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Cyclic AMP-Dependent Protein Kinase Regulates Sensitivity of Cells to Multiple Drugs

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The isolation of mutant cell lines affecting the activity of cyclic AMP (cAMP)-dependent protein kinase (PK-A) has made it possible to determine the function of this kinase in mammalian cells. We found that both a CHO cell mutant with a defective regulatory subunit (RI) for PK-A and a transfectant cell line expressing the same mutant kinase were sensitive to multiple drugs, including puromycin, adriamycin, actinomycin D, and some antimitotic drugs. The mutant and transfectant cells, after treatment with a concentration of the antimitotic drug colcemid that had no marked effect on the wild-type parent cell, had a severely disrupted microtubule network. The phenotype of hypersensitivity to the antimitotic drug colcemid was used to select revertants of the transfectant and the original mutant. These revertants simultaneously regained normal multiple drug resistance and cAMP sensitivity, thus establishing that the characteristics of colcemid sensitivity and cAMP resistance are linked. Four revertants of the transfectant reverted because of loss or rearrangement of the transfected mutant RI gene. These revertants, as well as one revertant selected from the original mutant, had PK-A activities equal to or higher than that of the parent. In these genetic studies, in which linkage of expression of a PK-A mutation with drug sensitivity is demonstrated, it was established that the PK-A system is involved in regulating resistance of mammalian cells to multiple drugs.

The functions and importance of cyclic AMP (cAMP)-dependent protein kinase (PK-A) have been studied extensively (9, 15, 18, 19, 26, 40). The holoenzyme consists of two regulatory and two catalytic subunits. Most cells have two main types of PK-As, (types I and II) that differ from each other in their regulatory subunits. Results of recent studies of the protein structure (8, 30) and isolation of cDNA clones for the subunits (16, 22, 31, 37) suggest that additional forms of both regulatory and catalytic subunits are present.

Many cultured fibroblasts, such as CHO cells, undergo many striking changes after they are cultured in cAMP (11), including a decrease in membrane blebbing (13); stimulation of ornithine decarboxylase (23), transglutaminase (29), and phosphodiesterase activities (C. S. Klee and M. M. Gottesman, unpublished data); formation of gap junctions (41); changes in cell morphology and arrangement; and growth inhibition (13) and decreased ν_{max} for glucose and amino acid transport (system A) (20). Most CHO mutants selected for resistance to the growth-inhibitory effects of cAMP have been shown to be defective in PK-A (12). In these mutants, as well as in those in other cell lines (11, 35), it has been established that the multiple changes that are apparent after culture with cAMP are mediated through PK-A.

When grown in normal media, these PK-A mutants have a normal growth rate and a morphological appearance very similar to that of the parental cell line. We present evidence here that CHO cells with a mutant gene for the RI regulatory subunit of PK-A are physiologically distinct from their wild-type parent in that they are more sensitive to a variety of drugs, including colcemid, adriamycin, and actinomycin D. These drugs do not share a common cytotoxic target but are included in a group of hydrophobic agents to which multi-drug-resistant cell lines are resistant (24, 25). The sensitivity of PK-A mutants to the antimitotic drug colcemid was used to select revertants which have normal resistance to colcemid and several other drugs and which no longer express the PK-A mutation. The results reported here with PK-A mutants and transfected cell lines provide strong genetic evidence for the linkage of the PK-A defect and the multidrug sensitivity of the mutants. We selected revertants from both the mutant 10248 and the transfectant 11564 using colcemid selection. This type of selection for revertants based on drug sensitivities may be applicable to other mammalian cell mutants as well.

MATERIALS AND METHODS

Actinomycin D, adriamycin, bleomycin, colcemid, colchicine, ethidium bromide, puromycin, and 8-bromo-cAMP (8-Br-cAMP) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Taxol was obtained from the National Products Branch, National Cancer Institute (Bethesda, Md.). G418 (geneticin) was obtained from GIBCO Diagnostics (Madison, Wis.).

Cell lines. All Chinese hamster ovary (CHO) cell lines were grown as described previously (12).

The wild-type parental cell line (10001) is a subclone of the CHO line Pro$^-$5 (34). The cAMP-resistant dominant mutant 10248 containing a mutant RI gene (2, 12, 32) was derived from the wild-type line 10001 after ethyl methanesulfonate mutagenesis. The cAMP-resistant transformant 11564 was derived by transferring genomic DNA from strain 10248 to wild-type cells (10001) (1, 2), and it also carried the bacterial gene *neo* which confers resistance to G418.

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Isolation of revertants. Isolation of revertants was based on the differential sensitivity of the mutant cells (10248), transfectant cells (11564), and wild-type cells (10001) to colcemid. By first selecting 11564 or 10248 cells in colcemid, colcemid-resistant colonies were obtained which were then screened for cAMP sensitivity. In the first step, 2.5×10^5 11564 or 10248 cells were plated in each of 6 or 10 dishes (diameter, 100 mm) at concentrations of colcemid that killed most of the mutant cells but that allowed the wild-type cell line to survive. The concentrations used were 0.025 μg/ml (for 10248) and 0.035 or 0.04 μg/ml for 11564. After incubation for 7 to 10 days, the colcemid-resistant colonies were isolated, and the cells were split into normal medium and medium containing 0.7 mM 8-Br-cAMP. After incubation for 2 to 10 days, the cells in 8-Br-cAMP were observed microscopically for revertants with the characteristic cAMP-sensitive morphology and slowed growth. The putative revertant cells were then grown from the corresponding well of cells in normal medium. TR1, TR2, TR3, TR4, and TR5 were selected from 11564; and MR1 was selected from 10248 by this method. TR1, TR2, and TR3 retained resistance to G418.

Revertants were also isolated directly from media containing 0.7 mM 8-Br-cAMP by using 10248 and UV-mutagenized 11564 transfectant cells. Cells were plated in 8-Br-cAMP in 24-well plates at an average density of 1 cell per well (11564) or at low density on plates (diameter, 100 mm) (10248). After incubation for 10 to 20 days, the plates were observed microscopically for cAMP-sensitive colonies. Once a sensitive colony was identified, the 8-Br-cAMP medium was replaced with growth medium that allowed the colony to return to normal growth. If the colony survived it was picked and divided into growth medium and 8-Br-cAMP medium to retest the initial observation of cAMP sensitivity. MR2 was selected from 10248 and TR6 was selected from 11564 by this method.

All revertants were subcloned from single cells. Revertants derived from the transfectant 11564 were also tested for their ability to grow in G418 at concentrations of 400 to 1,600 μg/ml.

UV mutagenesis was performed on 11564 cells with an 8-kW germicidal UV lamp. An exposure of 10 s at a distance of 50 cm resulted in a survival of approximately 20%, after which cells were trypsinized and replated in four dishes (diameter, 100 mm) at 5×10^5 cells per dish for a 3-day expression period. UV irradiation resulted in an 11-fold increase in resistance to 2 mM ouabain.

Growth tests and drug resistance. Growth curves were obtained by plating 10^4 cells per well in 2 ml of normal or test medium in 24-well plates as described previously (2). To test for the ability of cells to grow and form colonies during continuous exposure to various concentrations of drugs, 200 cells in 2 ml were seeded in dishes (diameter, 60 mm) and grown for 7 days in the desired drug concentration. After staining with 0.5% methylene blue, colonies were counted with a counter (Artek) that was calibrated to exclude colonies smaller than approximately 50 cells. Average colony counts from treatment plates were normalized to the average untreated controls for each cell line, and the percent survival at each treatment was plotted on a semilogarithmic scale. The drug concentration at which half the cells died (LD_{50}) was determined for each cell line by probit analysis of the dose-response curves. For comparisons of the drug sensitivities of the various cell lines with those of wild-type cells, a ratio of the LD_{50} for each test cell line to the LD_{50} of wild-type cells was calculated.

Indirect immunofluorescent staining for tubulin. Observations of microtubules and the degree of tubulin polymerization in intact cells were made by indirect immunofluorescence as described previously (3). Cells were seeded either on glass cover slips (diameter, 18 mm) or in 8-well microscope slides (Flow Laboratories, Inc., McLean, Va.) and were allowed to grow for 3 days. They were fixed in 3.7% formaldehyde for 10 min and then in 80% acetone for 10 min. Microtubules were visualized after incubation with monoclonal rat anti-yeast α-tubulin (Sera-Lab) and rhodamine-conjugated affinity-purified goat anti-rat immunoglobulin G (Cappel Laboratories, Cochranville, Pa.).

Protein kinase assays. Protein kinase activity was determined by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to histone at various cAMP concentrations (7) as described previously (32). Histone phosphorylation was measured by spotting 30-μl fractions of the reaction mixture on 2.5-cm squares of phosphocellulose paper, and then the phosphocellulose paper was washed in water and then in acetone (43). Incorporation on a time zero enzyme blank was subtracted from the total incorporation. All values were corrected for activity in the absence of cAMP. One unit of protein kinase activity was defined as the amount of the enzyme required to catalyze the incorporation of 1 pmol of ^{32}P into histone per min at 30°C.

DEAE-cellulose chromatography. Preparation of cell homogenates and DEAE-cellulose chromatography was performed as described previously (32).

Phosphorylation. Intact cells were grown with or without 1 mM 8-Br-cAMP and labeled with ^{32}P as described previously (21). The samples were analyzed on 10% sodium dodecyl sulfate (SDS)-acrylamide gels (21).

Southern analysis. DNA was isolated and digested with restriction enzymes by the instructions of the manufacturer and as described previously (4). Restriction fragments were precipitated and run on agarose gels and blotted onto GeneScreen (New England Nuclear Corp., Boston, Mass.) as described previously (2, 33). Filters were probed with a 770-base-pair *Pst*I fragment of the pRI clone, a cDNA clone homologous to mRNA for the RI gene of PK-A from bovine testes (22). The pRI clone was kindly provided by S. McKnight. The same filter was washed free of probe and rehybridized with a 0.8-kilobase (kb) *Pst*I fragment of a cDNA human β-tubulin cDNA clone (14). (C. Whitfield provided the β-tubulin clone, which was isolated in the laboratory of N. Cowan.) The probes were labeled with ^{32}P by nick translation (27). Quantitative analysis of the hybridization to the RI sequences was performed by scanning the X-ray film in a soft laser microdensitometer (LKB Instruments, Inc., Rockville, Md.) and by measuring the peak area. The areas of the peaks in the Southern blot probed with the RI sequences were compared with those of the same filter probed with the tubulin sequences.

RESULTS

Mutant and transfectant cells are more sensitive to some drugs than are the wild-type cells. The mutant CHO cell 10248 has a defective RI subunit for the type I PK-A (2, 22). The transfectant was derived by transferring genomic DNA from 10248 to the wild-type genomic strain 10001, producing the line 11564 (2). The transfectant also carried the bacterial gene *neo* which confers G418 resistance. Both the mutant and the transfectant were more sensitive to several drugs, such as colcemid, colchicine, and puromycin, than was the wild type (Table 1). They were relatively insensitive to

TABLE 1. Drug sensitivities and resistance of wild-type, mutant, and revertant cells

Cell	LD_{50} ratio of the following drugs[a]:									
	Colcemid (0.0147 ± 0)	Colchicine (0.087 ± 0.001)	Taxol (0.48 ± 0.45)	Puromycin (5.26 ± 0.045)	Adriamycin (0.059± 0.0011)	Actinomycin D (0.0038 ± 0.0001)	Ethidium bromide (1.56 ± 0.003)	Vinblastine (0.31 ± 0.001)	Bleomycin (1.02 ± 0.075)	Ouabain (261 ± 0.20)
Wild type 10001	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
cAMP mutant 10248	0.73 ± 0.01	0.72 ± 0.02	0.78 ± 0.01	0.86 ± 0.02	0.67 ± 0.02	0.76 ± 0.02	0.89 ± 0.02	0.60 ± 0.04	0.90 ± 0.10	0.98 ± 0.10
Transfectant 11564	0.88 ± 0.01	0.79 ± 0.03	0.68 ± 0.01	0.68 ± 0.01	0.65 ± 0.01	0.79 ± 0.02	0.86 ± 0.02	0.70 ± 0.07	1.10 ± 0.14	1.14 ± 0.11
Revertants										
MR1	1.38 ± 0.15	1.5 ± 0	ND[b]	ND	ND	ND	ND	ND	ND	ND
TR1	1.40 ± 0.06	>1.21	0.95 ± 0.00	1.41 ± 0.05	1.00 ± 0.01	1.14 ± 0.04	0.88 ± 0.02	ND	ND	ND
TR2	1.26 ± 0.05	>1.21	1.20 ± 0.19	1.38 ± 0.03	1.10 ± 0.08	1.27 ± 0.03	>1.3	ND	ND	ND
TR3	1.33 ± 0.06	1.08 ± 0.25	1.10 ± 0.03	1.37 ± 0.04	1.06 ± 0.09	1.34 ± 0.04	1.3 ± 0.03	ND	ND	ND
TR5	1.21 ± 0.06	>1.21	0.95 ± 0.02	1.03 ± 0.05	1.17 ± 0.08	1.16 ± 0.04	>1.3	ND	ND	ND

[a] LD_{50} ratio = test cell line LD_{50}/wild-type LD_{50}. Values in parentheses are the LD_{50}s for the wild type (in micrograms per milliliter).
[b] ND, Not determined.

ouabain and bleomycin. The growth curves (Fig. 1) also show that the mutant and transfectant were more sensitive than the wild-type cell line to the microtubule-depolymerizing drug colcemid. The fact that both the original mutant and the transfectant showed similar drug sensitivities is evidence that the increased sensitivity is due to the mutant RI gene.

Microtubule stability. Indirect immunofluorescent studies were performed to visualize the microtubules in each cell line without treatment and after a 4-h treatment with either colcemid or colchicine. The microtubule network of untreated wild-type cells appeared as distinct strands of polymerized tubulin in a clean background. The microtubules were denser toward the center of cells and finer but still discrete toward the periphery (Fig. 2). In both the untreated mutant 10248 and transfectant 11564, the microtubules were present, but in some cells they appeared to be less distinct, especially toward the periphery of the cell. There also appeared to be some increased hazy background, suggesting the possibility of unpolymerized tubulin. After 4 h in 0.025 μg of colcemid per ml, some wild-type cells showed a slightly depolymerized microtubule network but still contained many distinct strands of polymerized tubulin in the form of microtubules. The microtubules in wild-type cells did not become generally depolymerized until a concentration of 0.075 μg colcemid per ml (data not shown). Under the same conditions that left the wild-type microtubules mainly intact (0.025 μg/ml), however, the mutant 10248 and transfectant 11564 showed a totally depolymerized microtubule network. Some cells appeared to have shrunk and had aggregates or globules of tubulin scattered throughout the cell. The tubulin appeared to have depolymerized first at the outer edges of the cell. These data suggest that the increased sensitivity of the mutant and transfectant cells results from an effect of the colcemid on microtubules.

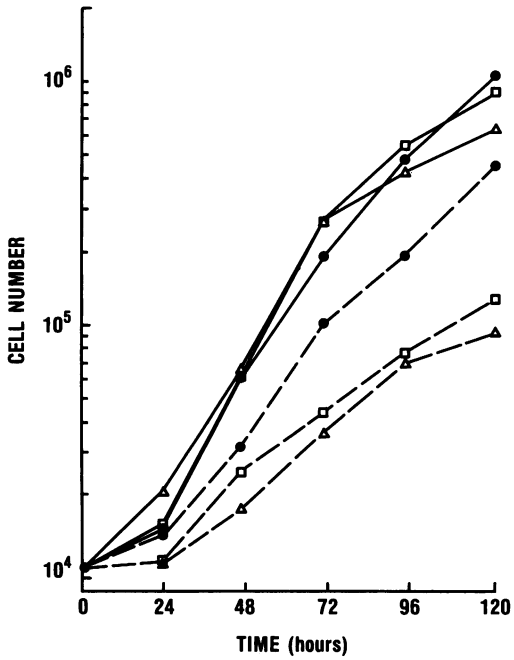

FIG. 1. Growth of wild-type, mutant, and transfectant cells in the absence and presence of colcemid. Cells were grown in normal media (—) or in the presence of 0.025 μg of colcemid per ml (– – –). Symbols: ●, wild-type 10001; △, mutant 10248; □, transfectant 11564 carrying mutant gene from 10248.

Revertants can be selected in colcemid. Because it was possible to differentiate between wild-type and mutant cells on the basis of their growth in colcemid, this characteristic was used to isolate revertants from the PK-A mutant or transfectant. Mutant cells were plated in the presence of 0.025 μg of colcemid per ml. Resistant colonies arose at a frequency of 1.6×10^{-6}. Surviving colonies were picked and grown on normal medium and normal medium–0.7 mM 8-Br-cAMP. Of the 23 colonies selected with colcemid, 4 had elongated cells when grown in 0.7 mM 8-Br-cAMP and therefore reverted to the cAMP-sensitive morphological phenotype of wild-type cells. In this study we focused on revertants that regained both cAMP-induced growth inhibition and a change in morphology. Three of the four potential revertant colonies were tested for growth on cAMP. Of these three, MR1 appeared to be a revertant for growth on cAMP as well as morphology of cells. The change of doubling time for MR1 after growth on cAMP compared with growth on normal media was similar to that for the wild-type 10001 cells (Table 2).

We also isolated revertants from the transfectant 11564 (Table 2). We found a much higher percentage of normally

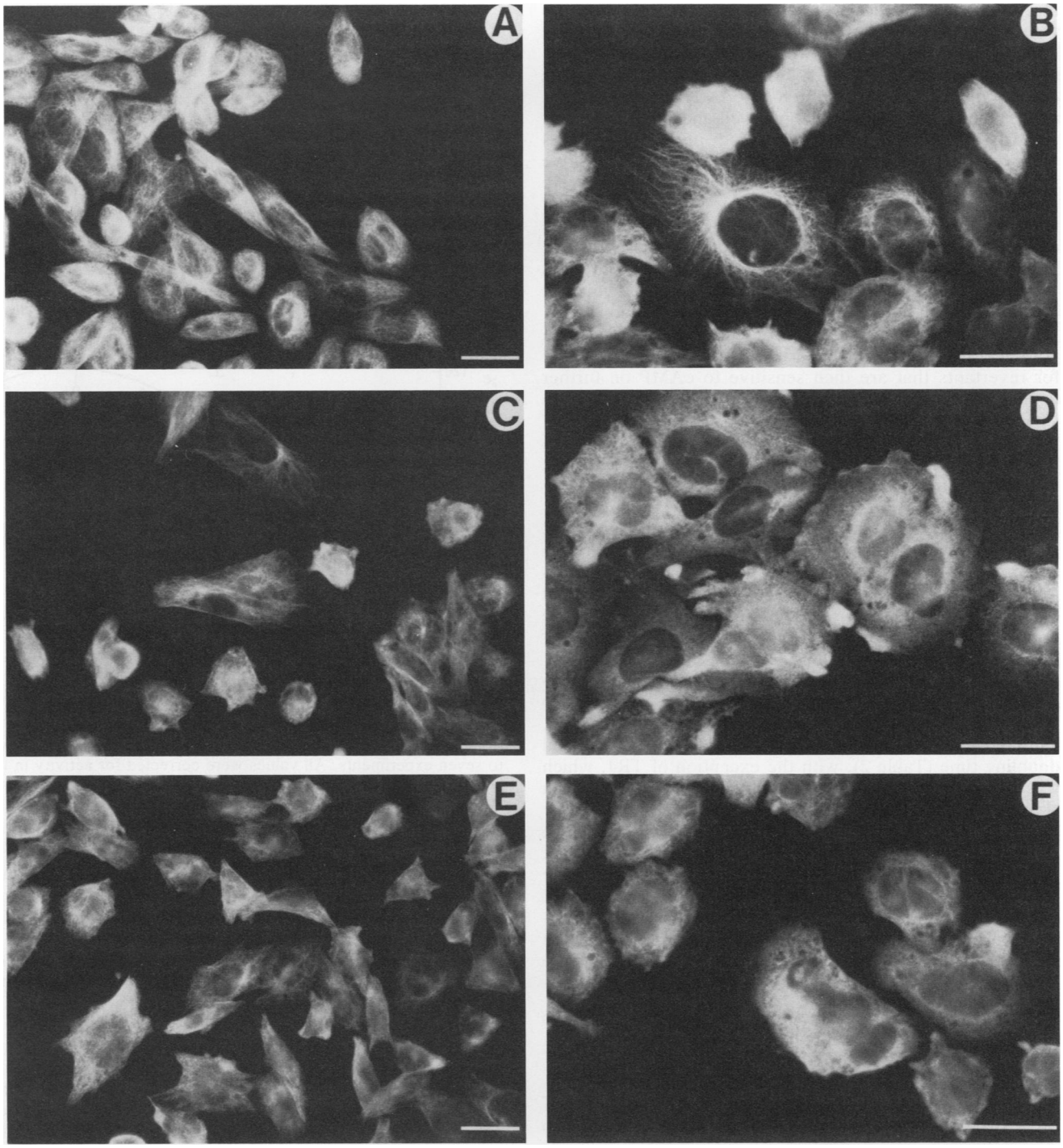

FIG. 2. Organization of microtubules in wild-type, mutant, and transfectant cells in the presence or absence of 0.025 μg of colcemid per ml. Cells were stained with monoclonal antitubulin and a rhodamine-labeled second antibody after growth for 4 h in colcemid-containing or normal medium. (A) Wild type, normal medium; (B) wild type, colcemid; (C) mutant, normal medium; (D) mutant, colcemid; (E) transfectant, normal medium; (F) transfectant, colcemid. Bars, 20 μm.

colcemid-resistant cells with transfectant 11564 than with parental mutant 10248. Of 10×10^6 11564 cells plated in colcemid, approximately 6,000 colcemid-resistant colonies grew. Of these, 245 were picked and tested for 8-Br-cAMP sensitivity. Three 8-Br-cAMP-sensitive colonies (TR1, TR2, and TR3) were identified on the basis of altered morphology and slowed growth in 8-Br-cAMP. UV irradiation did not appear to increase significantly the recovery of revertants from 11564, but two additional revertants (TR4 and TR5) selected after UV irradiation were chosen for further study. The parental line 11564 carried the *neo* gene and was resistant to G418. Revertants TR1, TR2, TR3, and TR4 retained G418 resistance, while TR5 lost resistance.

Revertant selection directly in 8-Br-cAMP. To show that

the revertants isolated in the colcemid selection procedure were not isolated because of random recovery of revertants that lost cAMP resistance, we selected revertants of UV-irradiated 11564 cells directly in 8-Br-cAMP without using colcemid. Cells were plated in 8-Br-cAMP in 24-well plates at an average density of 1 cell per well and were grown for about 10 days. After this the morphology of the colonies was observed, and those that had a typical cAMP-sensitive morphology, like that of the wild-type cell line 10001, were picked and allowed to continue to grow in normal medium. Of the 724 single cells that were cloned individually and examined, only 1 (TR6; 0.14%) was stably cAMP sensitive based on morphology. This is in comparison with 6 colonies that were stably cAMP sensitive from a total of 421 colonies that were selected for normal resistance to colcemid (1.4%). It thus appears that selection for colonies of 11564 with normal resistance to colcemid is enriched by about 10-fold for revertants that are then sensitive to cAMP on further testing. We found similar results with mutant 10248 in a smaller experiment (0.2% frequency of revertants obtained by direct selection in 8-Br-cAMP versus 4.3% frequency of revertants from colcemid-resistant cells). The revertant MR2 was isolated from this experiment. TR6 showed an extreme sensitivity to cAMP, growing over three times more slowly in cAMP than even the wild type (data not shown). MR2 showed the least sensitivity to cAMP of any of the revertants and retained colcemid sensitivity (data not shown). This result suggests that these revertants that were selected directly in 8-Br-cAMP are pseudorevertants, whereas the normally colcemid-resistant revertants are true revertants of the PK-A mutation.

Growth characteristics of the revertants selected in colcemid. Growth studies were performed on the revertants to determine their growth rates in control medium, 8-Br-cAMP, and colcemid. All these revertants had a relatively normal doubling time (Table 2), with the exception of TR4, which was tetraploid (data not shown). All the revertants showed a slower growth rate in 8-Br-cAMP compared with their parent line. The revertants showed a uniform return to a colcemid sensitivity that was close to that of the wild type.

Drug sensitivity of the revertants. The sensitivity of the revertants to several drugs was tested (Table 1). The mutant and transfectant were hypersensitive to colcemid, colchicine, puromycin, adriamycin, and actinomycin D. These are all drugs to which multi-drug-resistant mammalian cells are resistant (24, 25). The colcemid-resistant revertants simultaneously regained resistance to all of these other drugs, demonstrating the linkage of this complex phenotype with cAMP sensitivity.

TABLE 2. Growth of wild-type, mutant, and revertant cells in 8-Br-cAMP or colcemid

Cell	Population doubling time (h [% relative growth rate[a]])		
	Control medium	8-Br-cAMP (0.7 mM)	Colcemid (0.015 μg/ml)
Wild type 10001[b]	13.2	27.5 (48)	16.9 (88)
cAMP mutant 10248[b]	14.1	13.3 (106)	24.4 (60)
Transfectant 11564	13.4	12.6 (106)	22.8 (63)
Revertants selected in colcemid			
MR1	14.8	31.7 (47)	19.8 (87)
TR1	13.5	21.7 (62)	16.6 (85)
TR2	13.3	28.3 (47)	15.9 (89)
TR3	13.2	35.0 (38)	15.9 (86)
TR4	16.5	36.3 (45)	20.8 (84)
TR5	14.4	34.2 (42)	17.5 (89)

[a] (Population doubling time after growth on control/population doubling time after growth on 8-Br-cAMP) × 100.
[b] Values are of two experiments.

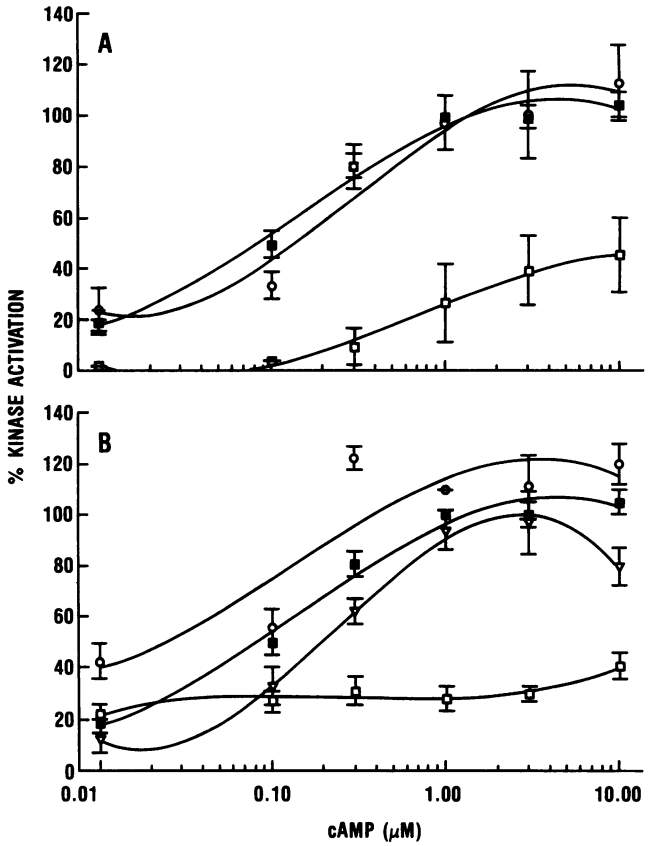

FIG. 3. Activation of PK-A by cAMP in wild-type, mutant, and revertant cells. Activation was measured relative to 100% activation of the wild type at 1 μM cAMP. Points represent the means of two to seven experiments. All values were corrected for activity in the absence of cAMP. (A) Symbols: ■, wild-type 10001; □, mutant 10248; ○, revertant MR1. (B) Symbols: ■, wild-type 10001; □, transfectant 11564; ▽, revertant TR2; ○, revertant TR3.

Protein kinase activity in the revertants. We next examined the activation of kinase activity by cAMP in the revertants. Activation of the revertant MR1 compared with that of the parental mutant 10248 and the wild-type control 10248 is shown in Fig. 3A. The cAMP-dependent activation of protein kinase activity of revertants TR2 and TR3 and that of the parental mutant transfectant 11564 and the wild-type 10001 control is shown in Fig. 3B. The mutant 10248 and transfectant 11564 had lower total activity and a maximal stimulation of kinase at a higher concentration of cAMP (10^{-5} M) than that of the wild type or the revertant. The revertants TR2, TR3, and MR1 (Fig. 3) and TR1 and TR5 (data not shown) were much more similar to the wild type in total cAMP-stimulated protein kinase activity than to the mutant or transfectant.

In a more detailed study, PK-A from revertant MR1 was fractionated on a DEAE column (Fig. 4). This separated two peaks of PK-A activity, PK-A types I and II. The wild-type enzyme characteristically fractionated as two peaks (Fig. 4A), as did the revertant MR1 (Fig. 4C). The mutant, on the other hand, had a reduced PK-A type I and very little PK-A type II (Fig. 4B) (32). Thus, the revertant regained cAMP

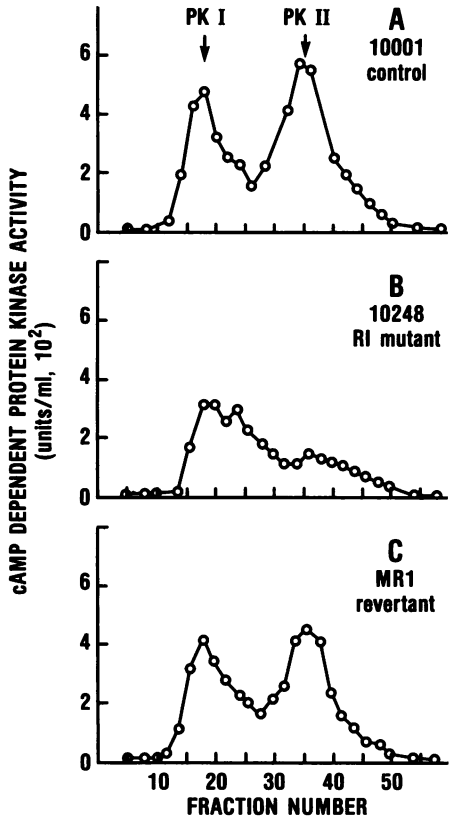

FIG. 4. DEAE-cellulose chromatography of PK-A from wild-type 10001, mutant 10248, and revertant 10248 MR1. Aliquots of 35 μl from every other fraction were assayed for protein kinase activity in the absence and presence of 10 μM cAMP. The PK-A activity, derived by subtracting the cAMP-independent activity from the total kinase activity, was plotted. (A) wild-type 10001; (B) mutant 10248; (C) revertant MR1. One unit is defined as the amount of enzyme required to catalyze the incorporation of 1 pmol of ^{32}P into histone per min at 30°C. PK I, PK-A type I; PK II, PK-A type II.

sensitivity and returned to the wild-type PK-A profile. Another characteristic of PK-A was examined. The wild-type enzyme is known to phosphorylate a specific 52,000-molecular-weight protein band in a cAMP-dependent manner (21). This phosphorylation was absent in the mutant. The ability of the kinase to phosphorylate the 52,000-molecular-weight protein band was regained in the revertant (Fig. 5).

Loss of an extra RI gene by revertants of the transfectant. Because the transfectant has at least one additional RI gene (2), we were able to determine whether the revertants derived from the transfectant lost or retained the extra gene copie(s). DNA was isolated from the revertant strains TR1, TR2, TR3, TR5, and TR6 and was examined for the presence of additional RI gene copies above those found in the wild type. Southern blots of restriction enzyme-digested DNA and filters from slot blots were made and were blotted with probes from fragments of the cDNA clones for the regulatory subunit of PK-A. An example of this Southern analysis with *Hin*dIII (Fig. 6A) shows that only the transfectant appears to have an extra copy of the RI gene (4.3-kb fragment). We checked this result by rehybridizing the filters containing DNA digested with *Hin*dIII (Fig. 6) or *Eco*RI (data not shown) with a probe for the β-tubulin gene. Filters were washed and reblotted with a probe from a fragment of a cDNA for a human β-tubulin gene (Fig. 6B). Because the

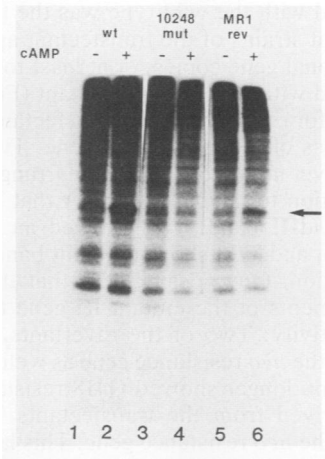

FIG. 5. Protein phosphorylation in intact wild-type (wt) 10001, mutant (mut) 10248, and revertant (rev) MR1 cells with and without 8-Br-cAMP. Cells were exposed for 3 h to 1 mM 8-Br-cAMP before they were labeled for 20 min with $^{32}P_i$. Fractions of 50-μl of sodium dodecyl sulfate cell lysates were applied to each lane of a one-dimensional sodium dodecyl sulfate-acrylamide gel and electrophoresed at 30 mA for 4 h. An autoradiogram of the gel is shown. The arrow indicates the position of the 52,000-dalton protein.

tubulin gene should be present in the same amount per cell in all the cell lines, the density of the bands was proportional to the total amount of DNA in each lane. We measured the density of the bands resulting from filters probed with the tubulin gene, compared it with the density of those probed with the regulatory subunit gene, and computed a ratio of DNA hybridization to pRI compared with hybridization to the control β-tubulin gene. The average ratio of pRI/tubulin DNA, normalized to the ratio obtained with the wild type as 1.0, was between 0.9 and 1.0 for all the revertant strains. It was 1.9 for the transfectant strain. From this result it is apparent that the only strain with additional copies of the RI

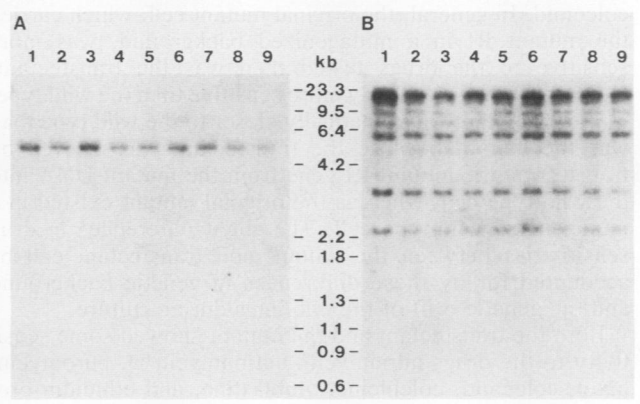

FIG. 6. Levels of RI subunit DNA. Hybridization of wild-type, mutant, transfectant, and revertant DNAs to the cDNA probe of the bovine testes RI subunit gene and to the human β-tubulin gene. DNA was extracted and digested with *Hin*dIII and electrophoresed on agarose gels. After electrophoresis the DNAs were transferred to GeneScreen (New England Nuclear) filters by the procedure described by Southern (33). Filters were probed with a ^{32}P-labeled *Pst*I fragment of the bovine testes RI cDNA clone pRI (A) or with a ^{32}P-labeled *Pst*I fragment of a cDNA clone for human β-tubulin (B). Autoradiograms of the filters are shown. Lanes: 1, wild-type 10001; 2, mutant 10248; 3, transfectant 11564; 4, revertant TR1; 5, revertant TR2; 6, revertant TR3; 7, revertant TR4; 8, revertant TR6; 9, revertant MR2.

gene compared with the wild type was the transfectant. All of the revertant strains of the transfectant appeared to have lost the additional gene copies, or at least to have a reduced level compared with that in the revertant (Fig. 6). Thus, the primary route for reversion in the transfectants appears to be through the loss of the transferred gene. Two of the revertants may have undergone some rearrangement of their DNA, in addition to having lost DNA that hybridized with the 4.3-kb *Hin*dIII band. TR2 showed an additional faint band at 0.8 kb, and TR3 showed a faint band at 7.0 kb after *Hin*dIII digestion. One possibility is that these new bands are rearrangements of the mutant RI gene that diminish or remove its activity. Two of the revertants, TR5 and TR6, may have lost the *neo* resistance gene as well as the pRI gene because they no longer showed G418 resistance. The other revertants derived from the transfectants TR1, TR2, and TR3 retained the *neo* resistance gene. This lack of pattern of retention suggests that at the initial transfection that resulted in strain 11564, the cotransformed genes pRI and *neo* did not become tightly linked.

DISCUSSION

Results of this study indicate that the functioning of the PK-A system in CHO cells has an effect on sensitivity to multiple drugs in these cells. Results of this study also demonstrate a method for isolating revertants of a mutant of PK-A carrying an altered RI gene by capitalizing on the sensitivity of this mutant or transfectants carrying this mutation to the antimitotic drug colcemid. Although the difference in sensitivity between the wild type and the mutant is not large, it is sufficient for revertant selection. Previously, the only method for isolating revertants from PK-A-deficient cells was a selection used with S49 cells for quiescent cells after growth in dibutyryl cAMP and bromodeoxyuridine (35, 39).

The PK-A mutant 10248 and transfectant 11564 were both tested for sensitivity to a variety of drugs, in addition to colcemid. In general, the original mutant cell, which carried the mutant RI in a mutagenized background, was more sensitive to the drugs tested than was the transfectant, although both were clearly more sensitive than the wild type. The transfectant was genetically closer to the wild type than was the mutant because the transfectant was derived by transferring the mutant RI gene from the mutant DNA into the wild-type cell, whereas the original mutant existed in a mutagenized wild-type cell. The slight differences in drug sensitivities between the mutant and transfectant can be accounted for by these differences in genetic background and the genetic drift of the two lines during culture.

Both the transfectant and the mutant showed some sensitivity to the drugs adriamycin, actinomycin D, puromycin, taxol, colcemid, colchicine, vinblastine, and ethidium bromide; but they exhibited little sensitivity to ouabain or bleomycin. Multi-drug-resistant cells are usually resistant to all of these drugs except ouabain and bleomycin (17, 24, 25, 42). Resistance of multi-drug-resistant lines results from increased expression of an energy-dependent drug efflux pump that is called P glycoprotein (M. M. Gottesman, I. B. Roninson, and I. Pastan, *in* D. Kessel, ed., *Resistance to Antineoplastic Drugs*, in press; 25). There are two possible explanations for the cross-sensitivity of PK-A mutants to these drugs. One is that the PK-A system is involved in the regulation of the intracellular levels of these drugs, as might occur if phosphorylation of P glycoprotein increased its efficiency as an efflux pump. P glycoprotein is known to be phosphorylated (5, 6, 10), and thus, it might be a substrate for PK-A. We have no direct evidence to support this hypothesis.

A second explanation for the multidrug sensitivity is that the sensitivity did not result from the effect of PK-A on one discrete mechanism, but that each specific sensitivity or class of sensitivities was the result of the pleiotropic effects of the PK-A enzyme on many specific and distinct cellular processes. For example, PK-A has been implicated as having a role in protein synthesis and is thought to have a role in the phosphorylation of the ribosomal protein S6 (28), which might explain the specific sensitivity to puromycin in the mutant cell lines. Results of previous studies (36, 38) have also shown the association of PK-A type II with microtubule-associated protein in the brain. If this type of association occurs in CHO cells, it might have functional significance and explain their sensitivities to the antimitotic drugs. That is, if PK-A has a role in microtubule function, we might expect cells that are deficient in PK-A to have an altered response to drugs that depolymerize microtubules, such as antimitotic drugs. At present, we cannot offer any conclusive evidence for the basis of the multiple drug sensitivity of these mutant and transfectant cells, except to conclude that it is caused by a defective PK-A.

We selected revertants to cAMP sensitivity at about a 10- to 20-fold higher frequency by using a colcemid selection process than by screening directly for poor growth in 8-Br-cAMP. This is further evidence that our colcemid selection method for revertants is effective. If we were merely randomly picking up revertants, our frequency of selecting cAMP-sensitive cells from the preselected cells should have been comparable to that which we observed in selecting directly for cAMP-sensitive cells in 8-Br-cAMP, that is, 10-fold lower. By selecting sublines of the transfectant or mutant cell that are normally resistant to colcemid, we simultaneously selected cells that were no longer resistant to cAMP. This strongly suggests that the two phenotypes of colcemid sensitivity and cAMP resistance are genetically linked and are both due to the mutant RI subunit gene. All the colcemid-selected revertants that arose from the transfectant appeared to have lost the additional RI gene(s) that were present in the transfectant. MR1 appears to have gone back to the identical PK-A activity as that of the wild type, based on the activation curve, DEAE separation of PK-A, as well as cAMP-dependent phosphorylation of a 52-kilodalton protein. Thus, the revertants simultaneously lost their colcemid sensitivity and cAMP resistance, and they also lost the additional RI gene(s) that was present in the parental transfectant cell and regained wild-type PK-A activity. This evidence strongly supports the argument that PK-A is responsible for all these phenotypes.

From the results of this study, we have shown that it is feasible to select revertants of PK-A mutants in CHO cells by two methods. This result may be generalizable for the isolation of PK-A revertants in other systems. The possibility of revertant selection in the CHO cell line will open the way to selecting second-site revertants that may have alterations in other enzymes or substrates that affect the PK-A pathway and help elucidate the downstream events that are necessary for growth inhibition after PK-A is stimulated in these cells.

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