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High-Level Expression of c-H-*ras*1 Fails To Fully Transform Rat-1 Cells

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Rat-1 cells were transfected with plasmids encoding normal (Gly-12), nonactivated (Pro-12), and activated (Val-12 and Ile-12) p21$^{H\text{-}ras}$ in the presence of an amplifiable dihydrofolate reductase marker. The introduced DNA was amplified by selection in methotrexate to establish the relationship between p21$^{H\text{-}ras}$ expression and various hallmarks of cellular transformation. The maximum level of p21$^{H\text{-}ras}$ (Gly-12) consistent with cell viability was approximately 0.13% of total cell protein (~ 60,000 molecules per cell); this is 44-fold greater than the level of the endogenous protein. The maximum tolerated level of a second nontransforming form of p21$^{H\text{-}ras}$ (pro-12) was about half of this. Amplification in Rat-1 cells of H-*ras* genes encoding the highly oncogenic Val-12 and Ile-12 forms of p21$^{H\text{-}ras}$ could not be achieved by methotrexate selection, providing strong evidence that synthesis of activated p21$^{H\text{-}ras}$ above a certain threshold (about 0.02% of total protein) in Rat-1 cells is incompatible with cell viability. Individual cell lines were isolated and their morphology, anchorage-independent growth, tumorigenicity, and response to and production of growth factors were studied. We report that cell lines expressing near-maximum tolerated levels of either the normal or pro-12 form of p21$^{H\text{-}ras}$ were not as transformed as cells expressing much more modest levels of the highly oncogenic (Val-12) form, suggesting that the complete elaboration of the transformed phenotype by *ras* depends, at least in part, on mutations that distinguish the cellular and viral proteins. We found that cells expressing elevated levels of the normal p21$^{H\text{-}ras}$ could be fully transformed by the activated (Val-12) form and that such cells continued to overexpress p21$^{H\text{-}ras}$ (Gly-12), arguing against a role for normal *ras* genes in suppression of the oncogenic potential of their mutationally activated counterparts.

The three mammalian *ras* genes, c-H-*ras*1, c-K-*ras*2, and N-*ras*, encode 21,000-dalton membrane-associated proteins (p21^{ras}) that bind and hydrolyze GTP (2, 21). The similarity of p21 sequences with those of G proteins, a superfamily of GTP-binding proteins involved in signal transduction (16), has led to the proposal that p21 acts as a coupling protein to relay extracellular messages to internal cellular effectors that control cellular proliferation (21). The polypeptides encoded by the *ras* gene family become oncogenically activated as a result of specific amino acid substitutions. These substitutions endow p21^{ras} with the potential to morphologically transform NIH 3T3 and other cell lines when transfected in culture. Such activated *ras* oncogenes have been detected in 20 to 30% of human tumor DNAs by DNA transfection of NIH 3T3 cells (reviewed in reference 2); by using a more sensitive hybridization analysis, the K-*ras* gene has been found activated in up to 40% of human colorectal cancers (3, 13). The amino acid positions within p21^{ras} most susceptible to oncogenic activation include Gly-12, Gly-13, and Gln-61, although substitutions at positions 13, 59, 63, 116, and 119 are also potentially activating (2). Activating substitutions in p21 generally (8, 15), but not always (19), diminish the intrinsic GTPase activity of the molecule, leading to suggestions that the GTP-bound form of p21 is the active component in the transformation process. Substitution of glycine at position 12 of H-*ras* with any amino acid except proline activates the transforming potential of p21$^{H\text{-}ras}$ (28); this is noteworthy in that proline is also the only amino acid known which, when substituted for glycine at this position, fails to decrease the intrinsic GTPase activity of p21 (8).

While the activation of *ras* genes through mutational alteration has been extensively documented, there have been reports that the overexpression of cellular *ras* genes encoding p21$^{H\text{-}ras}$ (Gly-12) similarly induces the transformation of NIH 3T3 cells as judged by morphological and tumorigenic criteria (6, 25). As such, cellular *ras* genes are commonly considered to belong to that class of proto-oncogenes whose malignant potential can be manifested by changes at either the quantitative or the qualitative level.

When transfected into established rodent cell lines, *ras* oncogenes transform them in an apparently dominant manner (reviewed in reference 2). On the other hand, it is clear that hybrids derived from the fusion of tumorigenic cells expressing H-*ras* oncogenes and normal cells exhibit a loss of in vivo tumorigenicity, although they remain morphologically transformed and express the *ras* oncogene (9, 14, 38); this raises the possibility that a gene product, present in the nontumorigenic parent, can at least partially suppress the action of the activated *ras* protein. It has also been noted that a number of transformed cell lines, such as T24 bladder carcinoma cells (4, 12), Calu-1 lung carcinoma cells (5, 30), A1698 and A2182 lung carcinoma cells (27), and SW480 colon carcinoma cells (5), are homozygous, hemizygous, or functionally hemizygous for transforming *ras* alleles. Furthermore, apparent allelic deletions of c-H-*ras* have been correlated with tumor progression (49). This suggests that selection against the expression of the normal *ras* alleles may be at least occasionally operative in tumor establishment, a surprising finding in view of observations that normal p21$^{H\text{-}ras}$ is itself oncogenic in cultured cells when expressed at high levels. To investigate the oncogenic capacity of c-*ras*

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genes more thoroughly, we introduced genes encoding nonactivated (Gly-12 and Pro-12) and activated (Val-12 and Ile-12) forms of $p21^{H\text{-}ras}$ into Rat-1 cells; expression levels were sequentially increased by appropriate selective conditions. The extent of transformation and tumorigenicity of cell lines overexpressing these p21s was compared with that of Rat-1 cells transformed by H-*ras*(Val-12). We found that overexpression of nonactivated forms of $p21^{H\text{-}ras}$ was unable to fully transform Rat-1 cells: by a variety of assays we found that structurally mutated forms of $p21^{H\text{-}ras}$ were more potent mediators of the oncogenic phenotype than the cellular proto-oncogene, even when expressed at much lower levels. We also found that cell lines expressing high levels of normal $p21^{H\text{-}ras}$ remained susceptible to the effects of oncogenically activated forms of $p21^{H\text{-}ras}$, suggesting that at least in Rat-1 cells normal $p21^{H\text{-}ras}$ does not seem to interfere with the oncogenic capacity of activated derivatives.

MATERIALS AND METHODS

Plasmids. The following plasmids were used: pSV2Neo, coding for neomycin and G418 resistance (34); pFD11, coding for dihydrofolate reductase (31); pSVEHygB, coding for resistance to hygromycin B; pc-H-*ras*, a plasmid with the 6.6-kilobase *Bam*HI restriction fragment containing the human H-*ras*1 gene in pBR322 with glycine, proline, valine, or isoleucine at amino acid position 12 (28).

Cell lines. All incubations with cells were at 37°C in an atmosphere with 5% CO_2. Rat-1 cells were cotransfected with 0.5 μg each of pSV2Neo and pFD11 and 5 μg of pc-H-*ras* (with glycine, proline, valine, or isoleucine at codon 12) by calcium phosphate precipitation (47). In controls, salmon genomic DNA replaced the *ras* expression plasmid. Transfectants were selected in standard medium (Ham's F12–low-glucose Dulbecco modified Eagle minimal essential medium, 50:50) containing 10% fetal bovine serum and 0.4 mg of G418 (Geneticin; GIBCO Laboratories) per ml. The introduced DNA in the pooled G418-selected colonies was amplified by plating 2×10^5 cells per 100-mm dish in standard medium without glycine, hypoxanthine, and thymidine and containing 150 nM methotrexate. The pooled colonies selected at 150 nM methotrexate were subsequently amplified by selection at 750 nM methotrexate. Individual colonies were isolated at each level of selection (G418, 150 nM methotrexate, and 750 nM methotrexate) with cloning cylinders after plating at low density. Clones were named according to the amino acid at position 12 of the transfected *ras* gene (G = glycine, P = proline, I = isoleucine, V = valine, and X = salmon DNA of controls), the level of selection (nanomolar methotrexate), and a clone number. Thus, V0C1 represents clone 1 of valine-12 *ras*-transfected Rat-1 cells selected in G418 only, P150C3 represents a clone of proline-12 *ras*-transfected cells selected at 150 nM methotrexate, and X750C1 represents a clone of salmon DNA-transfected cells selected at 750 nM methotrexate. When methotrexate selection failed to yield colonies, individual clones were subjected to selection until a few colonies were obtained. Thus, V750C1, V750C2, and V750C4 all derived from V0C4 (via pooled 50 and 150 nM methotrexate-selected colonies), G3000C1 derived from G750C4, G10000C1 and G10000C2 derived from G3000C1, P3000C1 and P3000C2 derived from P750C3, P3000C3 and P3000C4 derived from P750C1, and P10000C1 and P10000C2 derived from P3000C3. G150C1 cells were "supertransfected" by cotransfection with pc-H-*ras*1(Val-12) and pSVEHygB, and colonies were selected in standard medium containing 0.4 mg of hygromycin B per ml.

Analysis and quantification of H-*ras* p21 expression. Cells in culture were labeled with [^{35}S]methionine by a modification of the method previously described (28). For each line to be analyzed, 10^5 cells were plated per well of a 24-well dish (Costar) in standard medium with 10% fetal bovine serum and incubated for 20 h. Each well was rinsed twice with methionine-free medium (GIBCO selectamine kit) and incubated for 3 h in 200 μl of the same medium supplemented with 50 μCi of [^{35}S]methionine. Cells were lysed with 0.5 ml of lysis buffer and immunoprecipitated with an anti-H-*ras* polyclonal antiserum or preimmune serum (28). The amount of radioactive label incorporated into protein was determined by trichloroacetic acid precipitation of samples of cell lysates. When immunoprecipitates were to be scanned densitometrically to quantify p21 expression, the amount of precipitate applied to the 12% polyacrylamide gel was adjusted to normalize the p21 band signal intensity. The p21 bands of X-ray exposures were scanned with a laser densitometer (LKB 2202) linked to a Hewlett-Packard 3390A integrator. The ratio of p21 intensity/total protein label was calculated relative to that of untransfected Rat-1 cells. For greater accuracy in combining results of different gels, values were normalized to the average p21 level of the G750C2 cell line (34-fold that of Rat-1 cells). When this was done, the relative p21 expression of Rat-1 cells ranged from 0.5 to 2.

Determination of anchorage independence and tumorigenicity of cell lines. Cells, 3×10^4, were plated in 3 ml of modified Eagle medium (GIBCO) with 10% serum and 0.3% agar on a base of the same medium containing 0.5% agar. After 2 weeks, colonies were stained with p-iodonitrotetrazolium violet (Sigma Chemical Co.) and photographed, and the colonies were measured and counted. For the tumorigenicity determination, newborn Fischer rats were each injected with three different amounts of cells (5×10^4, 5×10^5, and 5×10^6) in groups of 10 for each cell line tested, and tumors were measured weekly for 6 weeks.

Determination of thymidine incorporation into DNA. Cells were plated in 96-well plates in standard medium with 10% serum and incubated for 16 h. Cells were then rinsed with and incubated in serum-free standard medium for 72 to 90 h. [6-^{3}H]thymidine was added (to 5 μCi/ml) with or without any growth factor being tested, and the cells were incubated for a further 15 h. The factors used and their sources were as follows: serum (GIBCO), Bombesin (Sigma), epidermal growth factor (EGF; Collaborative Research, Inc.), platelet-derived growth factor (PDGF; Collaborative Research), and transforming growth factor type beta (TGFβ; prepared from human platelets). Thymidine incorporation into DNA was determined with a cell harvester (PHD; Cambridge Technology Inc.).

Preparation and growth factor assay of conditioned media. Twenty-four-hour conditioned serum-free Waymouth medium was prepared essentially as described previously (10) from confluent cultures in 150-mm dishes, with 25 ml of medium per dish. The media were dialyzed against 1 M acetic acid, lyophilized, and reconstituted in one-tenth the original volume. The protein concentrations of the conditioned media were 68 (Rat-1), 59 (G3000C1), 74 (V0C1), and 7 μg/ml for the control conditioned medium. Epidermal growth factor-like activity was assayed by colony formation of NRK 49F cells in agar in the presence of 2 ng of TGFβ per ml (40). TGFβ concentration was determined by a radioreceptor assay developed by Venkat Mukka.

TABLE 1. Colonies obtained at each level of selection

Transfected DNA	pSV2Neo	pFD11 (*dhfr*)	No. of viable colonies per dish in:			
			G418 (0.4 g/liter)	Methotrexate		
				150 nM	750 nM	3,000 nM
pc-H-*ras* (Gly-12)	+	+	60	104	36	0
pc-H-*ras* (Pro-12)	+	+	45	69	46	0
pc-H-*ras* (Ile-12)	+	+	37	0		
Salmon DNA	+	+	121	87	74	81
pc-H-*ras* (Val-12)	+	+	31	0		
Salmon DNA	+	+	182	Not done		
Salmon DNA	−	−	0			
Salmon DNA	+	−	82	0		
Salmon DNA	−	−	0			

RESULTS

Amplification of H-*ras* gene expression in Rat-1 cells. To evaluate the consequences of overexpressing c-H-*ras* p21 and the nontransforming proline-12 mutation, we used a procedure that enables a systematic amplification of transfected genes. In brief, we simultaneously transfected cells with vectors that encode both a dominant selectable marker (in this case, the bacterial neomycin resistance gene [*neo*]) and an amplifiable gene (dihydrofolate reductase [*dhfr*]). Cells were initially selected on the basis of *neo* gene expression (G418 resistance); individual subclones were subjected to increasing concentrations of methotrexate in an effort to select cells in which an amplification of the transfected DNA occurred. Because the size of the amplification unit is so large (~1,000 kilobases) (39), in most cases in which the transfected *dhfr* sequences amplify the cotransfected sequences might concomitantly amplify. That this occurs is demonstrated in Table 1: no methotrexate-resistant colonies appear from populations transfected in the absence of the *dhfr* vector (pFD11), although many such colonies appear when the *dhfr* vector is cotransfected, indicating that the amplification of the transfected *dhfr* sequences provided resistance to the inhibitor. When the transfected G418-resistant H-*ras*(Gly-12) or H-*ras*(Pro-12) clones were selected in increasing concentrations of methotrexate, all 11 independent clones isolated and analyzed at the first two levels of amplification showed elevated expression of the *ras* p21 protein as documented by immunoprecipitation analysis (Fig. 1A). As expected, no increase in endogenous p21 expression was found in cell lines transfected with only salmon DNA and selected at either 150 or 750 nM methotrexate. A significant increase in expression of $p21^{H\text{-}ras}$ (Gly-12) and $p21^{H\text{-}ras}$ (Pro-12) was achieved at the first two levels of amplification. Amplification of the cell populations beyond 750 nM methotrexate was successful only in cells transfected with DNA preparations lacking *ras* genes (Table 1), indicating that expression of the transfected normal *ras* genes was toxic at levels commensurate with a further amplification of the *dhfr* sequences. Notably, cells transfected with the highly transforming valine-12 or isoleucine-12 form of H-*ras* yielded no colonies when subjected to even the first round of selection for amplified sequences (at 150 nM methotrexate), strongly suggesting that even a modest level of overexpression of these activated derivatives was inimical to cell viability.

Repeated efforts to select clones in higher levels of methotrexate (see Materials and Methods) yielded a rare colony

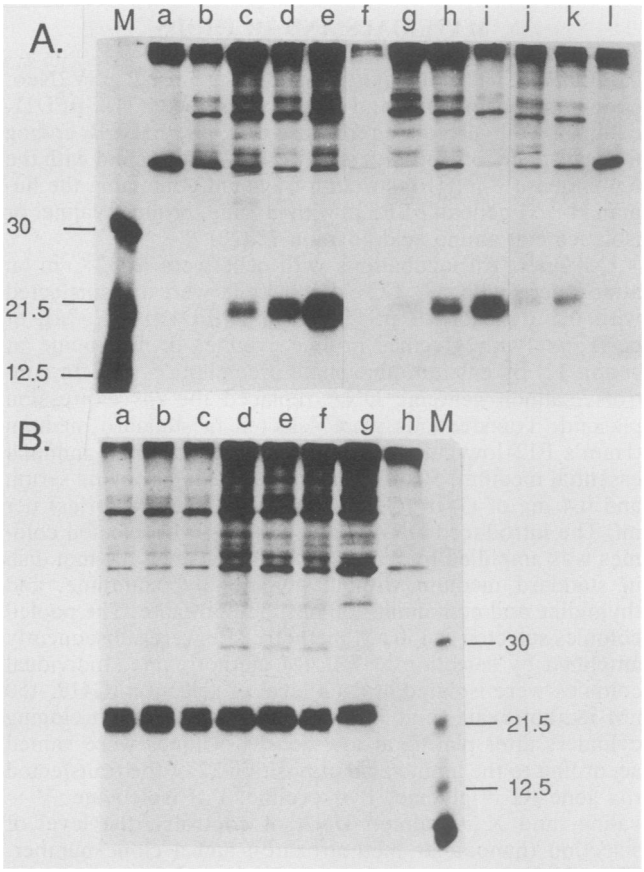

FIG. 1. Autoradiographs of [^{35}S]methionine-labeled cell proteins immunoprecipitated with anti-H-*ras* antiserum and electrophoresed in 12% polycrylamide gels. (A) Immunoprecipitations from equal amounts of total labeled protein from the following cell lines: lane a, Rat-1 (precipitated and preimmune serum); lane b, Rat-1; lane c, G0C3; lane d, G150C1; lane e, G750C2; lane f, G150C1 (precipitated with preimmune serum); lane g, P0C1; lane h, P150C3; lane i, P750C1; lane j, V0C1; lane k, V750C4; lane l, V0C1 (precipitated with preimmune serum). (B) Immunoprecipitations from cell lines selected at high methotrexate concentrations. Lane a, G750C2; lane b, G3000C1; lane c, G10000C1; lane d, P10000C1; lane e, P3000C3; lane f, P750C1; lane g, P150C3; lane h, P750C1 precipitated with preimmune serum. Lanes d, e, f, and h represent immunoprecipitates from twice as much, and lane g represents those from four times as much, cellular lysate as lanes a, b, and c. M, Molecular weight standards, × 10^3.

TABLE 2. Relative expression levels of H-*ras* p21

DNA transfected	Fold expression at given level of methotrexate amplification				
	0	150 nM	750 nM	3,000 nM	10,000 nM
H-Ras, Gly-12	1.6[a]	15.7	32.4	42.9	34.2
	0.8–2.4 (3)[b]	13.7–17.4 (3)	25.9–37.3 (3)		
H-Ras, Pro-12	1.0	6.4	9.5	15.3	14.4
	0.7–1.2 (2)	5.1–7.6 (2)	4.8–16.6 (3)	9.1–22.4 (4)	13.7–15.1 (2)
H-Ras, Val-12	3.2		3.0		
	0.9–5.6 (3)		1.5–3.8 (3)		

[a] Average value of individual colonies at each level of amplification.
[b] Range, with the number of colonies shown in parentheses when more than one colony was analyzed.

expressing H-*ras*(Val-12) at 750 nM methotrexate and Pro-12 and Gly-12 forms at both 3,000 and 10,000 nM methotrexate; this is consistent with the appearance of the rare cell in which the amplification of the selectable marker (*dhfr*) and *ras* was uncoupled. To examine this, p21 levels were determined by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 1B illustrates that p21(Gly-12) levels indeed do not significantly increase when cells are selected in levels of methotrexate above 750 nM. $p21^{H\text{-}ras}$ (Pro-12) expression was highest in cell line P3000C3; neither of the two colonies obtained by selection of this clone at 10,000 nM methotrexate displayed increased levels of $p21^{H\text{-}ras}$ expression (Fig. 1B, lanes d and e). Similarly, the three colonies expressing H-*ras*(Val-12) obtained by selection at 750 nM methotrexate showed no increase in expression of *ras* p21 over the G418-selected clones (Fig. 1A, lanes j and k). The level of $p21^{H\text{-}ras}$ expression was determined as described in Materials and Methods, and these results are summarized in Table 2. Overall, these data indicate that maximum tolerated levels of the normal and oncogenic forms of $p21^{H\text{-}ras}$ can be defined in Rat-1 cells, and these levels depend on the mutational state of the protein. Densitometric scans of autoradiograms of total protein and immunoprecipitated $p21^{H\text{-}ras}$ indicates that production of >0.12 and 0.018% of total protein as $p21^{H\text{-}ras}$ (Gly-12) and $p21^{H\text{-}ras}$ (Val-12), respectively, is not compatible with cell viability.

Influence of H-*ras*(Gly-12) and H-*ras*(Pro-12) overexpression on Rat-1 cells. To establish the relationship between p21 levels and extent of transformation, we used the following cell lines: Rat-1, G150C1, and G750C2 (the last two expressing $p21^{H\text{-}ras}$ [Gly-12] at 16- and 34-fold the level in Rat-1 cells, respectively), P750C1 (expressing $p21^{H\text{-}ras}$ [Pro-12] at 17-fold the endogenous level), and V0C1 (expressing $p21^{H\text{-}ras}$ [Val-12] at 3-fold the endogenous level). Photomicrographs illustrating the morphology of these cell lines and X750C2 (a Rat-1 cell line transfected with dihydrofolate reductase but no *ras* DNA sequences, yet nonetheless amplified by selection at 750 nM methotrexate) are presented in Fig. 2. Both the Rat-1 and X750C2 cells are flat and contact inhibited. In contrast, lines expressing elevated levels of $p21^{H\text{-}ras}$ (Gly-12), and particularly $p21^{H\text{-}ras}$ (Pro-12) are more refractile and spindle shaped, particularly after reaching confluence. The spindle-shaped morphology of these cells is similar to that seen in NIH 3T3 cells overexpressing H-*ras*(Gly-12) (25). The $p21^{H\text{-}ras}$ (Val-12)-expressing cells have the transformed morphology characteristic of cells expressing an "activated" *ras* protein, being very refractile and irregularly shaped, even at low cell densities (Fig. 1; data not shown). Thus, by the criterion of morphology, $p21^{H\text{-}ras}$ (Gly-12) possesses transforming potential, albeit weaker than that of activated derivatives.

As a more decisive criterion for transformation, we evaluated the ability of the *ras*-transfected cells to grow in an anchorage-independent fashion. Cells were plated in agar, and colony-forming ability was scored at day 14. The results of these growth studies are shown in Table 3. Rat-1 cells, X750C1 cells (transfected with salmon DNA and amplified) and G0C3 (an unamplified line expressing 2.4-fold the endogenous level of $p21^{H\text{-}ras}$ [Gly-12]) did not form colonies in soft agar. Cell lines expressing markedly elevated levels of $p21^{H\text{-}ras}$ (Gly-12) and $p21^{H\text{-}ras}$ (Pro-12) did form colonies, although at a lower efficiency than the $p21^{H\text{-}ras}$ (Val-12)-transformed cells (V0C1). When the scoring of colonies was confined to those >50 μm in diameter, the differences were even more marked; only cells expressing the structurally mutated form of p21 formed large colonies efficiently in agar. The finding that even G3000C1 (the cell line expressing the highest level of $p21^{H\text{-}ras}$ [Gly-12]) does not exhibit the same degree of anchorage-independent growth as the $p21^{H\text{-}ras}$ (Val-12)-transformed cells again argues that a robust transformation of Rat-1 cells cannot be achieved by overexpression of the normal c-H-*ras*.

A distinction can be made between transformed cells and tumorigenic cells (38). To evaluate the effect of c-H-*ras* overexpression on the tumorigenicity of Rat-1 cells, various clonal isolates of *ras*-transfected cells were injected subcutaneously into newborn rats and the growth of tumors was monitored over 6 weeks (Fig. 3). Cells derived from transfection with an activated *ras* gene (V0C1) formed rapidly growing tumors which killed all animals before week 4. In contrast, cell lines G150C1, G750C2, and P750C1, while oncogenic, caused significant tumor growth only after a 4- to 5-week delay. This indicates that there is a major difference in tumorigenicity between the cells overexpressing H-*ras*(Gly-12) or H-*ras*(Pro-12) and those transformed by H-*ras*(Val-12). The injection of vehicle alone resulted in no

TABLE 3. Growth of cell lines in soft agar

Cell line	Relative p21 synthesis (fold)	Plating efficiency	
		% Colonies	% Colonies > 50 μM
Rat-1	1	0	0
X750C1	0.5	0	0
G0C3	2.4	0	0
G150C1	16	2.4	0.1
G750C2	34	0.5	0.2
G3000C1	43	4.2	0
G10000C1	34	0.1	0
P750C1	17	1.9	0
P3000C3	22	0.8	0
P10000C1	15	0.1	0.1
V0C1	3.1	7.3	6.3

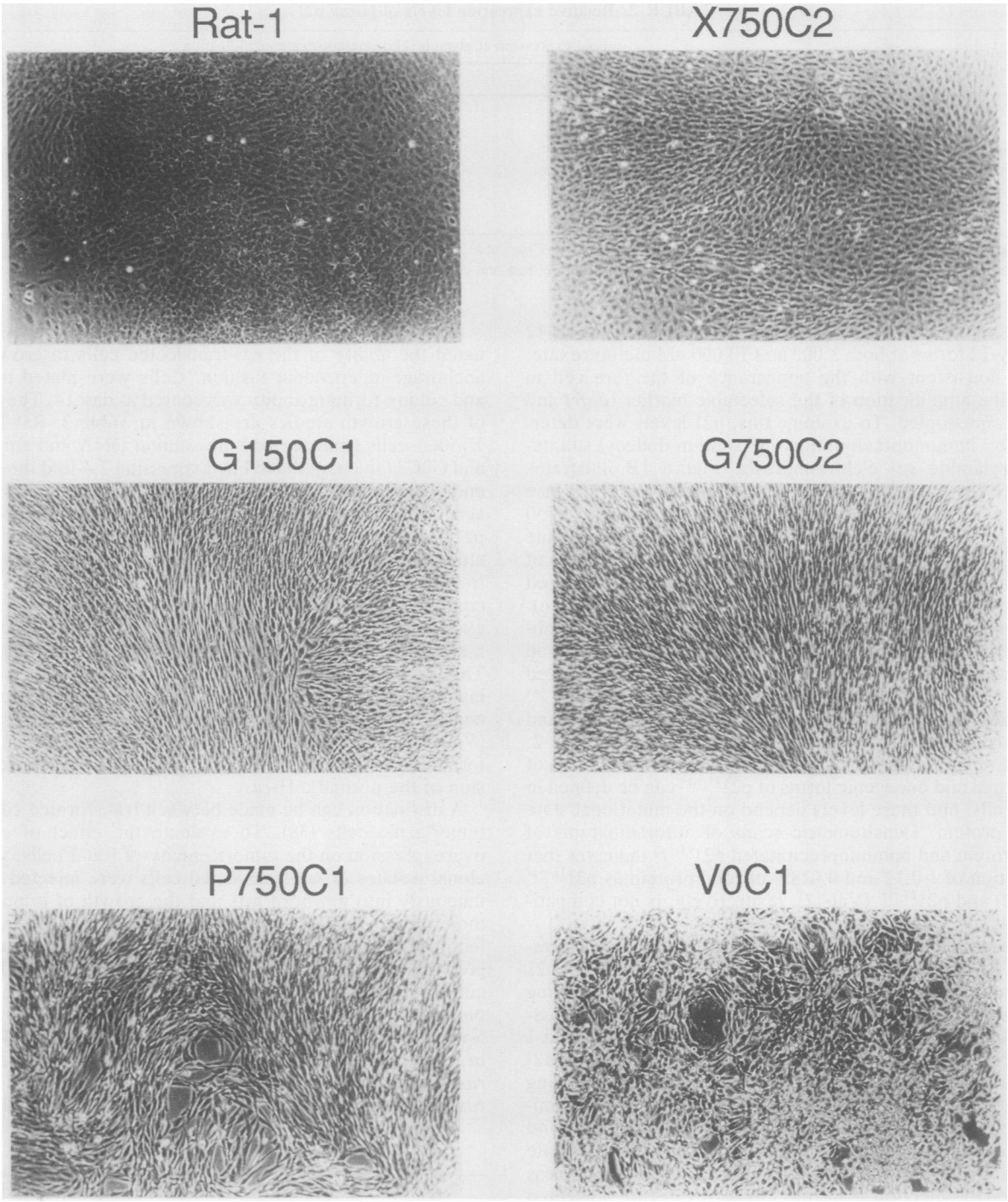

FIG. 2. Photomicrographs of the parental Rat-1 cells and some derived lines. A total of 10^5 cells were plated per 100-mm dish and maintained in standard medium supplemented with 10% fetal bovine serum. The cells were photographed after 8 to 14 days. Scale: 1 cm = 70 μM.

detectable growth in 10 animals. Tumor tissue was recovered from sacrificed rats and cultured in vitro. The cells resembled the original cells injected with respect to both morphology and resistance to methotrexate. The nature and quantity of *ras* p21 expressed in recovered G150C1 cells was analyzed by polyacrylamide gel electrophoresis. The amount of *ras* p21 synthesis was essentially the same as that of the original cell line and shows no evidence of any slower migrating p21 (data not shown); these findings indicate that the delay in growth of the tumors was not due to an initial selection for cells either not expressing elevated levels of H-*ras*(Gly-12) or expressing *ras* with a position-12 glycine-to-valine mutation.

Influence of growth factors on, and production of growth factors by, cell lines overexpressing H-*ras* p21. A number of transformed cell lines are known to produce growth factors (reviewed in references 10 and 37). These factors, later identified as primarily TGFα and TGFβ (1), were also found

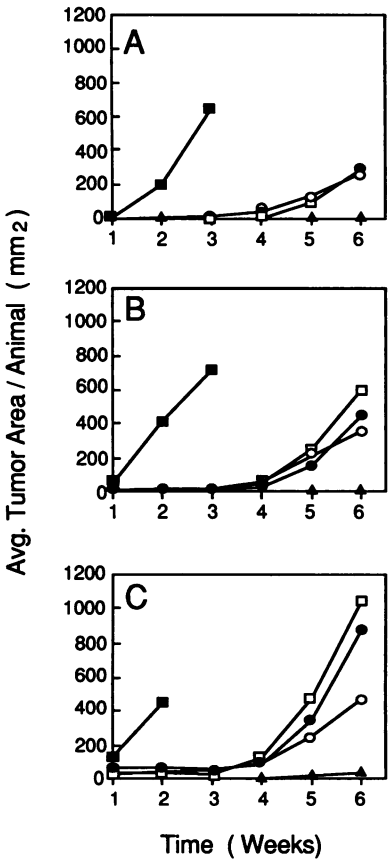

FIG. 3. Tumorigenicity of Rat-1 cells and derived cell lines in newborn rats injected subcutaneously with the following numbers of cells: (A) 5×10^4 cells per animal; (B) 5×10^5 cells per animal; (C) 5×10^6 cells per animal. Symbols: ■, V0C1; ●, G150C1; ○, G750C2; □, P750C1; ▲, Rat-1. Tumors were measured weekly [(length × breadth)/2], and the average for each group (of 10 original animals) is presented.

to be secreted by chemically induced mouse carcinoma cells (26), human tumor cells (44), and Fischer rat 3T3 cells transformed by a *ras* oncogene (40). To evaluate the effect of p21 on the production of these growth factors, we examined the ability of cell lines overexpressing $p21^{H\text{-}ras}$ (Gly-12) or $p21^{H\text{-}ras}$ (Pro-12) to grow in serum-free medium and determined the effect of added factors on these as well as Rat-1 and V0C1 cells by thymidine incorporation measurements (Table 4). In the absence of added factors, V0C1 cells incorporated 10-fold as much thymidine as the parental Rat-1 cells. Thymidine incorporation into G150C1, G750C2, and P750C1 cells was three- to fivefold that of Rat-1 cells. Serum (to 10%) caused a 2.8-fold increase in thymidine uptake in Rat-1 cells, a 1.4-fold increase in G750C2 and P750C1 cells, and only a 1.1-fold increase in V0C1 cells. A similar pattern of stimulation was seen when the cells in serum-free medium were exposed to EGF and PDGF. The PDGF stimulation of DNA synthesis in Rat-1 cells and absence of stimulation in *ras*-transformed Rat-1 cells parallel that reported in other studies (24). Bombesin, which may be linked via N-*ras* to inositol phosphate production (46), had no influence on the cell lines examined. TGFβ decreased DNA synthesis of V0C1 to 70% of the control level, but had no effect on the other cell lines.

TABLE 5. Influence of conditioned media on [^{3}H]thymidine incorporation in rat-1 cells

Addition to conditioned medium	cpm/10^5 cells ± SE	% Stimulation
Control	12,260 ± 325	
Rat-1	11,810 ± 233	−4
G3000C1	15,510 ± 845	27
V0C1	22,560 ± 70	84
None	8,690 ± 312	
PDGF (12.5 ng/ml)	19,770 ± 613	128

The high rate of DNA synthesis of the transformed V0C1 cells and its lack of stimulation in response to serum and other growth factors (e.g., EGF and PDGF) may indicate that these cells are producing one or more growth factors that act in an autocrine manner to maximally stimulate DNA synthesis. To examine this, serum-free conditioned medium was prepared from Rat-1 cells, G3000C1 cells, and the H-*ras*(Val-12)-transformed cell line V0C1; their ability to induce DNA synthesis in confluent cultures of Rat-1 cells was evaluated. While conditioned medium from Rat-1 cells was unable to stimulate DNA synthesis, medium derived from V0C1 cells stimulated DNA synthesis 84% (Table 5). Again consistent with previous findings that the expression of high levels of the normal c-H-*ras* proto-oncogene is only partially penetrant with respect to the induction of the transformed phenotype, conditioned medium from the G3000C1 cells stimulated DNA synthesis in an intermediate fashion (Table 5). TGFβ was detectable by a radioreceptor assay in the 10×-concentrated conditioned media of G3000 C1 (1.9 to 4.1 ng/ml) and V0C1 (02.0 to 3.1 ng/ml), but not from normal Rat-1 cells (<1 ng/ml) (data not shown); this is presumably unrelated to the effect of the conditioned medium on DNA synthesis as exogenous TGFβ does not stimulate DNA synthesis in Rat-1 cells (Table 4). To examine whether the growth factor could be related to TGFα, conditioned medium was tested in the NRK 49F soft-agar assay (40); no such activity was detected (<0.2 pmol of EGF per ml of conditioned medium; data not shown).

TABLE 4. Influence of factors on [^{3}H]thymidine uptake

Cell line	[^{3}H]thymidine uptake (cpm per well ± SE)[a] in the presence of[b]:					
	0	Serum	EGF	PDGF	TGFβ	Bombesin
Rat-1	3,429 ± 178	9,740 ± 682	9,067 ± 427	13,182 ± 515	3,797 ± 183	3,821 ± 96
G150C1	16,324 ± 496	29,700 ± 269	19,634 ± 1,045	27,813 ± 1,114	15,590 ± 930	14,914 ± 609
G750C2	10,587 ± 319	15,303 ± 815	13,185 ± 52	12,819 ± 279	9,701 ± 317	10,713 ± 589
P750C1	14,973 ± 777	20,900 ± 1,328	12,133 ± 1,408	20,665 ± 1,090	13,639 ± 1,351	16,321 ± 357
V0C1	35,609 ± 1,169	38,695 ± 1,449	39,840 ± 408	36,668 ± 1,380	24,890 ± 794	30,810 ± 639

[a] $n = 3$.
[b] The final concentrations of the added factors were: serum, 10%; EGF, 10 ng/ml; PDGF, 12.5 ng/ml; TGFβ, 2 ng/ml; bombesin, 100 nM.

TABLE 6. Transformation of cell lines by Val-12 H-*ras*

Cell line	Relative p21 synthesis (fold)	hyg^r colonies[a]	Foci[b]	Foci/hyg^r colonies
Rat-1	1	162	88	0.54
G150C1	16	201	73	0.36

[a] Cells were transfected with 1 μg of pSVHygB and carrier DNA to 10 μg and selected in 0.4 mg of hygromycin per ml.

[b] Cells were transfected with 4 μg of pc-H_1*ras* (Val-12) with carrier DNA to 10 μg. The plates were stained with crystal violet and foci were counted after 16 days.

Transformation of cells overexpressing H-*ras*(Gly-12) with H-*ras*(Val-12). The dominant transforming activity of activated *ras* oncogenes has been well documented (reviewed in reference 2), a result at some odds with the finding that many tumors that express activated p21^{ras}, and cell lines derived from them, express no detectable levels of the corresponding normal p21^{ras} (Gly-12) (4, 5, 12, 27, 30), suggesting that loss of the normal allele, or its expression, may be involved in progression to tumorigenicity. Having established that overexpression of p21$^{H\text{-}ras}$ (Gly-12) does not fully transform Rat-1 cells, we investigated whether cells overexpressing p21$^{H\text{-}ras}$ (Gly-12) were resistant to full transformation by activated *ras* oncogenes. When G150C1 cells (which express 16 times as much p21$^{H\text{-}ras}$ [Gly-12] as Rat-1 cells) were transfected with pc-H-*ras*1(Val-12), focus formation occurred at about the same frequency as in Rat-1 cells (Table 6); as expected, no foci formed when the cells were transfected with salmon DNA (data not shown). To verify that the transfection efficiencies of the two cell lines were comparable, cells were transfected with a selectable marker (hygromycin resistance); no significant difference in transfectability was observed (Table 6). Subsequently, G150C1 cells were cotransfected with pc-H-*ras*(Val-12) (or salmon genomic DNA as control) and pSVEHygB in a 10:1 ratio. Hygromycin-resistant colonies were isolated with cloning cylinders from both the control salmon DNA-transfected cells and the H-*ras*(Val-12) transfectants. While the control transfectants were morphologically similar to the parental G150C1 cells, clones supertransfected with pc-H-*ras*(Val-12) exhibited an extremely transformed morphology and formed large colonies in soft agar at a frequency similar to the V0C1 cell line; by immunoprecipitation analysis, the latter were found to still express elevated levels of p21$^{H\text{-}ras}$ as well as smaller amounts of p21$^{H\text{-}ras}$ (Val-12) (data not shown). These results indicate that cells can be fully transformed by an activated *ras* oncogene even in the presence of abundant levels of the normal H-*ras* gene product.

DISCUSSION

Perturbations in the expression of cellular *ras* genes have been firmly implicated in tumor development. The most thoroughly studied examples involve the mutational activation of *ras* genes, which occurs in about 25% of human tumors (2, 3, 13). Following reports that high levels of nonmutated p21 are potentially oncogenic in vitro (6, 7, 25), elevated levels of *ras* gene expression were detected in about 50% of tumors examined (33, 35, 36). Whether this merely reflects the high mitotic activity of tumor cells is unclear; it is known, for instance, that increased *ras* expression is found during tissue regeneration (17). In two studies amplification of K-*ras* gene sequences was found in 2 of 176 tumors examined (25, 49), while no amplification of H-*ras* genes was detected. The significance of the K-*ras* gene amplifications is unclear as it is not known whether the amplified genes are actively transcribed. In view of evidence that nonmutated p21 was capable of transforming NIH 3T3 cells (6, 25), it appeared that the differences in transforming potential between normal and activated *ras* genes were simply quantitative in nature; that is, a robust transformation could be achieved by nonmutated *ras* genes, if a sufficiently high degree of expression was attained.

We have addressed this issue by directly comparing the phenotype of cells expressing elevated levels of p21$^{H\text{-}ras}$ (Gly-12) with cells expressing more modest levels of the activated p21$^{H\text{-}ras}$ (Val-12) on Rat-1 cells. Our approach utilized a novel selection method, namely, the coamplification of target (*ras*) sequences with an amplifiable selectable marker (*dhfr*), after the introduction of both genes with a dominant selectable marker (*neo*). Previous efforts to evaluate the transforming capacity of other cellular proto-oncogenes relied on the initial ability of the expression vectors to direct the synthesis of elevated levels of the encoded polypeptide; in this way, for instance, it was demonstrated that pp60$^{c\text{-}src}$ at 10-fold normal levels were able to elicit a partially transformed phenotype in Rat-2 cells (23). By linking the target gene to be expressed to an amplifiable marker, one can achieve levels of target gene expression that appear to be limited only by the ability of the cells to tolerate the protein, even in cells (such as Rat-1) for which the amplifiable gene cannot be selected for directly (Table 1; unpublished results). Previously, all efforts to couple the amplification of a given gene to *dhfr*, for instance, have utilized a derivative Chinese hamster ovary cell line that lacks the *dhfr* enzyme (45); such cells would be unsuitable for the present analysis as they exhibit transformed characteristics. In this way we were able to isolate cell lines in which the level of p21 was 2- to 44-fold greater than endogenous levels. We find that, while normal p21 is able to promote a partially transformed phenotype in Rat-1 cells, it is a much less effective transforming protein than structurally activated derivatives, even when expressed at 40-fold-greater levels. This conclusion is valid insofar as transformation can be measured by focus formation, growth in soft agar, elevated production of growth factors, growth in the absence of serum factors, and tumorigenicity. The transforming potential of p21 cannot therefore be fully realized simply by its overexpression in Rat-1 cells, indicating that qualitative changes in the activity of p21$^{H\text{-}ras}$ accompany activating mutations. Although a qualitative difference in activity of the normal and the activated p21$^{H\text{-}ras}$ is therefore strongly suggested, quantitative effects are also operative. The level of activated p21 does play a role in the determination of cell transformation of both NIH 3T3 (32) and Rat-4 (48) cells, and we have shown in this study that overexpression of the normal allele does alter the properties of Rat-1 cells. Both qualitative and quantitative aspects of p21$^{H\text{-}ras}$ expression therefore appear to play a role in cell transformation and tumorigenesis. The finding that overexpression of the normal allele is not sufficient per se to fully transform cells can be compared to reports that c-*src* does not fully transform Rat-2 (23) or NIH 3T3 (29) cells.

The conclusion that there are essential qualitative differences between normal and structurally mutated forms of p21 differs from that of others (6, 25), who found that elevated levels of normal p21 were capable of the morphological and tumorigenic conversion of NIH 3T3 cells. While it is possible that the differences reflect the different cell types used (NIH 3T3 versus Rat-1 cells), the former studies did not compare per se the properties of cells expressing elevated levels of

normal p21 with cells expressing structurally activated p21. So while our findings confirm that Rat-1 cells can be rendered tumorigenic by the action of normal p21, it is clear that the degree of tumorigenicity promoted by normal p21 does not match that of a structurally mutated form of the protein.

The proline-12 form of p21 does not transform Rat-1 cells in a direct focus assay (28) and exhibits a higher GTPase activity than $p21^{H\text{-}ras}$ (Gly-12) in vitro (8). Our findings that at equivalent p21 levels cells expressing $p21^{H\text{-}ras}$ (Pro-12) are morphologically more transformed than those expressing the normal allele demonstrate that there is no absolute correlation between loss of GTPase activity and transforming potential, a finding in accord with recent published results (11, 19, 43); nonetheless, it is clear that significantly more p21(Pro-12) is required to morphologically transform Rat-1 cells than activated forms of the protein.

Although *ras* oncogenes are known to transform established cell lines in a dominant manner (2), a number of human tumors expressing activated *ras* genes have very little or no detectable normal p21 (4, 5, 12, 27, 30). In addition, somatic cell hybrids between tumor cells expressing an activated *ras* and normal *ras* are nontumorigenic (9, 14, 18, 38, 40). These results can be interpreted to mean that the loss of expression of the normal *ras* allele plays a role in tumorigenesis; as such, a high level of expression of the normal allele may protect cells from the full transforming effects of an activated *ras* allele. A partial understanding of this apparant paradox is provided by our finding that overexpression of the normal $p21^{H\text{-}ras}$ does not fully transform Rat-1 cells. Furthermore, cells expressing elevated levels of the normal allele can be fully transformed by H-*ras*(Val-12), a result in accord with work previously reported (42). This suggests that loss of expression of the normal *ras* allele seen in some tumor cell lines may be fortuitous; alternatively, it is possible that the threshold of $p21^{H\text{-}ras}$ (Val-12) required to fully transform cells may be lower in the absence of expression of the normal allele.

In summary, we have found that the complete elaboration of the transformed phenotype by *ras* depends on mutations that distinguish the cellular and viral forms of the polypeptide. While the expression of high levels of c-H-*ras* or the proline-12 mutant does cause partial morphological transformation of Rat-1 cells, the cells are not fully transformed when directly compared with H-*ras*(Val-12)-transformed cells when more rigorous standards of transformation such as anchorage-independent growth and tumorigenicity are applied. We conclude that qualitative changes in the function/activity of $p21^{H\text{-}ras}$ are necessary for the full elaboration of the transforming potential of the polypeptide. These could involve alterations in GTPase activity (2, 21); alternatively, as their tissue-specific expression pattern (20) and distinct evolutionary conservation (2, 21) imply distinct roles for the N-*ras*, K-*ras*, and H-*ras* proto-oncogenes, changes induced by activating mutations might directly affect the interaction of $p21^{H\text{-}ras}$ with one or more regulatory proteins.

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