MOLECULAR AND CELLULAR BIOLOGY, July 1992, p. 3117–3129
0270-7306/92/073117-13$02.00/0

Vol. 12, No. 7

Novel Revertants of H-*ras* Oncogene-Transformed R6-PKC3 Cells

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Rat 6 fibroblasts that overproduce protein kinase C β1 (R6-PKC3 cells) are hypersensitive to complete transformation by the T24 H-*ras* oncogene; yet T24 H-*ras*-transformed R6-PKC3 cells are killed when exposed to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (W.-L. W. Hsiao, G. M. Housey, M. D. Johnson, and I. B. Weinstein, Mol. Cell. Biol. 9:2641–2647, 1989). Treatment of an R6-PKC3 subclone that harbors a T24 H-*ras* gene under the control of an inducible mouse metallothionein I promoter with $ZnSO_4$ and TPA is extremely cytocidal. This procedure was used to isolate rare revertants that are resistant to this toxicity. Two revertant lines, R-1a and ER-1-2, continue to express very high levels of protein kinase C enzyme activity but, unlike the parental cells, do not grow in soft agar. Furthermore, these revertants are resistant to the induction of anchorage-independent growth by the v-*src*, v-H-*ras*, v-*raf*, and, in the case of the R-1a line, v-*fos* oncogenes. Both revertant lines, however, retain the ability to undergo morphological alterations when either treated with TPA or infected with a v-H-*ras* virus, thus dissociating anchorage independence from morphological transformation. The revertant phenotype of both R-1a and ER-1-2 cells is dominant over the transformed phenotype in somatic cell hybridizations. Interestingly, the revertant lines no longer induce the metallothionein I-T24 H-*ras* construct or the endogenous metallothionein I and II genes in response to three distinct agents: $ZnSO_4$, TPA, and dexamethasone. The reduction in activity of metallothionein promoters seen in these revertants may reflect defects in signal transduction pathways that control the expression of genes mediating specific effects of protein kinase C and certain oncogenes in cell transformation.

Protein kinase C (PKC) plays a central role in signal transduction, mediating, at least in part, the effects of several oncogene products, growth factors, and cytokines and serving as the cellular receptor for the phorbol ester class of tumor promoters (5, 53, 54). Activation of PKC has pleiotropic effects, and it has been difficult to determine which effects are most intimately linked to growth control and tumor promotion. Furthermore, the events occurring between activation of PKC and induction of PKC-regulated genes are largely unknown.

Dissection of the mechanisms by which PKC transduces signals and influences biological phenomena is complicated by the fact that PKC is encoded by a multigene family comprising at least eight members (4, 5, 53, 54, 57). One approach to this problem is the overproduction of single isoforms of PKC in various cell lines of interest (6, 7, 15, 29, 41, 58, 69). The results of these studies demonstrated that the effects of PKC are strongly cell type- and isoform-specific. We have found, however, that overproduction of even a single isoform (PKCβ1) in Rat 6 embryo fibroblasts causes multiple growth abnormalities. Rat 6 cells that overproduce PKCβ1 via a retroviral expression vector (R6-PKC3 cells) display exaggerated substrate phosphorylation and morphological alterations when treated with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), respond mitogenically to single-growth factors, form small colonies in soft agar and larger colonies in soft agar supplemented with TPA, and are weakly tumorigenic in nude mice (22, 28–30). Thus, R6-PKC3 cells are partially or weakly transformed. Consistent with this conclusion is the finding that the R6-PKC3 line is about 10 times more susceptible to complete transformation by transfection with the activated T24 c-H-*ras* oncogene than a control cell line, R6-C1 (30).

The synergism between PKC and T24 c-H-*ras* in the transformation of Rat 6 cells is of particular interest in light of the many observations suggesting a close interaction between PKC and *ras* in signal transduction and growth control (13, 18, 31, 32, 35, 36, 43–46, 50, 51, 59, 71). Transformation by activated *ras* oncogenes or introduction of mutant *ras* p21 protein into various cell types leads to elevated levels of diacylglycerol, an endogenous activator of PKC (16, 32, 44, 46, 59). Additionally, microinjection of anti-PKC antibodies or down regulation of PKC by prolonged treatment of cells with phorbol esters inhibits the mitogenicity of microinjected or scrape-loaded mutant *ras* p21 protein (18, 45, 50, 51). The majority of studies with activated *ras* oncogenes or mutant *ras* p21 place PKC downstream of *ras*-generated signals. Alternatively, two studies on the function of signals generated via endogenous, normal c-H-*ras*-encoded p21 place PKC upstream of this protein (14, 74).

Consistent with this close interaction between PKC and *ras*, we have observed that for transformation of Rat 6 cells, optimal synergistic effects require a critical balance between their respective functions (30). For example, high-level expression of T24 c-H-*ras* in R6-PKC3 cells appears to be cytotoxic, and R6-PKC3 cells transformed by this oncogene are killed when exposed to TPA (30). In the present study, we have exploited these findings to isolate nontransformed variants (revertants) of T24 H-*ras*-transformed R6-PKC3 cells by a novel procedure. The study of revertants is a

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classic and powerful approach to unraveling complex biochemical pathways. This rationale has recently been applied by several investigators to the study of cultured cells transformed by oncogenes (9, 20, 26, 38, 55, 70, 72, 73, 75 and references therein). In this report, we describe two revertant sublines of T24 H-*ras*-transformed R6-PKC3 cells that retain several features of the phenotype induced by overproduction of PKCβ1 but do not grow in soft agar and are resistant to retransformation by several oncogenes.

MATERIALS AND METHODS

Cell culture, transfection, and selection of revertants. R6-C1 and R6-PKC3 cells and derivatives of these cell lines were grown in Dulbecco modified Eagle medium (DMEM; GIBCO) plus 10% bovine calf serum (HyClone), penicillin (25 U/ml), and streptomycin (25 μg/ml). The cultures were maintained in a humidified incubator at 37°C with 5% CO_2 in air and fed twice a week with fresh medium.

pMT_{SB}*ras* (supplied by R. Lebovitz and M. Lieberman) contains the T24 H-*ras* oncogene under the transcriptional control of a truncated mouse metallothionein (MT) I promoter (positions −153 [*Sac*I] to +67 [*Bgl*II] [68]). Two micrograms of pMT_{SB}*ras* plus 1 μg of the hygromycin B resistance-conferring plasmid pRSV1.1 (52) and 17 μg of Rat 6 cell carrier DNA was transfected into R6-PKC3 cells by the calcium phosphate coprecipitation technique (31), and the cultures were selected in medium containing 300 μg of hygromycin B (Boehringer) per ml. Hygromycin B-resistant colonies were isolated with cloning cylinders, expanded into cell lines, and screened for complete morphological transformation when cultured in medium containing 100 μM $ZnSO_4$. The PKC3-A5 cell line was chosen for further study. To generate matched control cell lines, R6-C1 and R6-PKC3 cells were transfected with pRSV1.1 and Rat 6 cell carrier DNAs to yield the hygromycin B-resistant lines C1-D3 and PKC3-F4, respectively. C1-D3, PKC3-F4, PKC3-A5, and revertant lines were maintained in medium containing 50 μg of G418 per ml and 50 μg of hygromycin B per ml.

To isolate revertants of PKC3-A5 cells, cultures were typically seeded at 5×10^4 to 1×10^5 cells per 10-cm plate in medium containing 100 μM $ZnSO_4$. After 5 to 7 days, when the cells were fully morphologically transformed, the cultures were treated with 100 ng of TPA (LC Services) per ml. Within 48 h, virtually all of the cells were dead and had detached from the plate. The medium was removed, and the plates were rinsed and fed fresh medium containing 100 μM $ZnSO_4$. Seven days later, when colonies had clearly emerged, the cultures were treated again with 100 ng of TPA per ml. Forty-eight hours after this, colonies with a nontransformed morphology were counted and isolated with cloning cylinders.

To isolate mutagen-induced revertants, three 10-cm plates were seeded with 5×10^5 PKC3-A5 cells and the following day were treated with 500 μg of ethyl methanesulfonate per ml for 24 h. This resulted in 70% lethality. The cells were passaged and allowed to recover for 7 days and then were subjected to the selection protocol outlined above.

Assays for growth in 0.3% Noble agar (Difco) were performed as described by Housey et al. (29), except that 10% fetal bovine serum (HyClone) was used. After 3 to 4 weeks of growth, the plates were stained with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium hydrate (INT; Sigma Chemical Co.) for 48 h at 37°C, and the colonies were counted.

Retroviral infections were performed by seeding 5×10^5 cells onto 10-cm plates 24 h before infection with 3 ml of filtered viral suspension containing 8 μg of Polybrene per ml. Four hours later, 9 ml of DMEM plus 10% bovine calf serum was added, and the plates were refed with fresh medium again the next day. Cultures infected with oncogenic retroviruses were tested for their ability to grow in 0.3% agar 3 to 4 days after infection. The cell line GPE (R/−)−7, which produced recombinant retrovirus harboring the v-H-*ras* oncogene, was a gift from K. Marcu. A v-*raf* virus-producing cell line was kindly supplied by U. Rapp. v-*fos* viral supernatant from ψ-MoFBR cells (70) was a gift from R. Wisdom and I. Verma. Wild-type Moloney leukemia virus, recombinant v-*src* virus, and the histidinol resistance virus, MSVhis (25), were supplied by D. Glass, C. Granowitz, and S. Goff.

Nucleic acid and protein analyses. Southern and Northern (RNA) blot analyses of genomic DNA and total cellular RNA, respectively, were performed as previously described (42). Rat MT I and MT II probes (1) were kindly supplied by R. Anderson, the rat K*rev*1 probe (27) was from M. Gould, the c-*fos* and c-*jun* probes (61) were from T. Curran, the *junB* and *junD* probes (64, 65) were from the American Type Culture Collection (numbers 63025 and 63024, respectively), and the *fosB* probe was from R. Bravo.

Western blot (immunoblot) analyses of T24 H-*ras* p21 and v-H-*ras* p21 were performed as described by Guadagno et al. (22) with mouse monoclonal anti-H-*ras* p21 Val-12 antibody, DWP (New England Nuclear), and mouse monoclonal anti-*ras* p21 Arg-12 antibody, Ab-1 (Oncogene Science). ^{125}I-labelled sheep anti-mouse immunoglobulin G (Amersham) was used as the secondary antibody.

Total (i.e., membrane-associated plus cytosolic) PKC activity was determined by the method of Housey et al. (29). Briefly, cell lines were assayed for Ca^{2+}- and phosphatidylserine-dependent protein kinase activity present in partially purified extracts (i.e., the fraction eluted from a DEAE Sephacell column with 100 mM NaCl) with the synthetic peptide R-K-R-T-L-R-R-L as the phosphoacceptor substrate.

Somatic cell hybridization. R6-C1 and R6-C1/T24, an R6-C1 derivative transformed by transfection with the T24 H-*ras* oncogene (30), were infected with MSVhis. Histidinol-resistant sublines were selected in medium containing 10 mM histidinol (Sigma). C1-D3, PKC3-F4, PKC3-A5, and revertant lines were all resistant to hygromycin B. Cell fusions were carried out by the procedure of Davidson and Gerald (11), with some modifications. Briefly, 2.5×10^6 cells of each line to be fused were seeded onto a 6-cm plate and cultured for 24 h in nonselective medium. Fusions involving C1-D3, PKC3-F4, and PKC3-A5 were performed by exposing the cultures to 50% polyethylene glycol 1000 (PEG; Sigma) for 30 s. Revertant lines were very sensitive to the toxic effects of PEG. Fusions involving ER-1-2 were therefore performed with a 10-s exposure to 50% PEG, and fusions involving R-1a utilized a 10-s exposure to 45% PEG. The cultures were then washed four times and refed with nonselective medium. Twenty-four hours later, the cells were split into five 10-cm plates in medium containing 10 mM histidinol and 275 μg of hygromycin B per ml. Ten to 12 days later, individual colonies were pooled and assayed for growth in 0.3% agar.

RESULTS

Construction of PKC3-A5 cells and isolation of revertants. We hypothesized that the cytotoxicity of TPA to T24 c-H-*ras*-transformed R6-PKC3 cells is due to overstimulation of

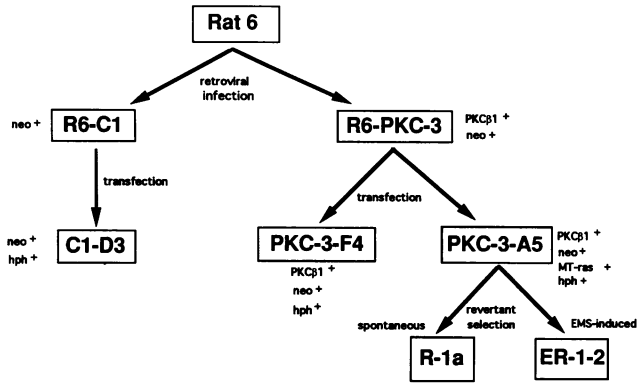

FIG. 1. Derivation and lineage of cell lines used in this study. neo, aminoglycoside phosphotransferase gene; hph, hygromycin B phosphotransferase gene.

a *ras*-PKC signalling pathway involved in growth and transformation. Thus, if R6-PKC3 cells could be transiently induced to synthesize high levels of mutant H-*ras* p21, such cells could then be treated with TPA, leading to very efficient killing of the cell population. Among survivors of such a selection would be cells that suffered mutations in a *ras*-PKC signalling pathway responsible, at least in part, for both the cytotoxic effect and transformation.

To test these hypotheses, we cotransfected R6-PKC3 cells with pMT_{SB}*ras* and a hygromycin B resistance plasmid. pMT_{SB}*ras* contains the T24 H-*ras* oncogene under the transcriptional control of a truncated mouse MT I promoter (21, 62). Hygromycin B-resistant colonies were isolated, expanded into cell lines, and tested for a $ZnSO_4$-dependent acquisition of a fully morphologically transformed phenotype. One such clone, which displayed gradually induced expression of the MT I-T24 H-*ras* gene during a 5-day period after the addition of $ZnSO_4$ (data not shown), was designated PKC3-A5 and used for further study. The slow induction of the transfected construct seen in this cell line, as opposed to the normally rapid induction of MT genes (~4 to 8 h [24]), suggests a possible position effect of integration on its expression. Similar results have previously been observed with this MT I-T24 H-*ras* gene (21), and several other R6-PKC3 cell transfectants showed the same effect (data not shown). To generate matched controls, R6-PKC3 cells and R6-C1, a vector control line (29), were transfected with the hygromycin B resistance plasmid alone, to yield the cell lines PKC3-F4 and C1-D3, respectively. A lineage of all of the cell lines used in this study is diagrammed in Fig. 1.

The scheme for selection of revertants of PKC3-A5 cells was as follows. Cultures were seeded at low density and grown in medium containing 100 μM $ZnSO_4$ for 7 days. Under these conditions, PKC3-A5 cells (but not the control PKC3-F4 cells) displayed an extremely transformed morphology (Fig. 2). Such cultures were then treated with 100 ng of TPA per ml for 2 days, at which point virtually all of the cells detached from the plate. The detached cells took up trypan blue dye and were not capable of reattaching when reseeded onto plates containing fresh medium lacking $ZnSO_4$ and TPA. Thus, this procedure was extremely cytocidal. When the culture plates treated with $ZnSO_4$ and TPA were refed for another week with medium containing $ZnSO_4$, isolated colonies emerged. These plates were then screened in the presence of both $ZnSO_4$ and TPA for colonies with a nontransformed ("flat") morphology. During the course of several experiments, this selection protocol yielded enrichments (i.e., number of colonies remaining on the plate per number of cells detaching from the plate) of between 4.3×10^{-4} and 1.2×10^{-5}. Approximately 1% of the survivors were flat revertants when scored by morphology. Thus, flat revertants arose spontaneously at a frequency of $\sim 10^{-7}$. When PKC3-A5 cells were first mutagenized with ethyl methanesulfonate (at a dose of 500 μg per ml, which caused 70% lethality) and allowed to recover for 6 days prior to selection by the procedure described above, the frequency of flat revertants rose to 3×10^{-6} to 4×10^{-6}, without significantly affecting the total number of survivors. The frequencies at which the flat revertants arose in the absence and presence of mutagen treatment are consistent with the interpretation that single gene mutations are the cause of reversion, but this remains unestablished. The morphologies of a spontaneously arising revertant, R-1a, and of an ethyl methanesulfonate-induced revertant, ER-1-2, when grown in the absence or presence of $ZnSO_4$, are shown in Fig. 2. It is apparent that, in contrast to the parental PKC3-A5 cells, both of these cell lines remained flat after treatment with $ZnSO_4$.

Revertants continue to express high levels of PKCβ1 and retain an intact MT I-T24 H-*ras* gene. We next analyzed the integrated state and expression of the exogenously introduced PKCβ1 and MT I-T24 H-*ras* genes in the two revertant cell lines. Southern blot analysis demonstrated that both R-1a and ER-1-2 cells retained the single copy of the integrated recombinant provirus carrying the PKCβ1 cDNA without any evidence of rearrangement (data not shown). Expression of the PKCβ1 cDNA is controlled by the Moloney murine leukemia virus (Mo-MuLV) long terminal repeat present in the retroviral vector, pMV7 (29). Northern blot analysis indicated that both revertant cell lines expressed a level of the vector-encoded PKCβ1 mRNA that was equivalent to that seen in the parental line, PKC3-A5 (data not shown). Partially purified extracts of C1-D3, PKC3-A5, R-1a, and ER-1-2 cells were also assayed for PKC enzyme activity. As shown in Table 1, PKC3-A5 cells expressed almost the same level of PKC activity as the original R6-PKC3 cell line, a level which was about 50-fold higher than that of the control C1-D3 cells (29). R-1a and ER-1-2 cells retained this very high level of PKC enzyme activity (Table 1). These results rule out the possibility that the revertant phenotype of R-1a and ER-1-2 cells is due to a mutation in the integrated PKCβ1 cDNA that results in loss of kinase activity. Several phenotypic features characteristic of overexpression of PKCβ1 were also retained in the R-1a and ER-1-2 cell lines (see below), suggesting that the overproduced enzyme was fully functional in these cells.

Southern blot analysis of the endogenous c-H-*ras* gene and the integrated MT I-T24 H-*ras* construct is presented in Fig. 3. Genomic DNAs from C1-D3 and PKC3-F4 cells cut with *Bam*HI and hybridized with a human H-*ras* probe displayed a prominent band of about 8 kb and a faint band of about 9 kb. These bands, which correspond to the endogenous rat c-H-*ras* gene, were also present in the PKC3-A5, R-1a, and ER-1-2 DNA samples. The PKC3-A5 DNA contained two additional bands of approximately 16 and 21 kb, representing multiple copies of the transfected MT I-T24 H-*ras* gene. These two bands were retained in unrearranged form in the R-1a and ER-1-2 DNA samples (Fig. 3). Additional digests with *Sst*I revealed an intact 2.5-kb band representing the unit length MT I-T24 H-*ras* gene in the PKC3-A5 cells and in each revertant (data not shown). Thus, both revertants retained the integrated MT I-T24 H-*ras* gene.

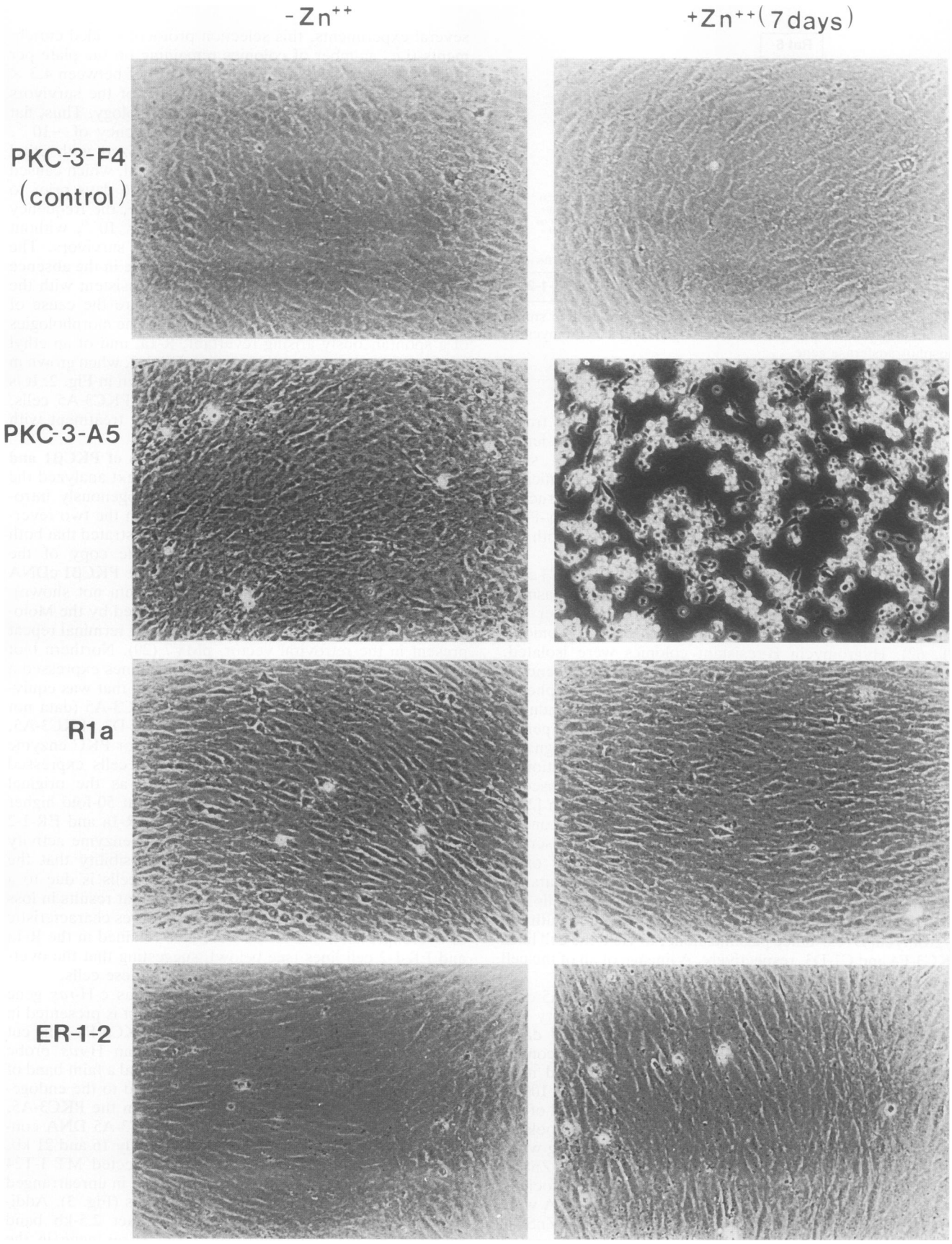

FIG. 2. Photomicrographs of the PKC3-F4, PKC3-A5, R-1a, and ER-1-2 cell lines. Cultures were seeded at 5×10^4 cells per 10-cm plate and were refed the following day with DMEM plus 10% bovine calf serum or DMEM plus 10% bovine calf serum and 100 μM $ZnSO_4$ ($+Zn^{++}$). Cultures were photographed after 7 days. Magnification, ×192.

TABLE 1. PKC activity in the C1-D3, PKC3-A5, R-1a, and ER-1-2 cell lines

Cell line	PKC activity (nmol/min/mg)[a]	Fold increase over PKC activity in C1-D3 cells
C1-D3	7.0	
PKC3-A5	358.2	51.4
R-1a	556.6	79.7
ER-1-2	412.0	59.1

[a] Total PKC activity was partially purified from each of the cell lines and assayed as described in Materials and Methods. Specific PKC activity is reported as the amount of phospholipid-dependent incorporation of ^{32}P into the synthetic peptide substrate per milligram of protein per minute. Values are averages of duplicate determinations, which differed by less than 10%. The experiment was performed twice on two separate extracts; representative data from a single experiment are shown.

In contrast to the parental PKC3-A5 cells, however, both revertant cell lines failed to express the transfected MT I-T24 H-*ras* construct, even in response to $ZnSO_4$. Subsequent analyses (see below) have demonstrated that the revertants have defects in expressing genes controlled by MT promoters. This defect may be an important factor in mediating the revertant phenotype (see Discussion).

Characterization of the revertant phenotypes of the R-1a and ER-1-2 lines. Serial passage indicated that R-1a and, to a lesser extent, ER-1-2 cells had a lower growth rate than those of the control cell lines studied (data not shown). We chose, therefore, to analyze these two cell lines in greater detail. R6-PKC3 cells form microscopic (0.10- to 0.15-mm) colonies in soft agar and larger (0.15- to 0.35-mm) colonies in soft agar supplemented with TPA (29). In contrast, R6-C1 cells do not form any colonies in soft agar, even in the presence of TPA, while R6-C1 or R6-PKC3 cells transformed by the T24 H-*ras* oncogene form very large (0.4- to 1.2-mm), macroscopically visible colonies (30). The C1-D3 and PKC3-F4 sublines were indistinguishable from R6-C1 and R6-PKC3 cells, respectively, in their abilities to grow in soft agar (Fig. 4). PKC3-A5 cells, however, formed considerably larger (0.3- to 0.9-mm) colonies than PKC3-F4 cells, even in agar lacking TPA or $ZnSO_4$ (Fig. 4). We presume that this was due to the low basal level of MT I-T24 H-*ras* expression in these cells (see below). Thus, while treatment of PKC3-A5 cells with $ZnSO_4$ was required to induce complete morphological transformation, very low levels of T24 H-*ras* expression were sufficient to cause transformation as defined by the formation of macroscopic colonies in soft agar. Supplementation of the agar with 100 μM $ZnSO_4$ further enhanced the anchorage-independent growth of PKC3-A5 cells (data not shown), presumably because of the increased expression of MT I-T24 H-*ras* induced by $ZnSO_4$. On the other hand, inclusion of TPA in the agar caused a marked reduction in the sizes and numbers of colonies formed by PKC3-A5 cells (Fig. 4), thus extending the cytotoxic effect of TPA seen in monolayer cultures. In contrast to the PKC3-A5 or PKC3-F4 cell lines, both R-1a and ER-1-2 cells failed to form colonies in soft agar, even when the agar was supplemented with $ZnSO_4$ (data not shown) or TPA (Fig. 4). Thus, these flat revertants have also lost the anchorage-independent growth property which is characteristic of transformed rodent fibroblasts.

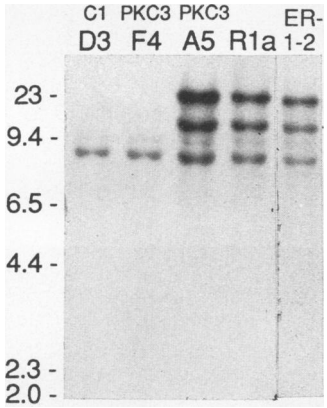

FIG. 3. Southern blot analysis of the transfected MT I-T24 H-*ras* genes in control and revertant lines. Ten micrograms of total cellular DNA was digested with *Bam*HI and then analyzed by Southern blotting techniques and hybridization to a ^{32}P-labelled T24 H-*ras* probe. Molecular size markers (positions indicated in kilobases) represent fragments from *Hin*dIII-cut lambda DNA.

Previous investigators have characterized revertant phenotypes as either dominant or recessive to the transformed phenotype. Somatic cell hybridization experiments were performed, therefore, to characterize this aspect of the phenotype of the R-1a and ER-1-2 cell lines. C1-D3, PKC3-F4, PKC3-A5, and the two revertant lines, each resistant to hygromycin B, were fused to histidinol-resistant derivatives of R6-C1 or T24 H-*ras*-transformed R6-C1 cells (C1/T24 cells). After fusion and double drug selection, hybrids were tested for their ability to form macroscopic colonies in soft agar (Table 2). Fusion of two R6-C1-derived cell lines to each other did not yield hybrids capable of growth in agar, whereas hybrids between C1-D3 and C1/T24 did form colonies in agar (Table 2; hybridizations 1 and 2, respectively). Fusion of various cell lines yielding hybrids expected to express both T24 H-*ras* and PKCβ1 formed colonies in agar 2- to 3.5-fold more efficiently than hybrids obtained from *ras*-expressing and control cell lines (compare hybridizations 3, 4, and 5 with hybridization 2 [Table 2]). Fusion of R-1a or ER-1-2 cells to control R6-C1 cells did not yield hybrids capable of growth in agar (hybridizations 6 and 8, respectively), indicating that the revertant phenotype probably did not result from loss of genetic information required for transformation. It is of interest that hybrids between R-1a or ER-1-2 cells and C1/T24 cells either failed to form colonies in agar or formed colonies with an extremely low efficiency (hybridizations 7 and 9, respectively [Table 2]). The revertant phenotype of the R-1a and ER-1-2 lines was therefore dominant, since it suppressed the transformed phenotype of cells carrying the activated *ras* oncogene. This suggests that these revertants arose because of a dominant mutation, but on account of the inherent limitations of somatic cell hybridization (i.e., segregation of unmarked chromosomes and high DNA content of hybrids, etc.), this interpretation must be confirmed by alternative approaches.

Revertants isolated from cells transformed by one oncogene are frequently resistant to transformation by other oncogenes (55, 73, 75). We therefore tested the susceptibility of C1-D3, PKC3-F4, R-1a, and ER-1-2 cells to transformation by four oncogenes. Each cell line was infected with recombinant retroviruses that express the respective oncogenes from the Mo-MuLV long terminal repeat. The cells were then assayed for formation of macroscopic colonies in soft agar (Table 3). Infection of C1-D3 and PKC3-F4 cells with a retrovirus containing the v-H-*ras* oncogene confirmed the hypersensitivity of PKC-overproducing cells to transformation by this oncogene. In contrast, infection of R-1a or ER-1-2 cells with this virus failed to elicit transformation,

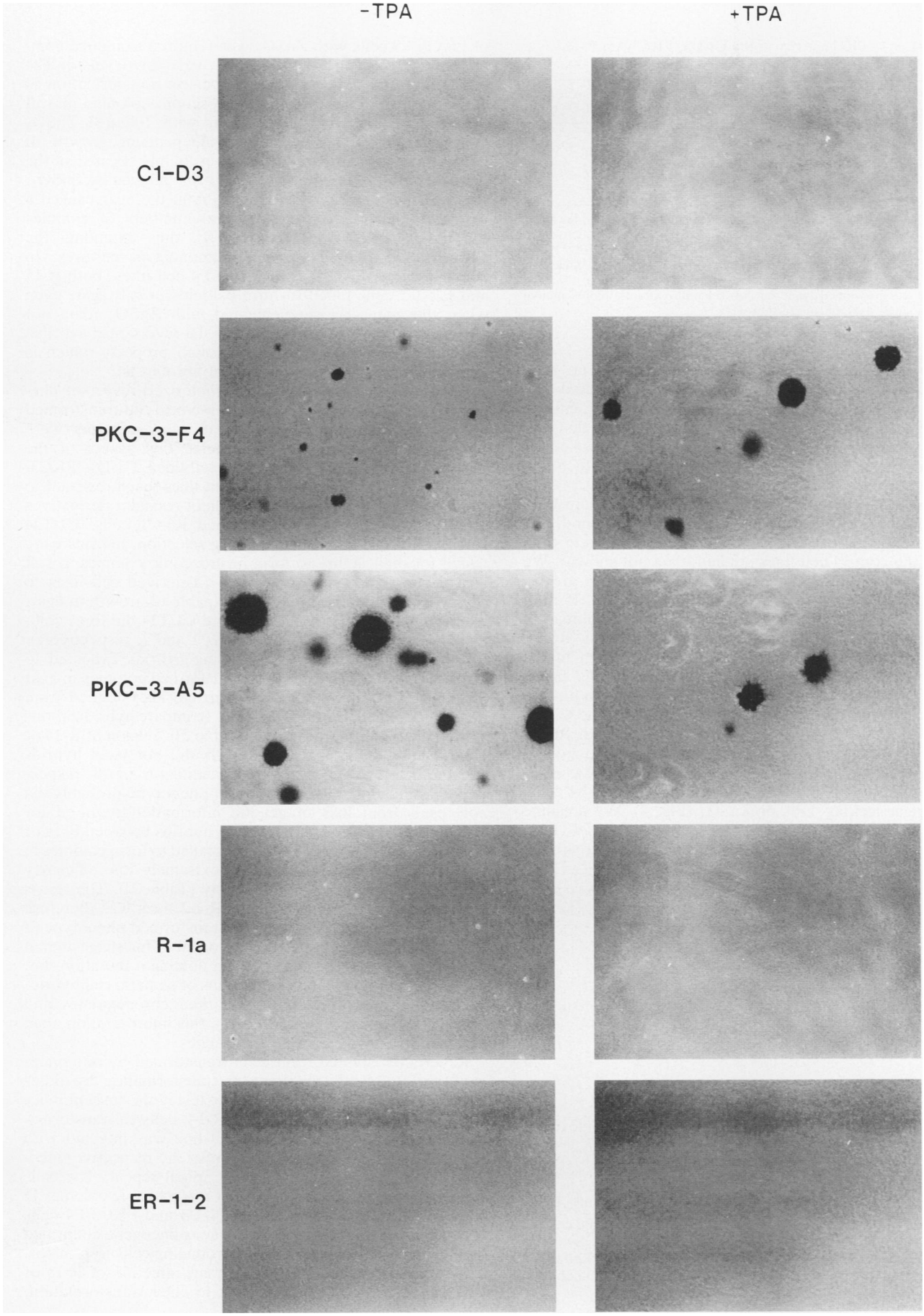
-TPA
+TPA
C1-D3
PKC-3-F4
PKC-3-A5
R-1a
ER-1-2

FIG. 4. Growth in soft agar of control and revertant lines. Growth in 0.3% agar in the absence or presence of 100 ng of TPA per ml was assayed as described in Materials and Methods. The plates were photographed after 4 weeks. Magnification, ×40.

despite detectable production of v-H-*ras*-encoded p21 after infection of these cell lines (data not shown). It was of interest to test v-*src* in this assay, since recent reports suggest that *src* exerts its effects, in part, through activation of PKC (56, 60). The C1-D3 and PKC3-F4 lines were about equally sensitive to transformation by v-*src*, but again both revertant lines were resistant to transformation by this oncogene. PKC3-F4 cells were much more susceptible to transformation by v-*raf* and v-*fos* than were C1-D3 cells, suggesting that PKC can synergize with these oncogenes, as well as with *ras*. R-1a cells were completely resistant to transformation by both v-*raf* and v-*fos*, while ER-1-2 cells were resistant to v-*raf* but could be transformed efficiently by v-*fos*. Thus, both revertants were resistant to transformation by at least three viral oncogenes, as determined by anchorage-independent growth. The differential response to transformation by v-*fos*, however, suggests that the R-1a and ER-1-2 lines have sustained different mutations, probably in different genes. It should be noted that the four cell lines studied in Table 3 were all approximately equally susceptible to infection by pseudotyped retroviruses, as determined by infection with a retroviral vector, MSVhis (25), that confers histidinol resistance (data not shown). This result demonstrates that the failure of the revertants to become transformed was not due to decreased infectivity.

Surprisingly, infection of the two revertant lines with the v-H-*ras* virus revealed morphological transformation when these cell lines were grown in monolayer cultures (Fig. 5). In this regard, they were similar to the control C1-D3 and PKC3-F4 cells (Fig. 5). When uninfected R-1a and ER-1-2 cells were treated with TPA, they also underwent the same transient and exaggerated morphological response seen in R6-PKC3 and PKC3-F4 cells (29; data not shown). Infection of the C1-D3 and PKC3-F4 cell lines with the v-*src*, v-*raf*, and v-*fos* viruses revealed a less pronounced morphological response than infection with the v-H-*ras* virus or treatment with TPA, but the smaller effect observed with these viruses was also seen in the two revertant cell lines (data not shown). Thus, in these two revertant lines, the signals generated by an activated *ras* oncogene or by overexpression of PKCβ1 that lead to morphological transformation in monolayer cultures were intact and could be dissociated from the signals required for a more stringent criterion of transformation, anchorage-independent growth.

Failure of revertants to display induced expression of MT I-T24 H-*ras* or endogenous MT I and MT II genes. The observation made above raised the following question: if R-1a and ER-1-2 cells could become morphologically altered following infection with a v-H-*ras* virus, why did they not do so when treated with $ZnSO_4$, which should lead to expression of the MT I-T24 H-*ras* construct? We therefore analyzed MT I-T24 H-*ras* expression by Western blotting with a monoclonal antibody directed against and specific for H-*ras* p21 Val-12 (data not shown). Cell extracts were prepared from cultures maintained under the same conditions as the cultures photographed in Fig. 2. PKC3-A5 cells expressed a small but detectable amount of T24 H-*ras* p21 when grown in control medium and a substantial amount of this protein when grown for 6 to 7 days in medium containing 100 μM $ZnSO_4$. In contrast, extracts from PKC3-F4 cells, which did not contain the MT I-T24 H-*ras* construct, gave no detectable signal in either type of medium. As mentioned above, neither R-1a nor ER-1-2 cells, both of which retained the transfected MT I-T24 H-*ras* construct (Fig. 3), synthesized any detectable H-*ras* p21 Val-12 protein, even when exposed to $ZnSO_4$ (data not shown). If the R-1a and ER-1-2 clones survived the selection protocol and failed to respond to treatment with $ZnSO_4$ simply because they had inactivated expression of the transfected MT I-T24 H-*ras* gene, both lines should have behaved like PKC3-F4 cells. But this is not the case, because R-1a and ER-1-2 cells were unable to grow in soft agar, suppressed the transformed phenotype in cell hybrids, and were resistant to the transforming effects of various oncogenes (Fig. 4; Tables 2 and 3). We hypothesized, therefore, that the R-1a and ER-1-2 lines were unable

TABLE 2. Growth in agar of hybrid cells derived from various cell fusions

Hybridization no.[a]	Cell hybrids		Colony-forming efficiency in soft agar (%)[b]
	Cells expressing histidinol resistance	Cells expressing hygromycin resistance	
1	C1	C1-D3	0.0
2	C1/T24	C1-D3	2.3
3	C1/T24	PKC3-F4	8.1
4	C1	PKC3-A5	5.0
5	C1/T24	PKC3-A5	7.1
6	C1	R-1a	0.0
7	C1/T24	R-1a	0.01
8	C1	ER-1-2	0.0
9	C1/T24	ER-1-2	0.6

[a] Hybridization numbers refer to fusions between the two cell lines indicated in the second and third columns, yielding different types of hybrids. These hybrids were then tested for growth in agar in the absence of $ZnSO_4$ or TPA.

[b] Macroscopic colonies were counted after 3 weeks of growth in 0.3% agar, and colony-forming efficiency was expressed as the percentage of cells originally plated. For additional details, see Materials and Methods and Results.

TABLE 3. Transformation of the C1-D3, PKC3-F4, R-1a, and ER-1-2 cell lines by various oncogenes

Source of infecting virus[a]	Oncogene	Growth in soft agar (CFU/ml/10^4 cells) of the infected cell line[b]			
		C1-D3	PKC3-F4	R-1a	ER-1-2
Mo-MuLV		0	0	0	0
GPE (R/−)−7	v-H-*ras*	97	550	0	2
RRSV-13	v-*src*	63	93	0	0
NIH/EH leukemia	v-*raf*	1	137	0	0
ψ-MoFBR	v-*fos*	0[c]	1,008	0	476

[a] v-H-*ras* and v-*fos* viruses were produced by packaging mutant helper lines; v-*src* and v-*raf* viruses were pseudotyped with wild-type Moloney murine leukemia virus.

[b] A total of 10,000 cells of each cell line were seeded into 0.3% agar 3 to 4 days after infection with the indicated virus. Macroscopic colonies were scored after 3 to 4 weeks of growth. Each infection was performed at least twice and gave similar results. Data from representative experiments are shown. For additional details, see Materials and Methods and Results.

[c] Microscopic colonies (0.05 to 0.1 mm) were formed at 685 CFU/ml/10^4 cells.

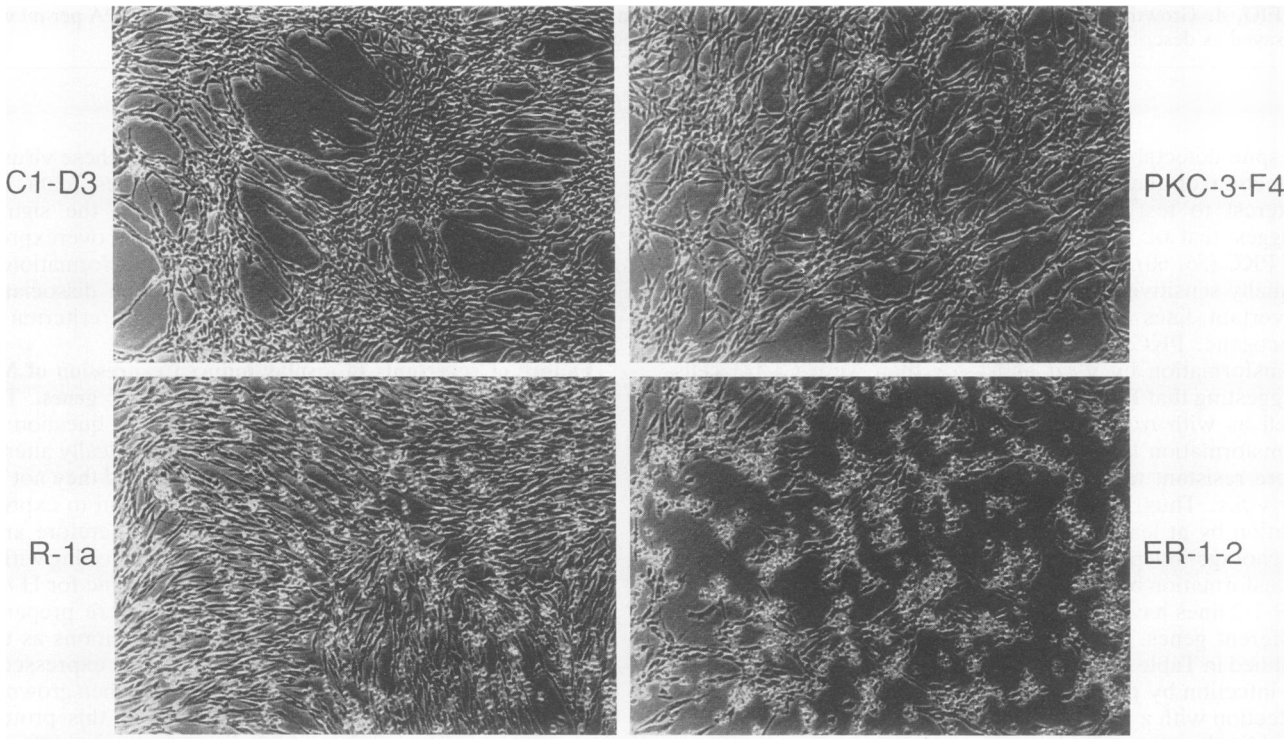

FIG. 5. Morphological response of control and revertant lines to infection with a recombinant v-H-*ras* virus. Cultures were infected as described in Materials and Methods. At 3 to 4 days after infection, the cultures were photographed (magnification, ×100). Mock-infected cultures or cultures infected with wild-type Mo-MuLV were morphologically indistinguishable from untreated cultures grown without $ZnSO_4$ (pictured in Fig. 2).

to express the MT I-T24 H-*ras* construct through an alternate mechanism. MT genes are broadly responsive, inducible by such diverse agents as heavy metals, TPA, glucocorticoids, interleukin-1, and interferons (17, 24, 33, 37, 63). It was conceivable, therefore, that the two revertants have a defect(s) in a pathway(s) that results in inhibition of expression of genes under the control of MT promoters, as well as in suppression of transformation. This would explain the lack of expression of the MT I-T24 H-*ras* construct in these cells.

To test this hypothesis, RNAs were isolated from the C1-D3, PKC3-F4, PKC3-A5, and the two revertant cell lines following treatment of the cultures with $ZnSO_4$, TPA, or dexamethasone. The RNA samples were then examined by Northern blot analysis for expression of MT I-T24 H-*ras* and also the endogenous MT I and MT II genes. As shown in Fig. 6A, PKC3-A5 cells synthesized a T24 H-*ras*-hybridizing RNA species of appropriate size (1.2 kb) that was inducible by treatment of the cells with 100 μM $ZnSO_4$ for 1 week (conditions similar to those used for Fig. 2). Consistent with the Western blotting data, the two revertant lines did not express T24 H-*ras* mRNA, even when exposed to $ZnSO_4$ for 7 days. Duplicate blots were hybridized with rat probes specific for either the MT I or the MT II gene. The C1-D3, PKC3-F4, and PKC3-A5 cells induced MT I, and to a lesser extent MT II, when treated with $ZnSO_4$ (Fig. 6A). In contrast, R-1a cells expressed virtually undetectable basal levels of both the MT I and the MT II genes and also showed negligible induction by $ZnSO_4$. ER-1-2 cells also had very low basal levels of MT I and MT II gene expression and reproducibly induced only about one-third to one-half the level of MT I and MT II mRNAs seen in PKC3-F4 cells. Because of the lack of T24 H-*ras* expression in the revertants, the PKC3-F4 cells were used as the most appropriate control for comparing MT gene expression. The more pronounced effect on the MT I-T24 H-*ras* gene relative to the endogenous MT genes in the ER-1-2 line may be due to position effects, the murine origin, and/or the truncated nature of the MT I promoter that controls the expression of the transfected H-*ras* gene. This failure to induce endogenous MT genes appropriately in the revertant lines was also observed when these cells and control cells were exposed to $ZnSO_4$ for 4 or 8 h (data not shown).

MT genes can also be induced by TPA treatment, apparently via an AP-1 site present in MT promoters (3, 33). Figure 6B shows the expression of MT I-T24 H-*ras*, MT I, and MT II in each of the five cell lines following treatment with 100 ng of TPA per ml for various lengths of time. The results were very similar to those found with $ZnSO_4$ exposure. The MT I-T24 H-*ras* gene was strongly induced by TPA in PKC3-A5 cells but not in either of the revertants. MT I and to a lesser extent MT II were also induced by TPA in PKC3-F4 and PKC3-A5 cells but were just barely induced in R-1a cells and only partially induced in ER-1-2 cells, compared with the PKC3-F4 line. Because of the high basal level of MT I and MT II expression in C1-D3 cells, it was difficult to demonstrate induction of these genes by TPA in this cell line. It is not clear why C1-D3 cells have higher basal levels of MT gene expression than PKC3-F4 or PKC3-A5 cells. Perhaps overproduction of PKCβ1 leads to partial down regulation of the *trans*-acting factors that regulate basal-level expression of MT promoters. Consistent with this possibility, we have observed a similar phenomenon with basal

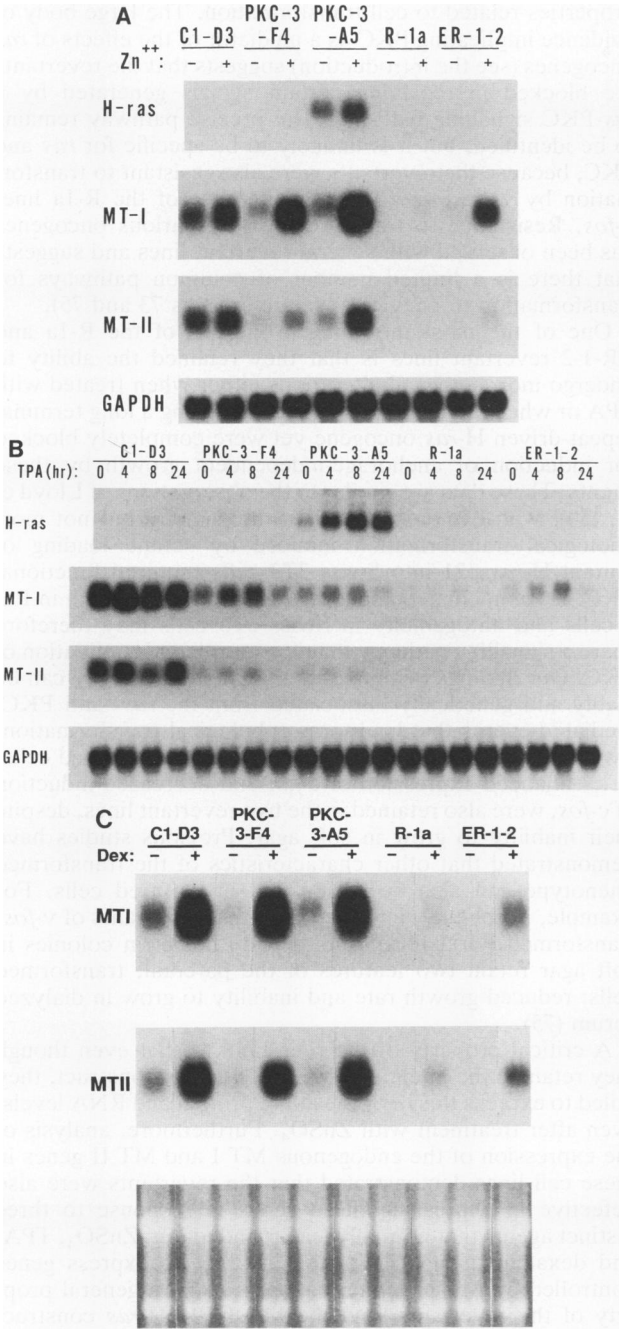

FIG. 6. Northern blot analysis of MT I-T24 H-*ras*, MT I and MT II gene expression in control and revertant lines. (A) Treatment of cultures with 100 μM $ZnSO_4$ for 7 days; (B) treatment of cultures with 100 ng of TPA per ml for the indicated lengths of time; (C) treatment of cultures with 1 μM dexamethasone for 4 hours. Ten micrograms of total cellular RNA was analyzed by Northern blotting and hybridization to ^{32}P-labelled probes as indicated. A rat glyceraldehyde phosphate dehydrogenase (GAPDH) probe was used as a loading control in panels A and B; the ethidium-stained gel, showing 28S and 18S rRNA bands, is pictured in panel C.

expression of the MT I-T24 H-*ras* construct in a panel of transfected R6-C1 and R6-PKC3 cells (22a).

MT genes are also inducible by dexamethasone via a glucocorticoid response element (24, 63). The MT I promoter that controls the T24 H-*ras* gene in the construct used in the present studies is truncated and lacks this element, so only MT I and MT II expression was analyzed in response to this compound. Figure 6C shows that 1 μM dexamethasone markedly induced the expression of both MT I and MT II in C1-D3, PKC3-F4, and PKC3-A5 cells. Once again, however, R-1a cells expressed only a barely detectable level of these genes, and ER-1-2 cells were only partially inducible, following treatment with dexamethasone.

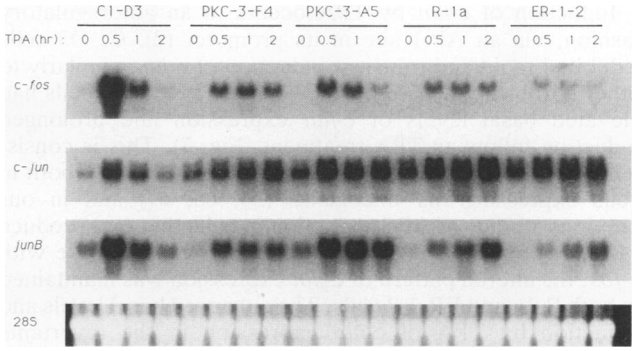

FIG. 7. Northern blot analysis of c-*fos*, c-*jun*, and *junB* gene expression in control and revertant lines. Cultures were treated with 100 ng of TPA per ml for the indicated lengths of time. Twenty micrograms of total cellular RNA was analyzed by Northern blotting and hybridization to ^{32}P-labelled probes as indicated. The ethidium-stained 28S rRNA band is shown as a loading control.

It should be noted that there was no alteration in band size or signal intensity of MT I and MT II genes in the revertants, relative to the control cell lines, when DNAs from each of these lines were analyzed by Southern blotting (data not shown). Thus, the R-1a and ER-1-2 lines were deficient in their ability to induce the expression of endogenous MT genes, in addition to their defect in expression of a transfected T24 H-*ras* oncogene under the control of a truncated MT promoter.

Expression of AP-1 family genes in control and revertant cell lines. As mentioned above, MT promoters contain AP-1 sites, and AP-1 activity is an important target for PKC activation (3, 48). It was of interest, therefore, to test for induction of several genes of the AP-1 family following treatment of the control and revertant cell lines with TPA. Further information about the regulation of the various signalling pathways that are responsible for induction of these genes in each of the different cell lines could also be gained from such studies. Figure 7 shows Northern blots analyzed for the expression of c-*fos*, c-*jun*, and *junB* in RNA samples from each of the five cell lines, following treatment with 100 ng of TPA per ml. C1-D3 cells showed the expected dramatic and transient induction of c-*fos*, as previously reported for other fibroblast cell lines (10). PKC3-A5 cells, however, displayed strongly dampened induction of c-*fos*, compared with C1-D3 cells. A similar phenomenon has been seen in other cell lines transformed by various *ras* oncogenes (8, 23, 49). The fact that this dampening was also seen in PKC3-F4 cells suggests that this effect of *ras* oncogenes may be exerted through activation of PKC. Interestingly, decreased induction of c-*fos* RNA was maintained in both the R-1a and the ER-1-2 lines. TPA induces c-*fos* via the serum response factor, SRF, and the serum response element in the c-*fos* promoter (19). Thus, aberrations in this pathway that were caused by overproduction of PKCβ1 remained altered in the revertants, despite their nontransformed phenotype.

Induction of c-*jun* by TPA occurs in an autoregulatory fashion, via an AP-1 site in its promoter (2). C1-D3 cells exhibited rapid and transient induction of c-*jun*, similarly to other fibroblast lines (47). PKC3-F4 and PKC3-A5 cells had elevated basal levels of c-*jun* expression and prolonged induction following TPA treatment (Fig. 7). This is consistent with the elevated AP-1 activity previously seen both in cells expressing *ras* oncogenes (34, 66, 67) and in our previous studies of AP-1 induction in cells that overproduce PKCβ1 (unpublished observations). As was the case with c-*fos*, the altered pattern of c-*jun* expression was maintained in both R-1a and ER-1-2 cells. The enhanced basal levels and induction by TPA of c-*jun* expression in the revertants suggest that their inability to induce MT genes in response to TPA (or other agents) was not due to loss of AP-1 activity.

The pathway controlling induction of *junB* is less well studied than those for c-*fos* and c-*jun* but appears to involve an inverted repeat sequence in the *junB* promoter that binds an uncharacterized 110-kDa protein (12). The pattern of *junB* induction was similar to that of c-*jun* in C1-D3 cells (Fig. 7). In PKC3-F4 and PKC3-A5 cells, induction of *junB* was prolonged, although the peak level of induction was somewhat reduced in PKC3-F4 cells. In contrast to the situation with c-*fos* and c-*jun*, the induction of *junB* was clearly different in the revertant lines from that in PKC3-F4 or PKC3-A5 cells (Fig. 7). Induction of *junB* was significantly delayed in both R-1a and ER-1-2 cells, since peak levels occurred in the control cell lines within 0.5 h but were not reached until 2 h after TPA treatment in the revertant cells.

We also studied expression of *junD* and *fosB* in each of these cell lines. As in previous reports on other cell lines (63), *junD* was constitutively expressed and not inducible by TPA in any of the five cell lines used in the present study, nor were there any significant alterations in expression among any of the five cell lines (data not shown). *fosB* was induced to very low levels in C1-D3 and PKC3-F4 cells by TPA and to somewhat higher levels in PKC3-A5 cells. Induction in both revertants was slightly less than in C1-D3 or PKC3-F4 cells (data not shown).

Stability of the revertant phenotype of R-1a and ER-1-2 cells. The R-1a and ER-1-2 cell lines were serially passaged for 5 months and were not overtaken by transformed variants. At the end of this time, each cell line was infected with the recombinant retroviruses harboring either the v-H-*ras* or v-*src* oncogene, as described in Table 3. Each cell line remained resistant to the induction of anchorage-independent growth by these oncogenes (data not shown). Thus, the revertant phenotype of both the R-1a and the ER-1-2 cell lines was very stable.

DISCUSSION

This paper describes a genetic approach to the dissection of signalling pathways utilized by PKC in growth control and transformation. The molecular analysis of revertants of transformed cells is a potentially powerful way of identifying critical components in the multibranched pathways that are presumably responsible for the pleiotropic effects of PKC on cell morphology and growth. The strategy employed in the present study was based on the fact that in a cell line (PKC3-A5) that stably overproduces high levels of PKCβ1 and carries a $ZnSO_4$-inducible MT I-T24 H-*ras* construct, treatment with $ZnSO_4$ plus TPA causes extensive cell death. With this as a selection procedure, we isolated and characterized two variants that are resistant to this treatment and demonstrated that they are reverted or blocked in certain properties related to cell transformation. The large body of evidence implicating PKC as a mediator of the effects of *ras* oncogenes (see the introduction) suggests that the revertants are blocked in receiving certain signals generated by a *ras*-PKC signalling pathway. The precise pathway remains to be identified, but it is unlikely to be specific for *ras* and PKC, because the revertants were also resistant to transformation by v-*src*, v-*raf*, and, in the case of the R-1a line, v-*fos*. Resistance to transformation by various oncogenes has been observed with several revertant lines and suggests that there is a limited number of common pathways for transformation (for reviews, see references 73 and 75).

One of the most intriguing properties of the R-1a and ER-1-2 revertant lines is that they retained the ability to undergo morphological alterations either when treated with TPA or when infected with a virus harboring a long terminal repeat-driven H-*ras* oncogene yet were completely blocked for induction of anchorage-independent growth by these agents. These data are similar to the observations of Lloyd et al. (50), who demonstrated that mitogenicity but not morphological transformation induced by scrape loading of mutant H-*ras* p21 into Swiss 3T3 cells required functional PKC. H-*ras*-mediated anchorage-independent growth in Rat 6 cells and mitogenicity in Swiss 3T3 cells may therefore share a signalling pathway that is controlled via activation of PKC. Our findings demonstrate that such a pathway can be stably and genetically dissociated from the *ras*- and PKC-mediated events that lead to morphological transformation. Two other properties of *ras*- and PKCβ1-transformed cells, i.e., enhanced expression of c-*jun* and decreased induction of c-*fos*, were also retained in the two revertant lines, despite their inability to grow in soft agar. Previous studies have demonstrated that other characteristics of the transformed phenotype can also be dissociated in cultured cells. For example, Zarbl et al. have reported that revertants of v-*fos*-transformed Rat 1 fibroblasts that do not form colonies in soft agar retain two features of the parental, transformed cells: reduced growth rate and inability to grow in dialyzed serum (75).

A critical property of the revertants is that even though they retained the integrated MT I-T24 H-*ras* construct, they failed to express this *ras* gene at the protein and RNA levels, even after treatment with $ZnSO_4$. Furthermore, analysis of the expression of the endogenous MT I and MT II genes in these cell lines demonstrated that the revertants were also defective in expressing these genes in response to three distinct agents that normally lead to induction: $ZnSO_4$, TPA, and dexamethasone. Thus, the inability to express genes controlled by MT promoters appears to be a general property of the revertants, and the MT I-T24 H-*ras* construct served as the first reporter for this defect. It seems unlikely that the inhibition of expression of MT genes is due to changes occurring in *cis* (i.e., mutations in MT promoter elements, altered chromatin structure, or DNA methylation), since the effect involved three distinct genes, the exogenous MT I-T24 H-*ras* construct and the endogenous MT I and MT II alleles. Additionally, the MT I-T24 H-*ras* construct contains a mere 220 bp of MT I promoter sequence, suggesting that the effect is mediated by a relatively short sequence of DNA. It seems more likely that this inhibition is due to alterations mediated in *trans*, for example, increased activity, or generation by mutation of a transcriptional repressor that binds to MT regulatory elements. Since it seems unlikely that the MT I and MT II genes themselves play a critical role in cell transformation (24), we postulate the existence of one or more other genes that do

play a critical role in transformation, especially anchorage-independent growth, and that these genes have MT-like regulatory elements that are inhibited by this putative repressor. This hypothesis would explain why the revertants isolated in the present study were resistant to transformation by the v-*ras*, v-*src*, v-*raf*, and, in the case of the R-1a cell line, v-*fos* oncogenes and why the revertants suppressed transformation in cell fusion studies. Since these revertants still display the typical morphological changes seen in cells treated with TPA or in cells transformed by *ras* oncogenes, we assume that these latter effects are mediated by functions independent of those required for anchorage-independent growth and expression of MT genes. Transient expression assays with the chloramphenicol acetyltransferase reporter gene driven by various MT promoter constructs and electrophoretic mobility shift assays on MT promoter fragments are in progress to test specific aspects of this hypothesis. Obviously, the present data do not rule out other mechanisms. In this regard, it is interesting to note that the R-1a and ER-1-2 revertant lines demonstrated delayed induction of *junB* in response to TPA. Kolch et al. Have recently observed that a revertant cell line derived from v-*raf*-transformed NIH 3T3 cells is almost completely blocked for induction of *junB* by serum or TPA (40). It is possible that certain common signalling mechanisms have been affected in each of these cell lines and that these defective pathways intersect with the pathways controlling induction of *junB*. Whether the alterations in *junB* expression are causally related to the revertant phenotype remains undetermined.

Two interesting genes that appear able to induce reversion when overexpressed (or reexpressed) in *ras*-transformed cells have recently been isolated. These genes, *Krev1* (39) and *rrg* (9), are not differentially expressed in our revertants, relative to PKC3-F4 or PKC3-A5 cells, indicating that they are probably not involved in the reversion seen in this system (42a). It is of obvious interest also to investigate the expression and regulation of the *p53* and *Rb1* gene products and other putative tumor suppressor genes in these revertants. Further studies are also required to determine whether the R-1a and ER-1-2 lines have mutations in the same gene or different genes. Any plausible scenario should, however, be able to account for the similar but distinct properties of the two revertant lines.

In summary, we have isolated interesting and distinctive variants of a derivative of the R6-PKC3 cell line with the use of a novel selection procedure. Molecular analysis of these cell lines should prove valuable for understanding the pleiotropic effects of PKC and its role in signal transduction, growth control, and cell transformation.

ACKNOWLEDGMENTS

We thank Steve Goff, Hamish Young, Max Gottesman, Martin Begemann, and Sylvie LeBlancq for critical comments on the manuscript; Russ Lebovitz, Esther Chang, and members of the Weinstein laboratory for useful discussions; and Ulf Rapp for communicating results prior to publication. We are grateful to the following people for their gifts of reagents: R. Lebovitz, M. Lieberman, D. Glass, C. Granowitz, S. Goff, R. Wisdom, I. Verma, U. Rapp, K. Marcu, R. Anderson, M. Gould, T. Curran, and R. Bravo. We are especially grateful to Sara Contente for assistance in analysis of the revertants for *rrg* expression.

This research was supported by NIH grant CA02656 to I.B.W. R.S.K. was supported during a portion of these studies by National Research Service award CA08346 from the National Cancer Institute.

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