monolayers (Fig. 4E). Contrary to the 10py clones, the ability of the Rpy lines to form foci overgrowing an R14 cell monolayer or to grow in an anchorage-independent manner was drastically reduced (Fig. 4A and D). Results similar to those shown in Fig. 4 were obtained when normal Fisher rat F111 (42) instead of R14 cells were used for the focus assay and for nine other Rpy and four 10py clones (not shown). In keeping with the high constitutive levels of expression of the *ras* antisense genome in the Rpy lines, this phenotype was evident in the absence of the inducer and changed very little upon induction when 50 μM $ZnSO_4$ was added (not shown).

Downregulating c-*ras* expression also greatly reduced the rate of cell proliferation. In 10% fetal calf serum, the doubling time of the parent C3H10T1/2 line was 22.7 h whereas that of the Ras-deficient R14 cells was 52.6 h (Fig. 5). Moreover, the polyomavirus T antigens could not significantly stimulate the proliferation of Ras-deficient R14 derivatives such as Rpy-1 cells (Fig. 5). Very similar results were obtained with 16 other, independently produced Rpy lines (not shown). This is a strong indication that the presence of the antisense *ras* is inhibiting the function of the early region of polyomavirus in the transformation of this line.

To ensure that the observed phenotype was indeed due to the downregulation of c-*ras* gene expression, an activated *ras* gene was introduced in Rpy cells under control of the strong Moloney murine leukemia virus promoter, the messages from which would be able to inactivate the MT promoter-driven antimessage and thus permit transformation:

Approximately 10^3 R14, C3H10T1/2, Rpy-1, Rpy-2,

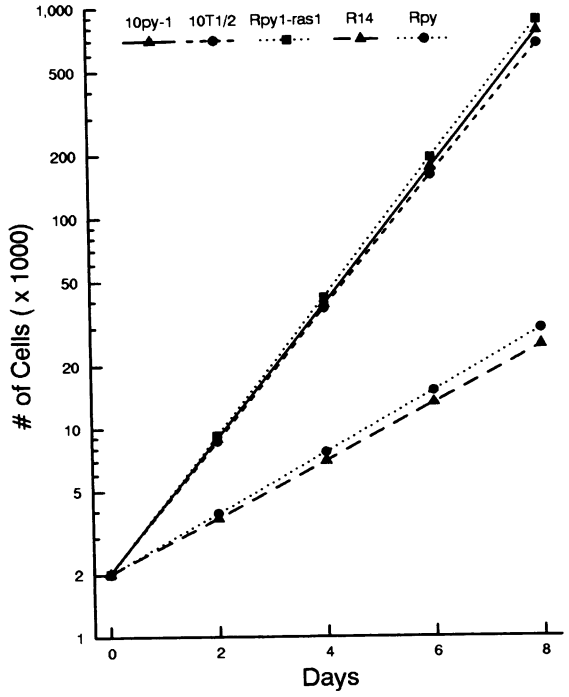

FIG. 5. Evidence that the growth rate of Ras-deficient Rpy-1 cells is substantially lower than that of 10py-1 cells, whereas the expression of an activated *ras* gene could reverse this effect. Two thousand cells of each of the indicated lines were plated in 3-cm dishes in 10% fetal calf serum and at the time points shown were harvested and counted.

Rpy-3, Rpy-4, or Rpy-5 cells, growing in a 6-cm petri dish, were infected with 3 ml of culture supernatant from the Nψ-6 packaging line, which secretes virus carrying an activated *ras* gene (see Materials and Methods). Ten days later, 12 individual clones were picked (2 from each of the R14 lines and the 5 Rpy clones, named Rras-1 and -2 and Rpy1-ras-1 and -2, respectively) and tested for mT:pp60$^{c\text{-}src}$ complex kinase and Ras protein levels, and their transformation properties were assessed. More than 90% of the R14 cells assumed a transformed morphology after infection, indicating that the procedure was effective in achieving expression of the activated *ras* gene. Although *ras* expression levels were approximately fivefold higher than for the original C3H10T1/2 line, evaluation of their mT:pp60$^{c\text{-}src}$ complex kinase activity levels revealed no measurable changes attributable to the expression of the activated *ras* gene, indicating that *ras* gene overexpression does not affect mT expression or its association with pp60$^{c\text{-}src}$. However, consistent with the notion that the anti-*ras* block is relieved by the excess *ras* message, all activated-*ras*-expressing cells grew in agar very effectively (Fig. 4C), formed foci overgrowing a monolayer of R14 (Fig. 4F) or F111 (not shown) cells, and had a doubling time slightly shorter than that of the 10py cells (Fig. 5).

Very similar results were obtained after infection of C3H10T1/2 cells and their derivatives with the culture supernatant from ψ2 cells transfected with the intron-containing c-H-*ras* retrovirus vector (see Materials and Methods). Presumably, the high expression levels of a combination of *ras* exons in the target lines under control of the strong murine leukemia virus long terminal repeat promoter would be responsible for inactivation of the MT promoter-driven antimessage and transformation by the polyomavirus early-region genes.

DISCUSSION

Recent results have shown that mT-antigen expression in rat F111 cells results in an increase in membrane-associated PKC activity (30a). On the other hand, introduction of v-Ras protein in quiescent Swiss 3T3 cells by scrape loading led to the immediate activation of PKC in the absence of protein synthesis (35). This observation, combined with recent reports on the interaction of membrane kinases with GAP (17, 22, 24, 34), suggests that Ras might be transducing signals from membrane-bound tyrosine kinases to PKC. Surprisingly, however, the Ras deficiency did not appear to suppress signals from membrane-associated mT:pp60$^{c\text{-}src}$ complexes to PKC in this study, as is indicated by the ability of polyomavirus to override the reduction of membrane-associated PKC activity caused by the Ras deficiency in R14 cells. These results show that, in contrast to rapid growth rate or the formation of foci in agar, low p21^{ras} levels are sufficient for the stimulation of membrane-associated PKC by polyomavirus. This stimulation could presumably be a consequence of the membrane-associated mT:pp60$^{c\text{-}src}$ complex protein-tyrosine kinase. The fact that pp60$^{v\text{-}src}$ has been shown to stimulate directly membrane-associated PKC activity (16a) is consistent with this interpretation. It was also shown previously that PKC is necessary for the association between mT and pp60$^{c\text{-}src}$ (32, 38, 40). The direct activation of PKC might explain the normal levels of mT:pp60$^{c\text{-}src}$ kinase activity under conditions of c-*ras* gene downregulation, as in the clone Rpy-1 (Fig. 1). However, the mechanism of this stimulation of PKC activity by mT remains to be elucidated.

Reducing c-*ras* expression drastically slows the proliferation of both the parent line (R14) and the line expressing the polyomavirus early region (Rpy-1), despite a persistent PKC signal from a normal complement of active mT:pp60$^{c\text{-}src}$ complexes. The Ras dependence of focus formation, soft agar growth, and proliferation could be due, at least in part, to the involvement of Ras activity in cell cycle transit in the later stages of the G_1 phase and perhaps in the G_2 phase, as demonstrated by Durkin and Whitfield (16), Mulcahy et al. (36), and Stacey et al. (45, 46). However, it was also shown that polyomavirus-transformed cells secrete autocrine factors such as transforming growth factors alpha and beta, which enable the cells to proliferate when suspended in soft agar (23, 37). Thus, Ras activity might also be needed in conjunction with mT to make and secrete these autocrine factors or to mediate the signals generated in the producer cell by the autocrine loop. The available data do not permit a distinction between these two possibilities.

It is known that only minor serological or electrophoretic differences exist between H-, K-, and N-*ras*, although the individually conserved *ras* genes are markedly divergent from one another on the nucleotide level (14). It is therefore possible that the antisense *ras* construct used in these studies has selectively hybridized to the H-*ras* species. However, further analysis utilizing *ras*-species-specific antibodies and oligonucleotide probes would be necessary to clarify this point.

The results presented here show that Ras proteins are needed for the full transformation of C3H10T1/2 mouse cells by polyomavirus. This conclusion is strengthened by the recent observation of Dobrovolny and Hassell (15a) that polyomavirus-induced transformation of mouse and rat cells is suppressed by overexpressing the Krev-1 gene, the product of which blocks Ras action by specifically interfering with the interaction of GAP and Ras proteins (18, 25, 31, 43).

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