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Biochemistry

Reduced hormone-stimulated adenylate cyclase activity in NIH-3T3 cells expressing the EJ human bladder *ras* oncogene

(cyclic AMP/transformation/guanine nucleotide-binding proteins)

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ABSTRACT **Recent studies have shown that the 21-kilodalton protein (p21) Ha-*ras* gene product shares sequence homology with and may exhibit biochemical properties similar to the mammalian guanine nucleotide-binding proteins. These data suggested that one of the biochemical functions of p21 in the vertebrate cell may be to regulate adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. We determined both in intact NIH-3T3 murine cells and in membranes isolated from these cells that the hormone-stimulated adenylate cyclase activity of cells expressing the EJ human bladder carcinoma oncogene (EJ-*ras*) is significantly reduced compared with control cells. Thus, the levels of cAMP measured in the EJ-*ras*-transformed cells by radioimmunoassay are reduced 78% and 93% after prostaglandin and isoproterenol stimulation, respectively, compared with the levels in control cells. Treatment of the EJ-*ras*-transformed cells with pertussis toxin or cholera toxin did not correct the alterations in adenylate cyclase activity. Cells expressing the normal human Ha-*ras* gene displayed intermediate levels of adenylate cyclase hormone sensitivity; these levels of adenylate cyclase activity were greater than those in the EJ-*ras*-transformed cells but lower than in control cells. Hormone-stimulated adenylate cyclase activities in cells transfected with Rous sarcoma virus DNA were similar to those in control cells. These data support the hypothesis that both the normal and mutated Ha-*ras* p21s are related to guanine nucleotide-binding proteins.**

Three human *ras* genes have presently been identified: Ha- (Harvey), Ki- (Kirstin), and N-*ras* (1, 2). Several laboratories utilizing DNA-mediated gene transfer have identified the presence of mutated *ras* genes in the DNA of a wide variety of histologically different human neoplasms (3–11). As a consequence of these mutations, the *ras* genes can morphologically and tumorigenically transform NIH-3T3 cells, an established cell line of murine origin.

Recent discoveries have improved our understanding of how the 21-kilodalton *ras* gene product (p21) may induce the transformation of cells. Hurley *et al.* (12) showed that guanine nucleotide-binding proteins (G-proteins), which transduce signals elicited by ligand binding to membrane receptors into intracellular changes in metabolism, share sequence homology in limited but presumably vital domains with the *ras* gene product; thus, the amino acid sequence corresponding to the first exon on the N-*ras* gene has 9 of 22 amino acids that are identical with both the α subunit of G_0 (13) and transducin, a G-protein analog (14). Other work indicated that mammalian *ras* and yeast *RAS* genes were functionally homologous, and that yeast strains carrying mutated *RAS* genes had increased activity of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] (15–17). These data suggested that p21 and G-proteins have similar biochemical functions. Since one function of G-proteins is to regulate adenylate cyclase in mammalian cells, these data suggest that the *ras* protein also modulates adenylate cyclase activity in cells.

In this paper we show that the hormone-stimulated adenylate cyclase activity of NIH-3T3 cells expressing the EJ human bladder carcinoma oncogene is markedly reduced compared to cells expressing the normal human c-Ha-*ras* gene or control cells. Our data indicate that expression of the normal c-*ras* p21 also influences adenylate cyclase activity, albeit at a reduced rate when compared to the EJ-*ras* protein.

MATERIALS AND METHODS

[2,8-^{3}H]Adenine (20.6 Ci/mmol; 1 Ci = 37 GBq), the 2′-*O*-succinyl[^{125}I]iodotyrosine methyl ester derivative of cAMP (2200 Ci/mmol), and adenosine 5′-[α-^{32}P]triphosphate (10–20 Ci/mmol) were purchased from New England Nuclear. Antiserum to cAMP was purchased from Collaborative Research, Waltham, MA. Guanosine triphosphate, imidazole, and isoproterenol·HCl were purchased from Sigma. 3-Isobutyl-1-methylxanthine was obtained from Aldrich. Cholera toxin and pertussis toxin were purchased from List Biological Laboratories (Campbell, CA).

Cells and DNA Transfections. Transfection of NIH-3T3 cells with cloned DNA was carried out by the calcium phosphate coprecipitation technique (11). Cotransfections were performed by coprecipitating 0.5–5 μg of *ras*-containing plasmid DNA in a 12:1 molar ratio with pUCNeo and calf thymus carrier DNA at 10 μg/ml. pEJ [American Type Culture Collection (ATCC)] is a pBR322 derivative that carries a 6.6-kilobase-pair *Bam*HI fragment containing a mutated c-Ha-*ras* gene isolated from the EJ human bladder carcinoma cell line [EJ-*ras* (18–20)]. pbc-N1 (ATCC) is a pBR322 derivative that carries a 6.4-kilobase-pair *Bam*HI fragment containing the normal human c-Ha-*ras* allele [glycine at position 12 (21)]. The transcription of the EJ-*ras* and the normal c-Ha-*ras* gene is under the control of the normal cellular promoter carried on the *Bam*HI fragments (22). pSRA-2 [ATCC (23)] is a permuted clone of Rous sarcoma virus (RSV) DNA. pSRA-2 DNA was digested with *Sal* I and ligated to form concatamers prior to transfection. pUCNeo, a pBR322 derivative that carries the neomycin gene from Tn*5* and the long terminal repeat from the Harvey murine sarcoma virus (Vince Groppi, personal communication), was used as a selectable gene in the transfection experiments.

Cyclic Nucleotide Measurement. Approximately 3×10^5 geneticin (G418)-resistant cells obtained from the cotransfection experiments (above) were plated into 35-mm wells in a total volume of 1.5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Twenty-four to

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Abbreviations: RSV, Rous sarcoma virus; PGE_1, prostaglandin E_1; PGI_2, prostaglandin I_2; G418, geneticin; G-proteins, guanine nucleotide-binding proteins.

48 hr later the percent conversion of ATP to cAMP was determined as previously described (24, 25). The levels of cAMP in the various cells were also determined by radioimmunoassay (RIA) according to the method of Steiner *et al.* (26), with the incorporation of the acetylation modification of Harper and Brooker (27). The results obtained from the RIA were qualitatively in agreement with those obtained by determining the percent conversion of ATP to cAMP. Because the latter assay was more facile it was routinely used. The data are presented as the percent conversion of ATP to cAMP per 15 min per 10^6 cells, mean ± SD of triplicate determinations.

Adenylate Cyclase Assay. Cells were harvested with a rubber policeman, centrifuged at 600 × *g* for 5 min at 4°C, and resuspended in 1.0 ml of ice-cold 1 mM $KHCO_3$. The cells were then homogenized by using a Teflon pestle in a glass tube, and the adenylate cyclase activity in the homogenate was determined according to the method of Salomon *et al.* (28). Agonists were added at the indicated concentrations and the reactions were initiated with 25–70 μg of cellular protein. Adenylate cyclase activity was calculated from triplicate samples and reported as the mean (±SD) pmol of [α-^{32}P]cAMP per mg of protein; protein was determined by the method of Lowry *et al.* (29).

Analysis of p21. Analyses of the expression of the EJ-*ras* or the cellular-*ras* genes were performed by immunoblotting essentially as described by Towbin *et al.* (30). The primary antibody was affinity-purified sheep antibody against the Ha-*ras* p21 obtained from Triton Biosciences (Houston, TX). A secondary antibody (rabbit anti-sheep IgG) was obtained from Zymed Laboratories (San Francisco, CA). Protein samples were obtained from cellular lysates as described by Furth *et al.* (31) and electrophoresed on a $NaDodSO_4$/15% polyacrylamide gel by the method of Laemmli (32).

RESULTS

NIH-3T3 Cells Transfected with EJ-*ras* DNA Accumulate Only Low Levels of cAMP after Hormone Stimulation. Fig. 1*A* shows the percent conversion of ATP into cAMP in 15 min in cells not exposed to hormone (basal) or hormone-stimulated EJ-*ras* DNA-transfected cells compared to the levels in control cells. In two experiments using cells derived from independent transfections the basal, PGE_1-stimulated (2.8 μM), and isoproterenol-stimulated (12 μM) levels of cAMP accumulation were reduced 65%, 50%, and 90%, respectively, in cells transfected with EJ-*ras* DNA compared to the levels in control cells.

Since the measurements of cAMP in cells determined by the percent conversion of ATP into cAMP can be influenced by different cellular pools of ATP, we also directly determined the amounts of cAMP in these cells by RIA (Fig. 1*B*). In the EJ-*ras* DNA-transfected cells, basal cAMP accumulation was inhibited on the average by 42%, PGE_1-stimulated accumulation was down 78%, and isoproterenol-stimulated accumulation was down 93% compared to the levels of cAMP that accumulated in the control cells. The reductions in isoproterenol and prostaglandin stimulation of adenylate cyclase in EJ-*ras*-transfected cells were always qualitatively similar but not identical; the isoproterenol response is more sensitive to inhibition than is the prostaglandin response. This may be explained by the fact that prostaglandins are generally more potent agonists of adenylate cyclase than isoproterenol. The EJ-*ras* DNA-transfected cells used in these experiments were morphologically transformed and expressed p21 as determined by immunoblot analysis (data not shown).

Adenylate Cyclase Activity in Membranes from NIH-3T3 Cells Transfected with EJ-*ras* DNA Is Reduced Compared with the Activity in Control Membranes. As expected from the data

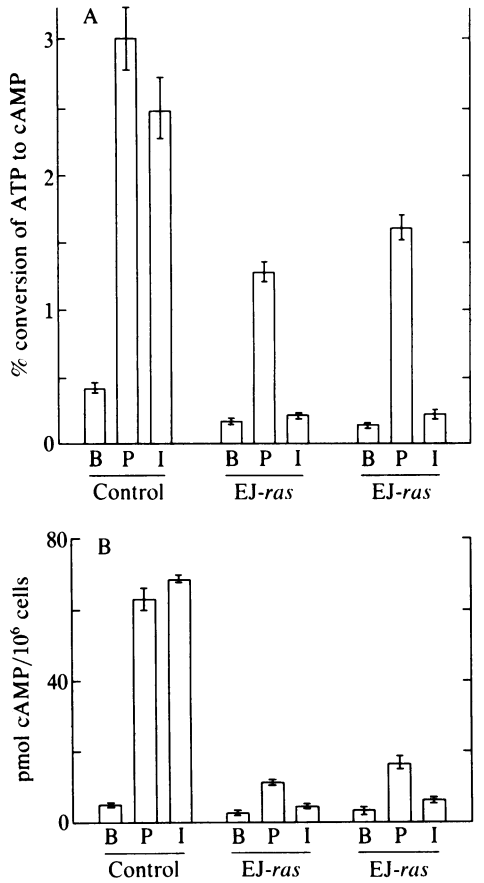

FIG. 1. Amounts of cAMP that accumulate in control or EJ-*ras* DNA-transfected cells. NIH-3T3 cells were cotransfected with pUCNeo and carrier DNA (control) or pUCNeo and EJ-*ras* DNA (EJ-*ras*; results of two independent transfections are shown). Twenty-four hours later selection with G418 was begun; 5–7 days later the G418-resistant cells were grown and the levels of cAMP were determined after no treatment (basal, B) or after stimulation with prostaglandin E_1 (PGE_1) (P; 2.8 μM) or isoproterenol (I; 12 μM). The data are presented as the mean ± SD of triplicate samples. (*A*) Conversion of ATP to cAMP was measured. (*B*) Direct determination of cAMP by RIA.

obtained with intact cells (above), the isoproterenol- and prostaglandin-stimulated adenylate cyclase activities were reduced by a factor of 2 to 3 in membrane preparations from EJ-*ras* DNA-transfected cells compared with the activities obtained in control cell membranes (Table 1). Surprisingly, however, the basal adenylate cyclase activity was not different in membranes from cells transfected with EJ-*ras* DNA compared with control cells. The addition of 100 μM GTP to the assay system significantly increased the basal and hormone-stimulated adenylate cyclase activities in membranes from both EJ-*ras* DNA and control transfected cells. However, the lower adenylate cyclase activities in membranes from EJ-*ras* DNA-transfected cells were not corrected by exogenous GTP (Table 1). Interestingly, adenylate cyclase activity in membrane preparations of EJ-*ras* DNA-transfected cells treated with NaF is about 2-fold greater than the activity in membranes from NaF-stimulated control cells. In contrast, membranes isolated from EJ-*ras* DNA-transfected cells treated with forskolin displayed reduced adenylate cyclase activities compared with controls, much like the hormone-stimulated responses (data not shown).

The decreased adenylate cyclase activities found in the membranes isolated from EJ-*ras* DNA-transfected cells are not artifacts of the adenylate cyclase assay. Both prostaglandin- and isoproterenol-stimulated (50 μM) adenylate

Table 1. Adenylate cyclase activities in membranes prepared from NIH-3T3 cells transfected with EJ-*ras* or control DNA

Treatment	pmol cAMP/12 min per mg of protein			
	Control	Control with GTP	EJ-*ras*	EJ-*ras* with GTP
None	64.82 ± 19.51	99.55 ± 14.74	62.89 ± 5.92	73.29 ± 4.37
50 μM isoproterenol	178.48 ± 12.17	267.38 ± 1.53	58.02 ± 2.93	85.50 ± 2.66
50 μM PGI_2	320.86 ± 22.15	507.45 ± 13.37	144.95 ± 23.99	214.50 ± 17.81
10 mM NaF	300.02 ± 30.43	378.96 ± 28.20	542.10 ± 27.93	532.68 ± 15.51

NIH-3T3 cells were cotransfected with pUCNeo and EJ-*ras* DNA or pUCNeo and carrier DNA (control). Twenty-four hours later selection of the cells in G418 was begun. After 7–10 days the G418-resistant cells were grown and membrane fractions were prepared and used in adenylate cyclase assays. GTP concentrations were 100 μM, and the data are presented as the mean ± SD of triplicate samples. PGI_2, prostaglandin I_2.

cyclase activities are linear for at least 15 min under our assay conditions in membranes from both control and EJ-*ras* DNA-transfected cells, and at all times the hormone-stimulated adenylate cyclase activities were markedly reduced in the membranes from the EJ-*ras* DNA-transfected cells compared with controls (Fig. 2).

Studies by Hurley *et al.* (12) demonstrated the sequence homology between mammalian G-proteins and p21. The α subunit of G_s (s is for stimulatory) and G_i (i is for inhibitory) mediate the hormonal activation and inhibition, respectively, of the catalytic moiety of adenylate cyclase (33, 34). Pertussis toxin and cholera toxin catalyze attachment of ADP-ribosyl groups to G_i and G_s, respectively. Treatment of cells with pertussis toxin generally induces a loss in their hormone-mediated inhibition of adenylate cyclase, while treatment with cholera toxin results in the persistent activation of adenylate cyclase. Because of these effects, we evaluated the influence of pertussis toxin and cholera toxin on the adenylate cyclase activity in membranes isolated from control and EJ-*ras* DNA-transfected cells. Incubation of control cells with pertussis or cholera toxin resulted in enhancements of the basal, isoproterenol-stimulated, and NaF-stimulated adenylate cyclase activities (1.4- to 9-fold) compared with control cells untreated with the toxins (Table 2). Similar

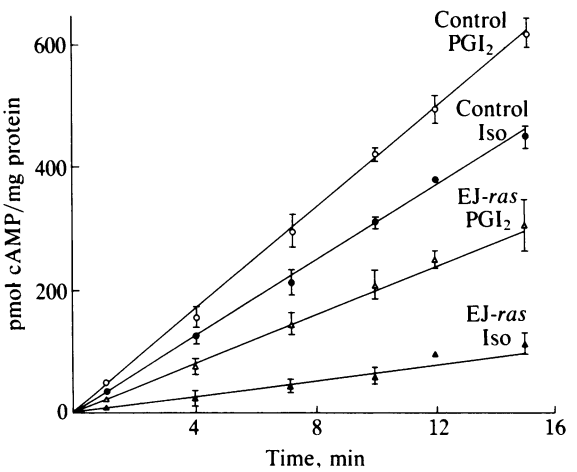

FIG. 2. Adenylate cyclase activities in membranes prepared from hormone-stimulated control (○, ●) or EJ-*ras* DNA-transfected (△, ▲) cells. NIH-3T3 cells were cotransfected with pUCNeo and carrier DNA (control) or pUCNeo and EJ-*ras* DNA (EJ-*ras*). Twenty-four hours later selection of the cells with G418 was begun; 5–7 days later the G418-resistant cells were grown and membrane fractions were prepared for adenylate cyclase activity assays. The assays were conducted in the presence of PGI_2 (50 μM) or isoproterenol (Iso; 50 μM). The data values are presented as the mean ± SD of triplicate samples.

enhancements of hormone-stimulated adenylate cyclase activities were also obtained in membranes prepared from EJ-*ras* DNA-transfected cells treated with pertussis or cholera toxin. In contrast, adenylate cyclase activity in membranes prepared from these cells incubated with cholera toxin and then stimulated with NaF was slightly lower (1.5-fold) compared to controls. We obtained an identical pattern of data as described above when the levels of cAMP accumulation were determined in experiments with intact cells (data not shown). Under the conditions used in these experiments we verified, using [^{32}P]NAD, that the treatment of cells with pertussis or cholera toxin resulted in the ADP-ribosylation of G_i and G_s proteins, respectively (data not shown). The above results indicate that neither pertussis nor cholera toxin treatment of cells corrected the EJ-*ras* DNA-induced reduction in adenylate cyclase activity. This was true despite the fact that the majority of the G_i and G_s proteins were successfully ADP-ribosylated.

Reduced Levels of cAMP Accumulation in Hormone-Stimulated NIH-3T3 Cells Expressing the EJ-*ras* Oncogene Compared with Cells Expressing the Normal Human *ras* Gene. To determine whether the low levels of cAMP accumulation in EJ-*ras* DNA-transfected cells compared with control cells were specific for cells expressing the EJ-*ras* gene, we determined the amounts of cAMP that accumulated in cells after transfection with EJ-*ras* DNA, with the normal human cellular *ras* gene DNA, or with DNA of a cloned RSV, which carries the v-*src* oncogene. In agreement with the data presented above, the amounts of cAMP that accumulated in EJ-*ras* DNA-transfected cells were much less after hormone stimulation compared with control cells (Fig. 3). Cells transfected with c-*ras* DNA had unchanged basal levels of cAMP accumulation compared with control cells; these cells did exhibit, however, reduced hormone-stimulated cAMP accumulation. The hormone-stimulated cAMP accumulation in c-*ras* DNA-transfected cells was greater than the corresponding accumulation in cells transfected with EJ-*ras* DNA. Agonist concentration response studies of PGE_1 and isoproterenol showed that the hormone-stimulated accumulations of cAMP in the c-*ras* DNA-transfected cells were always lower than the accumulations in control cells but were consistently higher than the corresponding levels in the EJ-*ras* DNA-transfected cells (data not shown). Immunoblot analysis of the total protein found in cell lysates of the EJ-*ras* DNA- and c-*ras* DNA-transfected cells indicated that these cells were synthesizing very similar levels of p21 (Fig. 4). Although the RSV DNA-transfected cells had the same maximal levels of hormone-stimulated adenylate cyclase activities as the control cells, the basal enzyme activities were consistently higher; similar increased basal levels of adenylate cyclase activities were found in membranes obtained from RSV DNA-transfected cells (data not shown). The RSV DNA-transfected cells used in these experiments were mor-

Table 2. Influence of pertussis toxin and cholera toxin on adenylate cyclase activity in NIH-3T3 cells transfected with EJ-*ras* or control DNA

	pmol cAMP/12 min per mg of protein					
	Control DNA			EJ-*ras* DNA		
Treatment	No toxin	Pertussis toxin	Cholera toxin	No toxin	Pertussis toxin	Cholera toxin
None	95.89 ± 5.66	244.34 ± 35.92	884.26 ± 2.83	102.28 ± 12.85	127.38 ± 12.34	729.71 ± 2.67
50 μM isoproterenol	211.43 ± 10.46	544.92 ± 5.37	1105.42 ± 6.79	107.93 ± 10.69	171.96 ± 32.95	718.98 ± 24.31
10 mM NaF	330.51 ± 18.66	498.56 ± 18.66	460.56 ± 9.33	907.98 ± 15.14	1110.33 ± 13.49	616.22 ± 2.93

NIH-3T3 cells were cotransfected with pUCNeo and EJ-*ras* DNA or pUCNeo and carrier DNA (control). Twenty-four hours later selection of the cells in G418 was begun. After 7–10 days the G418-resistant cells were grown and individual dishes of cells were incubated for 2 hr at 37°C in the presence or absence of either pertussis toxin at 100 ng/ml or cholera toxin at 10 μg/ml. After the incubation, membranes were prepared from the cells and adenylate cyclase activities were measured. All samples were tested in the presence of 100 μM GTP, and the data are presented as the mean ± SD of triplicate samples.

phologically transformed, expressed v-*src* RNA (as determined by RNA dot blot analysis using a v-*src* probe), and formed macroscopic colonies in soft agar (data not shown). We do not know the explanation for the increased basal adenylate cyclase activities in the RSV DNA-transfected cells, but the response is opposite to that observed with the *ras* DNA-transfected cells. The above data suggest that decreased adenylate cyclase hormone responsiveness is not a general phenomena of NIH-3T3 cells expressing an oncogene. Furthermore, since the cells transfected with c-*ras* DNA are morphologically normal but also display reduced adenylate cyclase responsiveness, the data suggest that aberrant adenylate cyclase regulation is likely the result of the presence of the *ras* genes rather than a secondary cellular response related to the transformed phenotype. However, since the suppressing activity of the EJ-*ras* gene on adenylate cyclase responsiveness is greater than that associated with the normal c-*ras* gene, the data also suggest that aberrant adenylate cyclase regulation may be an important consequence of the EJ-*ras* p21 in transformed cells.

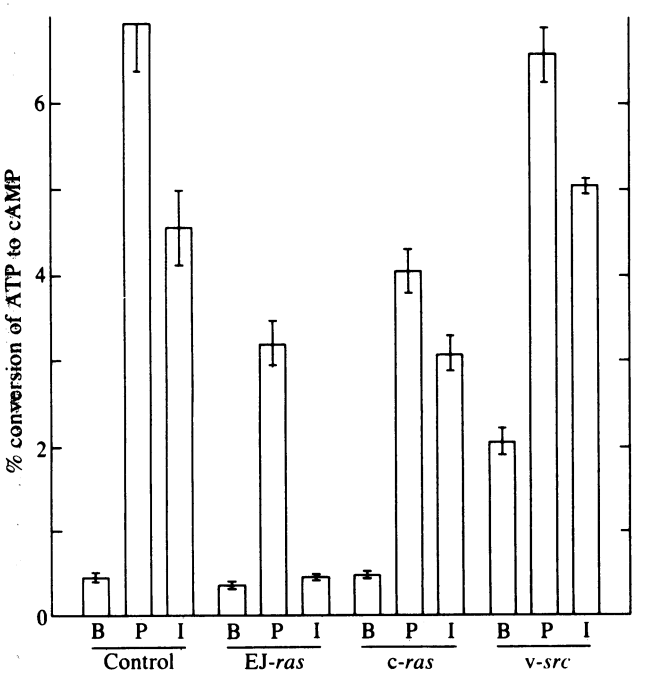

FIG. 3. Amounts of cAMP that accumulate in transfected cells. NIH-3T3 cells were cotransfected with pUCNeo and carrier DNA (control) or pUCNeo and the indicated viral or cellular oncogene DNA. Twenty-four hours later selection with G418 was begun; 5–7 days later the G418-resistant cells were grown and the amounts of cAMP were determined in cells after no treatment (basal, B) or after stimulation with PGE_1 (P, 2.8 μM) or isoproterenol (I, 12 μM). The data are presented as the mean ± SD of triplicate samples.

DISCUSSION

Several laboratories have reported that cAMP metabolism is altered in transformed cells (35–37). These findings have recently regained interest as a result of the reported sequence homology between p21 and G-proteins (12). Since one biochemical role of mammalian G-proteins is to regulate adenylate cyclase activity in cells (38, 39), these data suggested that an important biochemical role of *ras* p21 in transformed cells might be to inhibit adenylate cyclase responsiveness. Because of this we measured the cAMP in intact cells transformed by a mutated *ras* gene (EJ-*ras*) and determined the adenylate cyclase activities in membranes prepared from these cells under various conditions.

Since we have quantified alterations in cAMP metabolism in both intact cells and membranes isolated from these cells, the reductions in the levels of cAMP and adenylate cyclase activities in EJ-*ras* transformed cells and membranes, respectively, are not artifacts of any one assay. These differences in cAMP metabolism in EJ-*ras*-transformed cells are also not the result of a shift in the dose–response curves to hormone stimulation or because of an altered time course of maximal accumulation of cAMP in the various cells. We find that under the conditions used in our experiments the concentrations of PGE_1 and isoproterenol, 2.8 μM and 12 μM, respectively, are essentially maximally stimulating in intact cells and membranes. Moreover, concentrations of hormone up to 100 μM did not increase the hormone-stimulated response in the EJ-*ras* DNA-transfected cells compared with control cells (data not shown).

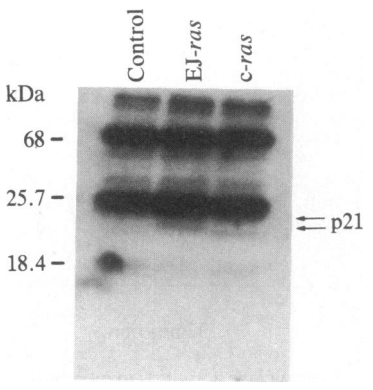

FIG. 4. Expression of *ras* genes in transfected NIH-3T3 cells. NIH-3T3 cells were cotransfected with pUCNeo and carrier DNA (control) or pUCNeo and the indicated *ras* DNA. G418-resistant cells were selected and the levels of p21 synthesized in the cells were determined by immunoblotting. Bars indicate the relative mobilities of marker proteins. The heavy bands of contaminating protein at 25 and 70 kDa most likely are light and heavy chains, respectively, of immunoglobulins present in the serum used to culture the cells.

To determine whether the reduced levels of cAMP or adenylate cyclase activities were specific to NIH-3T3 cells transformed by a mutated *ras* gene, we also performed similar analyses with cells transfected with the normal human c-*ras* gene DNA or RSV DNA. The amounts of cAMP that accumulated and the adenylate cyclase activities after hormone stimulation were greater in the cells transfected with the normal *ras* gene DNA compared with cells transfected with EJ-*ras* DNA. These adenylate cyclase activities and levels of cAMP were lower, however, in cells transfected with the normal c-*ras* gene DNA compared with control cells. These data may reflect the fact that high levels of c-*ras* expression are transforming to NIH-3T3 cells (40). Although the cells transfected with the normal c-*ras* DNA in our experiments were morphologically normal, they did contain very high levels of the *ras* p21 relative to the control cells (Fig. 4). Determining the hormone-stimulated responsiveness of adenylate cyclase in NIH-3T3 cells transformed by the normal c-*ras* gene whose expression is controlled by heterologous promoters should be informative.

The mechanism of the EJ-*ras* p21-induced inhibition of adenylate cyclase activity is not known. It does not seem to be simple replacement of normal cellular G-proteins with mutated *ras* p21. Treatment of EJ-*ras* DNA-transfected cells with either pertussis or cholera toxin did not correct the reduction in adenylate cyclase responsiveness. Since we were able to show effects of these toxins on both adenylate cyclase activities and ADP-ribosylation of the normal G-proteins, the α and β subunits of the G-proteins must be associated and functional in the EJ-*ras* DNA-transfected cells. Similar results were recently reported by Beckner *et al.* in reconstitution experiments (41).

Presently, several hypotheses exist to explain how the EJ-*ras* p21 inhibits adenylate cyclase activity in NIH-3T3 cells. It could interfere with coupling of the G-proteins to adenylate cyclase, have a direct effect on the catalytic subunit of adenylate cyclase, be a nonspecific membrane perturbent that uncouples hormone receptors from the catalytic subunit, or alter hormone receptor levels. Finally, the observation that cells transfected with c-*ras* DNA display an intermediate adenylate cyclase responsiveness compared with EJ-*ras* DNA- or control DNA-transfected cells suggests that the normal *ras* p21 has some regulatory role in the control of adenylate cyclase and cAMP levels in mammalian cells. In fact, we believe that the *ras* gene product may influence G-protein-regulated systems besides the adenylate cyclase.

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