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Immortalization by c-*myc*, H-*ras*, and E1a Oncogenes Induces Differential Cellular Gene Expression and Growth Factor Responses

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Early-passage rat kidney cells were immortalized or rescued from senescence with three different oncogenes: viral promoter-driven c-*myc*, H-*ras* (Val-12), and adenovirus type 5 E1a. The normal c-*myc* and H-*ras* (Gly-12) were unable to immortalize cells under similar conditions. Quantitation of RNA in the *ras*-immortalized lines demonstrated that the H-*ras* oncogene was expressed at a level equivalent to that of the normal H-*ras* gene in established human or rat cell lines. Cell lines immortalized by different oncogenes were found to have distinct growth responses to individual growth factors in a short-term assay. E1a-immortalized cells were largely independent of serum growth factors, whereas c-*myc*-immortalized cells responded to serum better than to epidermal growth factor and insulin. H-*ras*-immortalized cells responded significantly to insulin alone and gave a maximal response to epidermal growth factor and insulin. Several cellular genes associated with platelet-derived growth factor stimulation, including c-*myc*, were expressed at high levels in the H-*ras*-immortalized cells, and c-*myc* expression was deregulated, suggesting that the H-*ras* oncogene has provided a "competence" function. H-*ras*-immortalized cells could not be morphologically transformed by secondary transfection with a long terminal repeat–c-*myc* oncogene, but secondary transfection of the same cells with H-*ras* (Val-12) produced morphologically transformed colonies that had 20- to 40-fold higher levels of H-*ras* oncogene expression. Thus, transformation in this system is dependent on high levels of H-*ras* oncogene expression rather than on the presence of activated H-*ras* and c-*myc* oncogenes in the same cell.

Cellular oncogenes often have profound effects when transfected into recipient cells, namely, the induction of unrestrained growth and a transformed phenotype. However, under other circumstances oncogenes can induce more subtle alterations in growth, such as rescue from senescence or immortalization. Some oncogenes, e.g., H-*ras*, have been most frequently associated with transformation (19, 26), whereas others, e.g., c-*myc*, have been linked to immortalization (21, 27). However, activated c-*myc* oncogenes have been shown to induce a transformed phenotype in immortalized cell lines (15, 16; reviewed in reference 9), albeit less dramatically than with H-*ras*, whereas the H-*ras* oncogene was shown to have immortalizing activity (31). It is important to understand whether both the transforming and immortalizing activities of a given oncogene are the result of the same alteration in growth regulation manifest differently under the different cell backgrounds or whether there is some fundamental difference between the two processes. Furthermore, numerous investigations into the origin of cancer have suggested that multiple steps are required to generate fully malignant cells (reviewed in reference 37).

We were interested in immortalizing primary cells by using a selected number of known oncogenes for two reasons. First, in rescuing primary (early-passage rodent) cells from senescence, oncogenes would impart phenotypes to the recipient cells that were a direct, possibly unique, reflection of the function of the immortalizing oncogene. One could, therefore, choose to compare the phenotypes of such cells as a first step toward understanding how each oncogene affects the growth-regulatory network. The phenotypes of immediate interest to us were morphology, growth factor requirements, and patterns of gene expression that might be induced by the transfected oncogene. Our second goal was to determine whether the dosage of a particular oncogene could affect its ability to immortalize; i.e., was there a lower limit to the level of expression of an activated oncogene below which it would not have the ability to rescue cells from senescence? Likewise, was there an upper limit or threshold of tolerance for the expression of an oncogene in a primary cell background? In this study, we describe the influence of three different oncogenes on the growth factor requirements in immortalized cells, the dosage dependence of the H-*ras* oncogene for immortalization and transformation, and the influence of H-*ras* on the regulation of the c-*myc* proto-oncogene.

MATERIALS AND METHODS

Plasmid constructs and RNA analysis. The pLTRh*myc*, pSVm*myc*, p1A, and vector plasmids have been described previously (16). The pEJ*ras* and pEC*ras* clones were generous gifts of R. Weinberg. The *ras* first-exon clone, pEJ-BX, used for RNase protection assays, is a 1.9-kb *Bam*HI-*Xba*I fragment from pEJ inserted into the corresponding sites of pT7-2. p*neo*-HB (constructed by T. Jones) is a 320-base-pair *Bgl*II-*Hin*dIII fragment from the *neo* gene inserted into the corresponding sites of pT7-1. pr*myc*1.4, containing a 1.4-kilobase *Bgl*II fragment of the rat *myc* first exon inserted into the *Bam*HI sites of vector pGEM-4, was a gift from David Steffen.

Transfection, selection, and cell immortalization of primary cell cultures. Baby rat kidney (BRK) cells were derived from pooled kidneys from 6-day-old litters of rats; the kidneys were carefully teased and processed (26). Secondary cultures were used directly for transfection assays and RNA preparation. Cells were transfected by using the calcium phosphate protocol (12). To select cells that had acquired exogenous DNA, the plasmids to be assayed for immortalizing activity were cotransfected at a 10:1 ratio with

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TABLE 1. Immortalizing activities of oncogenes

Plasmid DNA	Immortalization	
	No selection	RSV*neo* coselection[a]
LTRh*myc*	+	11/17
H-c-*myc*	−	0/10
SVc-*myc*	+	7/12
EVX-*myc*XH	+	5/8
EVX	−	0/10
SV2neo	−	0/10
RSVneo	−	0/10
EJ*ras* (Val-12)	+	6/15
EC*ras* (Gly-12)	−	0/10
E1a (adenovirus type 5)	+ +	9/10

[a] Number of G418-resistant colonies which could be expanded into continuous cell lines/number of colonies tested.

pSV2*neo* (30) or pRSV*neo* (11). Cells expressing *neo* were selected in medium containing 300 μg of G418 (GIBCO Laboratories) per ml, beginning 48 h after transfection. G418-resistant colonies were scored after 3 weeks, isolated by using cloning cylinders, and transferred to individual wells of 24-well microtiter dishes. Those colonies that plated down and proliferated were expanded and characterized. Where transfections were performed without selectable markers, the plasmids (10 μg) were transfected as above, and the cells were split 1:10 after 24 h. Individual colonies were isolated after 2.5 to 3 weeks with cloning cylinders and expanded as described above.

Tumorigenicity assays and soft agar cloning. Tumorigenicity assays were performed essentially as described by Blair et al. (4). Cells were grown to mass culture, harvested by trypsinization, and washed at least twice in phosphate-buffered saline. Cell pellets were suspended at concentrations of 2×10^6 viable cells in 0.2 ml of sterile phosphate-buffered saline and injected subcutaneously into 6- to 8-week-old BALB/c athymic nude mice. Animals were monitored regularly, up to 12 weeks after injections, for the appearance of tumors. Characterization of the anchorage-independent growth properties of cells was performed by plating 5×10^4 cells in medium containing 0.26% agar layered over 0.65% agar medium in 60-mm culture dishes. Plates were scored for colonies 14 days after seeding.

Total cytoplasmic RNA was isolated and analyzed by Northern blot hybridization as described previously (15). Hybridization was carried out with the buffers of Church and Gilbert (7). Total or poly(A)$^+$ RNAs were analyzed by RNase protection essentially as described by Zinn et al. (39). Protected fragments were electrophoresed in 6% polyacrylamide–urea gels. Labeled, *Msp*I-restricted pBR322 markers were run in parallel.

Serum and growth factor response assays. Confluent cultures of secondary or immortalized BRK cells were washed with phosphate-buffered saline and then fed with serum-free Dulbecco modified Eagle medium. After incubation for 12 to 16 h, Dulbecco modified Eagle medium containing different concentrations of serum or growth factors was added to the quiescent cultures; after an additional 5 h, [^{3}H]thymidine (1 μCi/ml) was added. Incorporation of trichloroacetic acid-precipitable radioactivity was determined after 12 h.

RESULTS

Immortalization of early-passage rat kidney cells by the H-*ras* oncogene. We were interested in the interaction of oncogenes with the control of normal cell growth, in particular that of primary cells. The initial response of primary cells to many oncogenes is immortalization or rescue from senescence. We decided to characterize cell lines immortalized by different oncogenes with respect to oncogene dosage, response to secondary oncogene transfections, and growth factor requirements. To assay the ability of an oncogene to immortalize primary cells, secondary cultures of Fisher BRK cells were transfected with plasmids carrying three different oncogenes and, where possible, their normal proto-oncogene homologs. We used viral promoter-linked c-*myc* genes, the adenovirus type 5 E1a gene, and the H-*ras* oncogene (Val-12) (29) as well as the normal c-*myc* gene with its own promoter and the normal H-*ras* (Gly-12) gene. The assays were carried out with or without a drug resistance marker as described in Materials and Methods. The results for several oncogenes and control plasmids are shown in Table 1. As expected from previous studies (14, 21, 27), viral promoter-linked c-*myc* genes and E1a had immortalizing activity, whereas the normal human c-*myc* gene with its own promoter, vector DNA, RSVneo, and SV2neo were negative. Similarly, the activated H-*ras* gene (Val-12) induced immortalized colonies as reported previously (31), whereas the normal H-*ras* gene (Gly-12) did not. It should be noted that the mutant H-*ras* construct used in this study harbors the gene with its own promoter and no enhancing sequences. Depending on the oncogene, from 40 to 90% of the G418-resistant colonies could be expanded into established lines. Most of our further studies were carried out on G418-selected lines, and the cell lines used were derived from individual colonies resulting from independent, primary transfections.

Cell morphology. The cell lines established by c-*myc*, H-*ras*, and E1a each had a characteristic morphology (Fig. 1); the E1a morphology was the most recognizable, with small polygonal cells (34). At high densities, E1a cells tended to pack closely together but did not overgrow the monolayer at any stage. c-*myc*-immortalized lines released cells into the medium at high density but, like the E1a cells, did not overgrow the monolayer. H-*ras*-immortalized lines had the typical morphology of established cultures, with flat cells that exhibited a swirling pattern at high densities. It should be pointed out that H-*ras* oncogene-transformed cells generally exhibit a very different morphology. Such cells are rounded, refractile, and attached loosely to the surface of the culture dish (data not shown). In the early stages of the immortalization procedure with EJ*ras*, foci resembling those in a *ras*-transformed culture appeared at a low frequency in the culture dishes, but these foci seldom progressed beyond 100 to 150 cells, i.e., they either senesced before the first passage or did not replate on passaging.

Expression of the immortalizing oncogenes. In H-*ras*-immortalized cells, preliminary analysis with Southern blots and Northern blots (RNA blots) showed one to five copies of the transfected plasmid and levels of H-*ras* expression that were equivalent to those found in established lines (data not shown), but it was impossible to distinguish between endogenous and exogenous H-*ras* expression. To determine whether the transfected gene is transcribed correctly and to compare levels of transcription with other cells, we carried out RNase protection analyses by using a probe that spanned the first exon of the human H-*ras* gene. Figure 2A shows an analysis of poly(A)-containing RNA from one immortalized line, BRKneo*ras*1, the established rat fibroblast line Rat2, and the human cell line HeLa. The human H-*ras* RNA protects a 130-base fragment, whereas the protected endog-

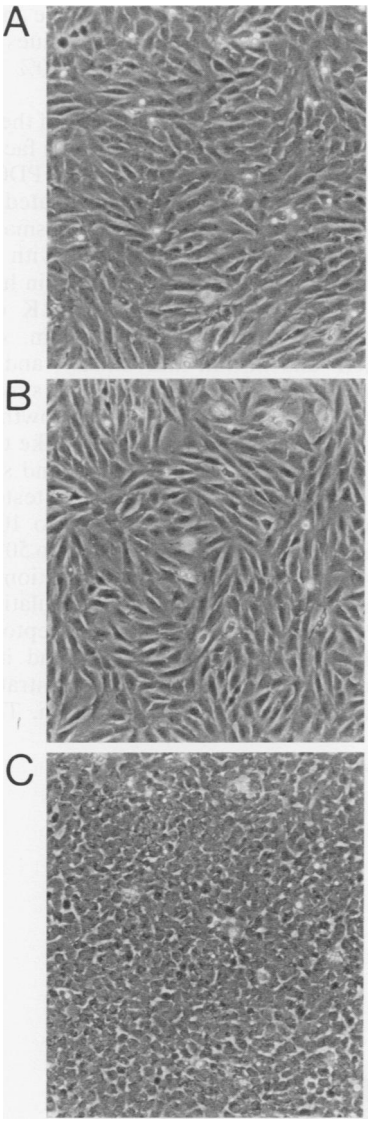

FIG. 1. Phase-contrast micrographs showing the morphology at confluence of BRK cells immortalized by activated oncogenes. (A) BRKnh*myc*3, immortalized with a long terminal repeat-driven human c-*myc* gene. (B) BRKn*ras*3, immortalized with an activated H-*ras* (Val-12) gene. (C) BRKnE1a3, immortalized with the adenovirus type 5 E1a gene.

enous rat fragment is somewhat smaller (96 bases) due to sequence differences. The level of expression of the transfected H-*ras* oncogene (Val-12) in the H-*ras*-immortalized cells is equivalent to that in HeLa cells and very similar to the level of the endogenous rat H-*ras* RNA found in either Rat2 cells or in the *ras*-immortalized lines. The same level of H-*ras* oncogene (Val-12) expression was found for every *ras*-immortalized line analyzed (Fig. 2B), independent of whether immortalization was carried out with G418 selection. Thus, the dosage of H-*ras* oncogene (Val-12) RNA that promotes immortalization of rat kidney cells is equivalent to the level that is normally expressed in established cell lines. Similarly, the level of c-*myc* expression in the *myc*-immortalized lines was one to five times the level found in established lines, and the E1a gene expressed both 13S and 12S RNAs as expected (35; data not shown).

Expression of endogenous genes in immortalized lines. Further characterization of the immortalized cell lines was provided by the analysis of the steady-state expression of seven cellular genes, many of which have been linked to cell proliferation, by using RNA from 80% confluent cultures growing in standard medium with 10% fetal calf serum (Table 2). The results for the endogenous c-*myc* gene are discussed in detail below. c-*fos* was expressed at a low level in all of the immortalized lines, comparable to the low level observed in Rat2 cells (data not shown). RNA levels were also analyzed for two genes, *JE* and *KC*, which were characterized as part of a platelet-derived growth factor (PDGF)-inducible "competence" gene family in fibroblasts (8). Both of these genes were expressed at the highest levels in the BRK-*ras* lines, expressed at a three- to fourfold lower level in BRK-*myc* cells, and undetectable in the BRK-E1a cells (data not shown). Expression of *MHC* genes was not suppressed (but was, in fact, slightly elevated) in the E1a lines, an observation that agrees with earlier reports that adenovirus type 12 E1A protein, and not adenovirus type 5 E1a protein, could suppress expression of *MHC* genes in rodent cells (28). β-Actin and p53 RNA levels were constant among all cells analyzed.

Differential growth factor responses of oncogene-immortalized lines. Initially, the dependence of the different immortalized cell lines on serum concentration was analyzed by growth curves (data not shown). In 10% fetal calf serum (FCS), the growth of all of the lines plateaued at confluence. On the other hand, in 0.5% FCS cells immortalized by E1a

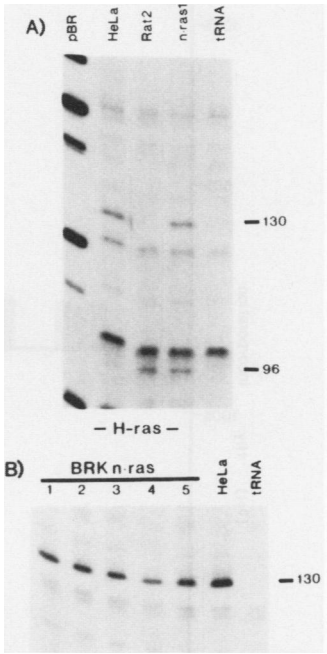

FIG. 2. Expression of H-*ras* in oncogene-immortalized cells. (A) RNase protection assay with 0.1 μg of poly(A)$^+$ RNA from HeLa, Rat2, and BRKn*ras*1 (a *ras*-immortalized line) cells. The labeled RNA probe, pEJ-BX (linearized with *Sma*I), protects 130 base pairs of human H-*ras* oncogene RNA and 96 base pairs of the endogenous (rat) H-*ras* RNA. This probe consistently produced a small number of RNase-resistant fragments with no hybridizable RNA, as evident in the tRNA lane. The markers were derived from *Msp*I-digested pBR322. (B) RNase-protection assay of the transfected H-*ras* oncogene RNA in five independently derived H-*ras*-immortalized lines compared with RNA from HeLa cells. Equivalent amounts (10 μg) of total cytoplasmic RNA were used for each.

TABLE 2. Comparative levels of cellular gene expression in primary and oncogene-immortalized BRK cells

Cellular gene	Relative levels[a] the following cell lines:		
	BRK-*myc*	BRK-*ras*	BRK-E1a
c-*myc*	+	+ + + +	+
p53 gene	+	+	+
c-*fos*	+	+	+
JE	±	+ +	−
KC	±	+ +	−
MHC	+	+	+ +
β-Actin gene	+	+	+

[a] Poly(A)-containing RNA was isolated from 80% confluent monolayers and subjected to Northern blot analysis with probes as described in Materials and Methods. Two lines of each type were analyzed. The RNA levels were compared with those found in Rat2 cells as follows: (−) no expression, (+ to + + + +) levels equal to or higher than those in Rat2 cells.

and H-*ras*, but not those immortalized with c-*myc*, grew to a limited but significant degree. To better understand the growth factor requirements of each cell type, we analyzed the growth response to purified factors in a short-term assay by monitoring [^{3}H]thymidine uptake. Two to four individual lines immortalized by each oncogene were tested for their growth response to different factors; comparable results were obtained for different lines immortalized by the same oncogene, although absolute values varied from line to line. Since the total number of cells at confluence and the absolute growth rates varied between the lines, values were normalized to [^{3}H]thymidine incorporation in 10% serum, which was taken as a 100% response.

The short-term growth factor response of the cell lines was analyzed for two factors, epidermal growth factor (EGF) and insulin (Fig. 3), which promote entry of PDGF-stimulated cells into the S phase (6, 24). When incubated in serum-free medium, BRK-E1a cells showed only a small decrease in [^{3}H]thymidine incorporation compared with that for 10% serum, and the addition of EGF and insulin had little effect (Fig. 3). In contrast, the secondary BRK cultures were severely suppressed in serum-free medium, showed no response to EGF and insulin individually, and were weakly stimulated when both factors were added simultaneously.

The most interesting response to growth factors was exhibited by the BRK-*ras* lines (Fig. 3). Like the BRK cells, the *ras*-immortalized lines failed to respond significantly to EGF alone at any of the concentrations tested. However, high concentrations of insulin alone (1 to 10 μg/ml) were found to stimulate incorporation up to 40 to 50% of the value observed for 10% serum. At this concentration of insulin, the factor is almost certainly binding and stimulating cell growth through the insulinlike growth factor I receptor (6). Furthermore, simultaneous addition of EGF and insulin gave a growth response that, at the highest concentration of insulin, was equal to that exhibited in 10% serum. Thus, the pres-

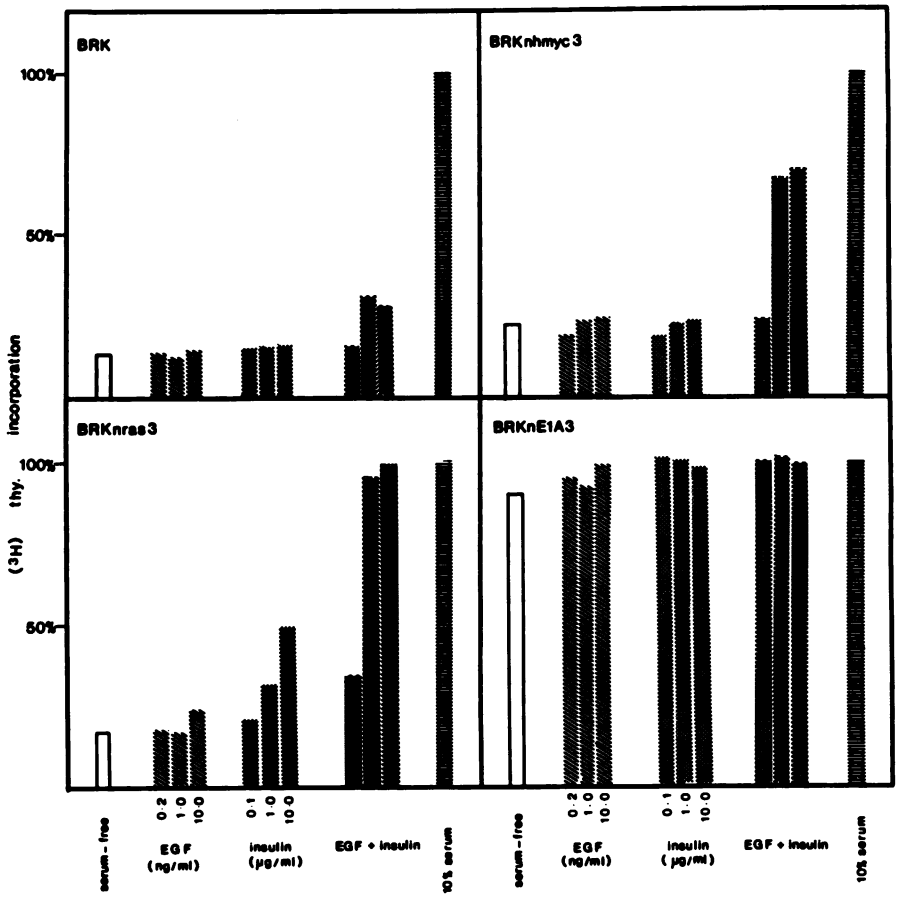

FIG. 3. Growth response of secondary and oncogene-immortalized BRK cells to EGF, insulin, and serum. The specified growth factors (at the indicated concentrations) were added to quiescent cultures in serum-free medium. For the response to both insulin and EGF added together, a constant amount of EGF (10 ng/ml) was used and the insulin concentrations were varied (0.1, 1.0, and 10.0 μg/ml). The highest concentration of each growth factor gave the maximum possible response in separate titration experiments.

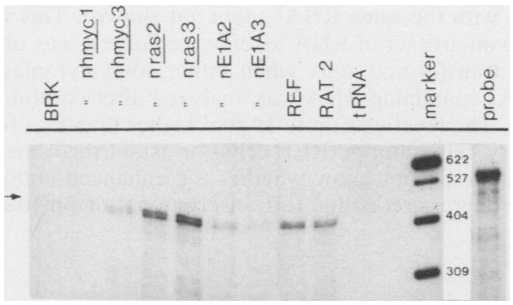

FIG. 4. Expression of the endogenous c-*myc* gene in BRK cells immortalized by different oncogenes. The steady-state expression of c-*myc* was analyzed by the RNase protection protocol. Samples (10 μg) of total cytoplasmic RNA from 80% confluent cultures of different immortalized lines and from secondary BRK, REF, and Rat2 cells were hybridized with a labeled RNA probe, pr*myc*1.4 (linearized with *Bam*HI), specific for the rat first c-*myc* exon and protected fragments (403 base pairs), electrophoresed in a 6% polyacrylamide–urea gel. Labeled, *Msp*I-restricted fragments of pBR322 were used as molecular weight markers.

ence of an H-*ras* oncogene (Val-12) rendered cells capable of responding to a single growth factor (insulin), whereas established lines and the secondary BRK cultures require multiple factors for a response. Moreover, in this short-term assay, additional growth factors that are present in serum, such as PDGF and fibroblast growth factor, appeared not to provide any added stimulation for BRK-*ras* cells beyond that provided by EGF and insulin.

The BRK-*myc* lines exhibited a growth factor response that was different from either the E1a- or *ras*-immortalized cells. No stimulation was observed when either EGF or insulin was added individually, but the addition of both factors stimulated the cells to a value that was 55 to 60% of that observed for 10% serum, conditions which failed to significantly stimulate the secondary BRK cultures. This result is consistent with the ability of constitutive c-*myc* expression to partially relieve the PDGF requirement for fibroblast growth (1).

Deregulated c-*myc* expression in H-*ras*-immortalized cells. Since the expression of the c-*myc* gene has been tightly linked to growth factor responses (5, 17), the distinctly different responses of the oncogene-immortalized BRK cells to added growth factors prompted us to speculate that the regulation of the c-*myc* gene might be altered. We therefore analyzed the levels of c-*myc* after the addition of serum and different growth factors for each type of cell. RNA was isolated from either subconfluent cells or cells that were grown to confluence and incubated for 16 h in serum-free medium, after which medium was added with 10% FCS or with specific growth factors.

Somewhat surprisingly, we found that the level of c-*myc* RNA was consistently elevated two- to threefold in all subconfluent H-*ras*-immortalized lines when compared with the level in established lines such as Rat2. Figure 4 shows an analysis of two independently derived H-*ras*-immortalized lines; similar results were obtained with four additional lines that were tested (data not shown). In contrast, the endogenous c-*myc* gene was expressed at very low, but detectable, levels in all c-*myc*- and E1a-immortalized lines, approximately equivalent to that found in primary BRK cells.

We next analyzed whether the c-*myc* gene was regulated normally in the H-*ras*-immortalized lines. In most fibroblasts, the growth arrest associated with contact inhibition and depletion of serum growth factors leads to a downregulation of the c-*myc* gene (5, 17). In the H-*ras*-immortalized cells, however, the c-*myc* gene was expressed at a high level in quiescent, confluent cells that had been incubated for 24 to 48 h in serum-free medium (Fig. 5). Moreover, the addition of medium containing 10% FCS to serum-deprived cells gave no more than a transient twofold increase in c-*myc* RNA. The endogenous c-*myc* gene exhibited a very different response in the c-*myc*- and E1a-immortalized lines. In confluent, serum-deprived cells, the low level of endogenous c-*myc* expression decreased to virtually undetectable levels but could be transiently induced 10- to 20-fold by the addition of medium with 10% FCS (more clearly evident in the longer exposure in Fig. 5). Thus, immortalization of early-passage rat kidney cells by the H-*ras* oncogene appears to induce deregulation of the c-*myc* gene. Deregulation of c-*myc* was not the result of c-*sis* (PDGF) activation (which could potentially activate an autocrine loop), since no c-*sis* mRNA was detected in any of the cell lines (data not shown).

Secondary oncogene transfection of immortalized lines. The cell lines created by the immortalizing activity of different oncogenes provide unique targets for studies of the influence of a second oncogene. Both c-*myc*- and H-*ras*-immortalized lines were transfected with the H-*ras* (Val-12) or long terminal repeat-driven c-*myc* oncogenes along with a selectable marker, and the colonies were scored for morphological transformation after 2 weeks. Interestingly, no transformed colonies were observed after transfection of LTRh*myc* into either *myc*- or *ras*-immortalized lines (Table 3); several colonies were expanded, and the expression of the transfected c-*myc* oncogene was verified (data not shown). Thus, the presence of a constitutively expressed c-*myc* oncogene is

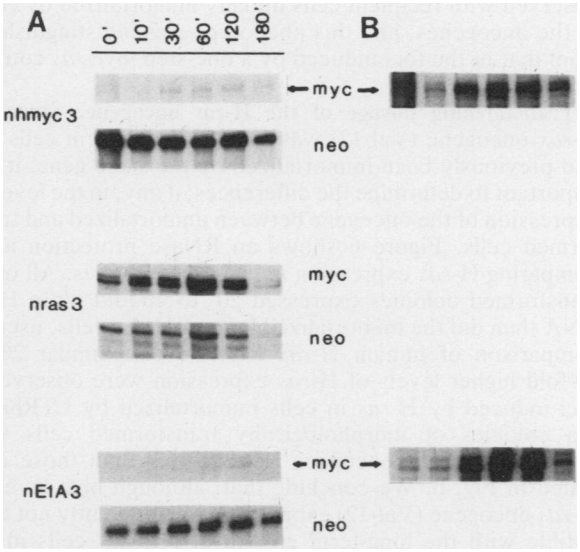

FIG. 5. Serum stimulation of endogenous c-*myc* RNA in oncogene-immortalized lines. Oncogene-immortalized cells were grown to confluence and incubated overnight in serum-free Dulbecco modified Eagle medium. The cells were then fed with medium containing 10% FCS, and total cytoplasmic RNA was isolated after the indicated times (10, 30, 60, 120, and 180 min). RNA (10 μg) was hybridized with labeled pr*myc*1.4 and p*neo*-HB (for RNA quantitation) and subjected to RNase protection. (A) Two-hour exposures of serum-stimulated BRKnh*myc*3, BRKn*ras*3, and BRKnE1a3 cells. Different amounts of RSVneo are expressed in each line. (B) Twenty-four-hour exposure of c-*myc* response in BRKnh*myc*3 and BRKnE1a3 cells.

TABLE 3. Secondary transfection of oncogene-immortalized cells[a]

Recipient cells	Transfected oncogene	Colonies/dish	Colonies with transformed morphology[b] (%)
BRK-*myc*	LTRh*myc*	22	0
BRK-*myc*	H-*ras*	38	70
BRK-*ras*	LTRh*myc*	28	0
BRK-*ras*	H-*ras*	54	92

[a] Three to four different cell lines immortalized with either the c-*myc* or H-*ras* oncogene were further transfected with LTRh*myc* or EJ*ras* (Val-12) oncogenes along with a selectable marker (pSV2neo) in a 10:1 ratio, and drug-resistant colonies were counted and scored after 15 days. The number of colonies per dish represents the average of three or four dishes for each line.

[b] Morphologically transformed colonies were scored as rounded, refractile cells typical of H-*ras* transformation. Representative colonies were found to exhibit anchorage-independent growth and to be tumorigenic in nude mice (data not shown).

not sufficient to cooperate in the morphological transformation of early-passage rodent cells with the H-*ras* oncogene (Val-12) when the latter is expressed at a low but biologically active (i.e., immortalizing) level.

Quite different results were obtained when the c-*myc*- and H-*ras*-immortalized lines were transfected with the H-*ras* oncogene. In each case, colonies of morphologically transformed cells were observed which could be isolated and analyzed (Table 3). Similarly, foci of morphologically transformed cells could be induced in monolayers of immortalized cells after transfection of the H-*ras* oncogene (data not shown). Cells derived from the colonies had a typical "*ras*-transformed" phenotype: rounded cells which were loosely adherent, anchorage independent, and rapidly tumorigenic in nude mice (data not shown). The same phenotype was observed with recipient cells initially immortalized by either of the oncogenes, and this phenotype was indistinguishable from that of the foci induced by a one-step *myc-ras* cotransfection (19).

Transforming dosage of the H-*ras* oncogene. Since the H-*ras* oncogene (Val-12) was found to transform cells that had previously been immortalized by the same gene, it was important to determine the differences, if any, in the levels of expression of the oncogene between immortalized and transformed cells. Figure 6 shows an RNase protection assay comparing H-*ras* expression in the two cell types. All of the transformed colonies expressed 20- to 40-fold more H-*ras* RNA than did the immortalized lines or HeLa cells, used for comparison of human H-*ras* RNA levels. Similar 20- to 40-fold higher levels of H-*ras* expression were observed in foci induced by H-*ras* in cells immortalized by LTRh*myc*. No colonies of morphologically transformed cells were found with H-*ras* oncogene levels lower than those illustrated in Fig. 6. We conclude that, although high levels of H-*ras* oncogene (Val-12) expression are apparently not compatible with the long-term growth of primary cells in this system, they induce transformation of BRK cells established or immortalized by either c-*myc* or H-*ras*. The same high levels of H-*ras* oncogene expression were found in foci induced by cotransfection of H-*ras* and activated c-*myc* oncogenes (Fig. 6).

It should be noted that, as is evident in Fig. 6 from the intensity of the 96-base protected fragment, we consistently observed a significant increase in the level of the endogenous rat H-*ras* RNA in the morphologically transformed cells. This band is somewhat obscured by breakdown products of the probe in Fig. 6 but was clearly evident in other experiments with the same RNAs (data not shown). This was not due to an artifact of RNA loading, because it was observed in all transformed cells when either total cytoplasmic or poly(A)-containing RNA was analyzed after careful quantitation. The level was up to 10-fold higher than that found in primary cell cultures (REF cells) or established lines (Rat2 cells). We do not know whether the enhanced endogenous H-*ras* expression is due to transcriptional or posttranscrip-

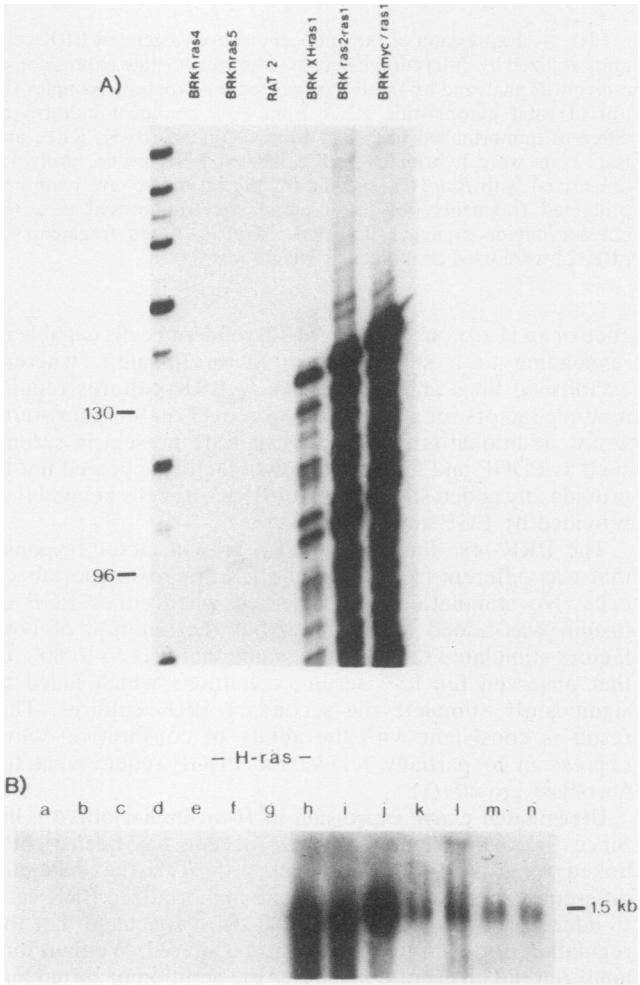

FIG. 6. H-*ras* RNA levels in immortalized and transformed cells. (A) RNase protection assays with a labeled H-*ras* first-exon probe. Levels of H-*ras* RNA in immortalized (BRKn*ras*4 and BRKn*ras*5) and established cells (Rat2) were compared with those in cell lines transformed by secondary transfection of the H-*ras* oncogene. BRKXH*ras*1 derives from a focus selected by secondary transfection of pEJ*ras* onto an *myc*-immortalized BRK line. BRK*ras*2*ras*1 derives from a focus from secondary transfection of pEJ*ras* onto a *ras*-immortalized BRK line. BRK*myc*/*ras*1 is a focus from cotransfection of pSVc-*myc* and pEJ*ras* onto early passage rat kidney cells. Samples (10 μg) of HeLa and RAT2 cell RNAs were used as controls for human and rat H-*ras* transcripts, respectively. The bands between the 130- and 96-base-pair protected bands arose from a small amount of nuclease activity in this particular experiment. (B) Northern blot showing the comparative levels of H-*ras* oncogene expression in *ras*-immortalized and *ras*-transformed lines. Lanes: a through g, 10 μg of total RNA from seven *ras*-immortalized lines; h through n, 10 μg of total RNA from *ras*-transformed lines (by cotransfection of primary cells with *myc* or sequential transfection of immortalized lines).

tional effects, and the level of this RNA was always found to be less than that of the transfected H-*ras* oncogene RNA.

DISCUSSION

The genesis of malignant tumor cells in vivo is a multistep process, probably involving two or more genetic lesions and an unknown number of epigenetic changes. The transfection of cellular and viral oncogenes into early-passage cells in culture allows an examination of the influence of a single genetic lesion on cell growth, where oncogenes can induce two distinct but probably related phenotypes: transformation and immortalization. In particular, the H-*ras* oncogene (Val-12) has usually been associated with morphological transformation in both primary and established cells. However, we show that expression of the H-*ras* oncogene at levels equivalent to those found in established cell lines leads to the immortalization of early-passage rat kidney cells, without any evidence of transformation. This result is similar to that reported earlier for early-passage rodent cells (31). The efficiency of immortalization by H-*ras* was lower than that by E1a and c-*myc* oncogenes, but we observed no spontaneous immortalization under our assay conditions. Typical foci of *ras*-transformed cells appeared on the dish in the initial stages of the assay (19), but these cells failed to grow and were unable to escape what may be regarded as terminal differentiation. This observation suggests that a high level of H-*ras* oncogene expression either is lethal or induces differentiation in primary cells. Consistent with this interpretation are reports that infection of the PC12 (pheochromocytoma) cell line with Harvey sarcoma virus (23) or microinjection of *ras* protein (3) causes the cells to differentiate. Furthermore, overexpression of mutant H-*ras* in REF52 cells (a rat fibroblast line that has many properties of primary cells) induced crisis and cell growth arrest rather than stable transformation (10). The stable REF52 H-*ras* oncogene transfectants that could be isolated contained low amounts of H-*ras* protein. Similarly, the H-*ras*-immortalized cell lines in this study expressed the transfected and endogenous genes at the same level (Fig. 2). Thus, only low levels of H-*ras* expression appear to be compatible with the immortalization of early-passage cells in this system.

The process of immortalization, however, rendered the cells susceptible to transformation by the H-*ras* oncogene, whether the BRK cells were first immortalized by H-*ras* or by c-*myc*. In both cases, transformation involved 20- to 40-fold higher levels of H-*ras* RNA than in the immortalized cells. Thus, if the explanation presented above for the inability of morphologically transformed cells to grow were correct, then immortalization would render the cells tolerant of the high levels of H-*ras* that are apparently lethal or induce terminal differentiation in early-passage cells. No colonies of transformed cells were found that had less than 20-fold higher levels of H-*ras* oncogene expression than those observed in the immortalized cells. We cannot exclude the possibility that additional genetic or epigenetic changes occur during the expansion of the immortalized clones before secondary transfection. It should also be noted that the colonies of transformed cells with high levels of H-*ras* expression arise at high frequency and can be propagated stably without any evidence of crisis, unlike the rare H-*ras*-transformed foci derived from REF52 cells, which exhibit only a limited lifespan in culture (10). Somewhat surprisingly, secondary transfection of H-*ras*-immortalized cells with the c-*myc* oncogene yielded no transformed colonies, which would have been predicted if transformation required only the expression of two cooperating oncogenes in the same cell (19). This result is different from that reported previously for secondary transfection of H-*ras*-immortalized cells with the E1a oncogene (10, 31), which may be due to differences in the transforming and immortalizing activities of c-*myc* and E1a and to E1a being able to induce elevated H-*ras* expression.

The dosage dependence of the H-*ras* oncogene (Val-12) in transformation is likely to vary with the recipient cell background. For example, low levels of the H-*ras* oncogene appear to be sufficient to transform NIH/3T3 cells (33). With rat embryo fibroblasts, relatively high levels of H-*ras* can induce fully transformed cells, but only when the background monolayer has been suppressed by the use of selectible drug resistance markers (18, 25, 31). Viral promoter-linked H-*ras* genes have also been found to induce complete transformation of early-passage cells (31).

Each oncogene-immortalized cell type was found to have distinct growth-response characteristics. The BRK-E1a lines appear to be largely growth factor independent as indicated by their inability to be serum suppressed under the same conditions that inhibit growth of *myc* and *ras* cells. There are two possible mechanisms by which this growth factor independence might occur; (i) E1a cells might produce small amounts of EGF-like or insulinlike growth factors that stimulate their own growth in an autocrine fashion, or (ii) E1a might promote entry into the S phase by bypassing the requirements for cellular components (such as *myc*) that are normally induced by exogenous factors (20). The results from this study are most consistent with the latter mechanism, because several cellular genes usually associated with progression through the cell cycle (Table 2) are not expressed in E1a cells. Moreover, we have been unable to observe a mitogenic effect on either secondary *myc*- or *ras*-immortalized BRK cells with conditioned medium from E1a or H-*ras* cells (data not shown).

The H-*ras* oncogene (Val-12) appears to provide recipient cells with a competence function (6, 24), which is suggested by the mitogenic response to EGF and insulin that is equal to that for 10% FCS and by the expression of several cellular genes associated with the competent state (c-*myc*, *JE*, and *KC*). In addition, H-*ras* may induce an "early progression (I)" state (5), as indicated by the response of the serum-suppressed cells to high concentrations of insulin alone.

The observation that low levels of H-*ras* oncogene expression deregulate endogenous c-*myc* expression was unexpected and quite interesting. Similar effects have not been observed in H-*ras* transformation of established cells or in a study of H-*ras* in REF52 cells (10). However, our data suggest that H-*ras* exerts part of its activity through the activation of c-*myc* in the immortalization of primary BRK cells. One objection that might be raised against this interpretation is that cells constitutively expressing c-*myc* could have been selected for in a *ras* immortalization assay and, therefore, that it is c-*myc* and not the H-*ras* oncogene that is providing the immortalizing function in these cells. We suggest that this is unlikely because cotransfection of H-*ras* with RSV*neo* and subsequent selection in G418 would require that a high percentage of G418-resistant cells also undergo some spontaneous deregulation of c-*myc* expression to exhibit the high efficiency (40%) of immortalization observed with H-*ras*, a situation rather difficult to envisage. In addition, we would then have expected a high percentage of spontaneously immortalized colonies, which was not observed. In studying gene expression in the H-*ras*-immortalized cells, we observed higher levels of several genes that

are associated with cell proliferation. These observations are consistent with recent studies that demonstrate the ability of H-*ras* to alter cellular gene expression (36). It is important to point out that both the H-*ras*- and c-*myc*-immortalized cells exhibit growth arrest at confluence despite the constitutive expression of either the endogenous or exogenous c-*myc* genes, respectively.

The immortalization of primary cells by acquisition of a mutation in the H-*ras* gene could clearly play a role in the genesis of tumors. Indeed, it has been shown that immortalization is an early response after carcinogen treatment of primary cells in culture, and these cells can subsequently be transformed by a second carcinogen treatment, although the cellular target(s) for mutation has not been defined (22). H-*ras* mutations have also been found in benign tumors arising from carcinogen treatment in vivo (2). Thus, the cellular response to the intracellular signal provided by a H-*ras* mutation may be pleotropic and may depend both on dosage and on cell background.

It is tempting to speculate that the phenotype associated with *ras*-immortalized cells in this study may have a direct bearing on the studies of Barbacid's group (39), in which chemical mutagens that induce mutations in the H-*ras* gene in vivo potentiate tumor formation only after an extended latency period. The implication of these studies is that the *ras* mutation occurs immediately after chemical treatment, but some secondary event is required to induce ultimate malignancy. Our studies would predict that cells with a *ras* mutation in vivo would exhibit deregulated c-*myc* expression and increased sensitivity to growth factors associated with progression through the cell cycle and still be subject to contact inhibition.

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