DNA-mediated Gene Transfer of a Mutant Regulatory Subunit of cAMP-dependent Protein Kinase*

Irene Abraham, Steven Brill, Jennifer Hyde, Robert Fleischmann, Margaret Chapman, and Michael M. Gottesman‡

From the Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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We have used DNA-mediated gene transfer of genomic DNA to introduce into wild-type Chinese hamster ovary (CHO) cells a mutant gene that confers resistance to the growth inhibitory effect of cAMP. This dominant mutation in CHO cell line 10248 is responsible for an alteration in the R1 subunit (R1*) of the type I cAMP-dependent protein kinase (Singh, T. J., Hochman, J., Verna, R., Chapman, M., Abraham, L., Pastan, I. H., and Gottesman, M. M. (1985) J. Biol. Chem. 260, 13927-13933). The transformant 11564 which was studied in detail, has the same characteristics as the original mutant 10248 including continued growth in medium containing 8-Br-cAMP, an increase in the Kₐ for cAMP activation of the kinase, a greatly reduced amount of type II protein kinase activity, an altered incorporation of the photoaffinity label 8-N₆[^32P]cAMP into the R1* subunit of PKI, and an absence of cAMP-dependent phosphorylation of a Mₑ = 52,000 protein in intact cells. In addition, analysis of the DNA of the transformant indicates the presence of an increased amount of DNA for the R1 gene. These results are consistent with the transfer of a mutant gene for the R1* subunit of the cAMP-dependent protein kinase and its phenotypic expression in the transformant and also support the hypothesis that the mutation responsible for the defect in cell line 10248 is due to an alteration in the gene for R1.

Cyclic AMP-dependent protein kinases are found throughout the animal kingdom and play a central role in metabolism of many tissues (1, 2). The effects of cAMP on cultured mammalian cells have been studied by isolating mutants resistant to cAMP analogs (reviewed in Ref. 3). We have isolated and characterized several independent Chinese hamster ovary (CHO) cell mutants that are resistant to the growth inhibitory effects of cAMP (4, 7, reviewed in Ref. 8). Most of the mutants analyzed to date in this cell line have alterations in cAMP-dependent protein kinase activity. There are two cAMP-dependent protein kinases in the CHO cell as are found in many other cells (2), type I kinase and type II kinase. Each kinase is composed of two regulatory subunits, R1 or RII, and two catalytic subunits (C). The catalytic subunits appear to be identical in both type I and type II kinases (9). One mutant studied in detail, 10248, has an R1 with reduced affinity for cAMP (R1*) so that R1 is not easily dissociated from C by cAMP (7). In this mutant, type I kinase is present but very little or no type II kinase is found. The cAMP-resistance phenotype is expressed even in the presence of a wild-type RI subunit (4) and it is hypothesized that under equilibrium conditions in the cell the mutant R1* subunit, which cannot be dissociated from the C subunit, excludes both wild-type RI and RII from binding to C (7).

We are ultimately interested in cloning the normal and mutant genes for cAMP-dependent protein kinase from CHO cells in expression vectors. The dominant mutations such as found in mutant 10248 are of special interest since they might be able to confer their phenotype on a variety of cells. As an initial step toward this goal, we have transferred the R1* gene from mutant 10248 via DNA-mediated gene transfer. The successful gene transfer reported here formally proves the genetic nature of the mutation, confirms its phenotypic expression in the presence of a wild-type RI gene, and through use of a cDNA probe for the bovine testes RI (22) which detects transfer of the CHO RI gene, confirms that the mutation is linked to the RI gene. In order to perform the DNA-transfer experiments we have used the CaPO₄ technique developed by Graham and van der Eb (10) and Wigler et al. (11). We have previously shown that it is feasible to transform CHO cells with both purified plasmid DNA and genomic DNA at relatively high frequencies (12). In this work, we have further refined this procedure to eliminate the background of spontaneous cAMP-resistant mutants by using a co-transformation protocol with a two-step selection suggested by Kavathas and Herzenberg (13).

MATERIALS AND METHODS

Cell Lines—CHO cell line 10001 was derived from a CHO Pro-5 strain (14). Cells were grown in monolayer culture as previously described (4). The protein kinase characteristics of the wild-type strain and cAMP-resistant mutant selection and characterization of the dominant cAMP-dependent protein kinase mutant 10248 have been described (4, 7). 10248 was selected with 1 μg/ml chola toxin and 1 mM theophylline, but is also resistant to 8-Br-cAMP (4), which was exclusively used as the selective agent in this study.

DNA Isolation and Cell Transformation—High molecular weight DNA was isolated from monolayer cultures as described (12). pSV2-neo plasmid (15) was kindly supplied by B. Howard (National Institutes of Health) and plasmid DNA was isolated by standard procedures (16, 17). CHO cells were transformed by the DNA-CaPO₄ precipitate technique (10, 11) but the precipitate was allowed to remain on the cells from 16 to 20 h (12). We used a co-transformation strategy (13) to select the cAMP-resistant cells. Cells were transformed with 1.0 μg of pSV2-neo and 20 μg of mutant (10248) or wild-type DNA/2.5 × 10⁵ cells/10-cm tissue culture plate. A total of 5 × 10⁴ cells were transformed with wild-type DNA, and 5 × 10⁴ cells were transformed with mutant DNA. The DNA-CaPO₄ precipitate

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‡To whom correspondence and reprint requests should be sent.

The abbreviations used are: CHO, Chinese hamster ovary; RI and RII, regulatory subunits of type I and type II protein kinases; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MES, 2-(N-morpholino)ethanesulfonic acid.
was applied for 16 h and then replaced with normal medium 24 h later. Cells were selected in 800 μg/ml of the neomycin analog G418 (Gibco, and a gift from Schering Corp.) for 10 days. G418-resistant colonies arose at a frequency of 7 × 10−6 after transformation with pSV2-neo with either mutant or wild-type genomic DNA. At this stage, many large colonies were selected, and several were subcultured. All tubes were harvested at 100% for 3 min and the samples were electrophoresed on SDS-acrylamide gels.

Results

DNA-mediated Transfer of the cAMP-resistant Phenotype—Wild-type CHO cells can be transformed to cAMP resistance with DNA from cells carrying the RI mutation (10248) (Table I). CHO cells were co-transformed with the plasmid pSV2-neo and high molecular weight DNA from either the wild-type 10001 cells or the mutant 10248. The DNA was left on for 16 h and then replaced with normal medium. 24 h later, the normal medium was replaced with medium containing 800 μg of the neomycin analog G418. After 10 days the G418-resistant colonies were pooled and plated at 106 cells/cm2 plate in 1 mM 8-Br-cAMP in agar (8). 12 days later, 24 colonies were picked from the plates of cells that had received wild-type or mutant DNA. The colonies on the plates of cells that had received the mutant DNA had many large colonies which were not seen on plates of the control cells that had received the wild-type DNA. Wild-type CHO cells will form small colonies in agar in the presence of cAMP. Mutant, CAMP-resistant cells, on the other hand, will form much bigger colonies. We would therefore expect a true transformant to form a large colony, since 10248 carries a dominant mutation (4). Since the G418-resistant colonies were trypsinized and pooled before selection in 8-Br-cAMP agar, CAMP-resistant colonies do not necessarily represent unique events. That is, several CAMP-resistant colonies might be derived from the same G418-resistant colony. For this reason, we cannot determine the true frequency of cAMP-resistant transformants among the G418-resistant colonies.

Table 1

| DNA | Cells plated | G418 colonies | Growth response to cAMP |
|-----|--------------|----------------|-------------------------|
| Wild-type, 10001 | 24 | 0 | 0 2 6 |
| pSV2-neo | | | |
| Mutant, 10248 | 0 | 24 | 6 2 4 |
| pSV2-neo | | | |

* Growth response to cAMP was measured by the change in doubling time after growing with 1 mM 8-Br-cAMP as compared to growth with normal medium (see “Materials and Methods”). R = resistant; change in doubling time of less than 20%, I = intermediate; change in doubling time of between 20 and 50%, S = sensitive; change in doubling time of greater than 50%. Wild-type, 10001 cells, showed a 70% increase in doubling time after growth on cAMP; mutant 10248 cells showed a change of −3.7%.
but there must have been at least one cAMP-resistant colony/3500 G418-resistant colonies, for an overall frequency of \( \geq 2 \times 10^{-7} \) original transformed cell.

Since colony size is a relatively subjective measure and influenced by various factors, including the microenvironment in the agar, we tested these colonies farther for growth as monolayers in 1 mM 8-Br-CAMP. The results are also shown in Table I. Six of the colonies derived from the transformation with the mutant DNA were resistant to the growth inhibition of 8-Br-cAMP in monolayer culture. We have chosen one of these transformants, 11564, for further analysis. A growth curve, comparing growth of this transformant with one of the control transformants, 11572 (G418-resistant clone transformed with 10001 DNA and pSV2-neo), is shown in Fig. 1. The parental 10001 strain has a growth response to cAMP very similar to the control 11572 (data not shown). It is clear that the transformant is very resistant to inhibition of growth by 8-Br-cAMP. Two clones derived from this transformation and two derived from the transformation with wild-type DNA showed slight resistance to 8-Br-cAMP. These variants might be the result of spontaneous mutations causing a very marginal CAMP resistance. Alternatively, the transfer of wild-type DNA sequences themselves may in some way alter the CAMP resistance phenotype possibly by a gene dosage effect.

Protein Kinase Activity in the Transformants—Transformants selected on G418 and then 1 mM 8-Br-cAMP were tested for the level of CAMP-dependent protein kinase activity in extracts of whole cells (Fig. 2). The transformant 11564, transformed with the mutant DNA, has a CAMP activation curve more like that of the mutant cells than that of wild-type cells. Both the mutant and transformant show a decreased sensitivity to CAMP which is shown by the rightward shift of their CAMP activation curves. This increased \( K_c \) for CAMP is characteristic of mutant 10248 (7).

DEAE Analysis of Type I and Type II Protein Kinases—
The wild-type CHO cell has two types of CAMP-dependent protein kinase, type I and type II, that are present in many other cells (2, 23). The mutant 10248, however, has mainly type I (7) (Fig. 3). The protein kinase profile of the transformant 11564 looks very similar to the mutant, having also very little type II kinase.

Analysis of CAMP-dependent Phosphorylations in Intact Cells—Previous studies have demonstrated that the mutant cell 10248 does not phosphorylate a 52,000-dalton protein in a CAMP-dependent manner (19) although the wild-type does.

To test for this defect in the transformant, we labeled intact cells with CAMP. Homogenates of the mutant, wild-type, and transformant 11564 were electrophoresed on one-dimensional SDS-acrylamide gels. The results (Fig. 4) show the presence of a phosphorylated band at 52,000 daltons in the wild-type. This band is not phosphorylated in mutant 10248 and is also not phosphorylated in the transformant. With respect to phosphorylation, the transformant is behaving like the mutant, as would be expected if we have transferred the gene responsible for the behavior of the mutant.

The Regulatory Subunit of the Transformant Is like That of the Mutant—The amount of RI present in each of the fractions eluted from the DEAE column can be measured by the incorporation by the RI subunit of the photoaffinity label, \( -\text{N}_{3}\)\(^{32}\text{P}\)-cAMP. When the RI linked to the photoaffinity label from representative fractions is electrophoresed on SDS-acrylamide gels, a characteristic profile is seen. This is shown in Fig. 5. The largest peak of label represents free RI (6). The small peak that is evident in the wild-type, at fractions 16–20, represents the RI present in the holoenzyme PKI. Note that the mutant 10248 and the transformant 11564 do not have this peak of RI in these fractions. This absence of normally binding RI in the holoenzyme PKI is characteristic of the mutant 10248 (7) and has not been seen for any of our other protein kinase mutants. Under these labeling conditions, RII does not bind well to the photoaffinity label and the upper bands, representing RII, can only be seen faintly starting at fraction 28.

Presence of Excess Sequences for RI in the Transformant—Since the mutation in mutant 10248 was thought to be in the RI gene, we wanted to see if the transformant 11564 contained any extra sequences for this gene, or any new bands that hybridized to this sequence. In another transformant\(^2\) with a

*\(^2\) C. Whitfield, I. Abraham, and M. M. Gottesman, unpublished data.
mutant tubulin gene, we had observed an amplification of the transferred gene. To test for extra copies of the RI gene in the transformant, we isolated DNA from our transformant, wild-type cells, and mutant cells, digested with the restriction endonucleases HindIII, EcoRI, and BamHI, and electrophoresed the fragments on agarose. To probe the DNA fragments, we used a fragment isolated from a cDNA clone to bovine testis CAMP-dependent protein kinase type I (22). As can be seen in Fig. 6, in the transformant (lanes 3, 7, and 11) there appears to be an amplification of the one or two bands that hybridized to the cDNA clone of bovine testis RI. This amplification is not seen in either the wild-type cell, a mock transformant, or the mutant. We have verified that the amount of DNA in all of the lanes is approximately the same by removing the RI probe and hybridizing the filter with chicken actin probe (23). Since the actin gene should be present in the same amount per cell in the three cell lines, the density of the bands is proportional to total DNA in each lane. All bands hybridizing with this probe were of approximately equal intensity. This was confirmed by densitometry. Table II shows a quantitation by densitometer of the density of the bands on the filter after probing with actin or RI, normalized to the amount of actin DNA. The amplification of the band or bands that hybridized with the pRI probe is between 1.4- and 1.7-fold. While the amount of amplification is small, on the order of 50-100%, it is consistently present with all three enzyme digestions and on several blots.

### Table II
**Densitometry of Southern blot probed with pRI and actin DNA**

| Restriction enzyme | Transformant/ wild-type | Transformant/ mutant | Transformant/ control transformant | Average |
|--------------------|-------------------------|----------------------|-----------------------------------|---------|
| HindIII            | 1.9                     | 3.3                  | 1.7                               | 2.3     |
| EcoRI              | 1.6                     | 1.6                  | 1.6                               | 1.6     |
| BamHI              | 1.5                     | 1.3                  | 1.4                               | 1.4     |

![Fig. 3. DEAE-cellulose chromatography of cAMP-dependent protein kinase from wild-type (10001), mutant (10248), and transformant (11564) CHO cells. Chromatography was as described under "Materials and Methods." Aliquots of 35 μl from every other fraction were assayed for protein kinase activity in the absence and presence of 10 μM cAMP. The cAMP-dependent protein kinase activity, derived by subtracting the cAMP-independent activity from the total kinase activity, is plotted. A, 10001, wild-type; B, 10248, mutant; C, 11564, transformant. One unit is defined as the amount of enzyme required to catalyze the incorporation of a pmol of 32P into histone/min at 30 °C.](image-url)

![Fig. 4. Protein phosphorylation in intact wild-type (10001), mutant (10248), and transformant (11564) cells with and without 8-Br-cAMP. Cells were exposed for 3 h to 1 mM 8-Br-cAMP prior to labeling for 20 min with 32P. 50-μl aliquots of SDS cell lysates were applied to each lane of a one-dimensional SDS-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.](image-url)
FIG. 5. RI subunit profile in DEAE-cellulose chromatography fractions of mutant, wild-type, and transformant. Fractions from the DEAE-cellulose columns described in Fig. 3 were incubated with 8-N\textsuperscript{32}P\textsuperscript{cAMP} as described under “Materials and Methods.” These were then electrophoresed on SDS-acrylamide gels, and the RI protein visualized by autoradiography. A and B: wild type, 10001; C and D: mutant, 10248; and E and F: transformant, 11564. Autoradiograms showing incorporation of 8-N\textsuperscript{32}P\textsuperscript{cAMP} into RI are shown on the left (A, C, and E) and quantitation of densitometry tracings of autoradiograms are shown on the right (B, D, and F).

We confirmed this result by a slot blot analysis of DNA from wild-type cells and the transformant, 11564, shown in Fig. 7. In this experiment we used MEP cDNA as a control (24). For each level of DNA tested, the signal in the lanes probed with the RI cDNA probe is stronger for the transformant DNA (lane d) then for the wild-type DNA (lane c). These data were quantitated by densitometry and normalized to the small differences seen between transformant (lane b) and wild-type DNA (lane a) using the MEP cDNA probe. The signals were directly proportional to the amount of DNA applied. The relative amounts of RI DNA in the transformant compared to wild-type cells for each DNA level were: 1.9 (1 µg), 2.1 (2 µg), 2.2 (3 µg), 2.0 (4 µg), and 1.9 (5 µg), with an average of 2.0. An increase of 50–100% in the RI band could be accounted for by the transfer of one or two extra gene copies, assuming the CHO cell has two alleles of the RI gene.}

**DISCUSSION**

This work demonstrates that we have transferred the cAMP-resistant phenotype from mutant CHO 10248 cells to wild-type CHO cells. The transformant has the cAMP-dependent protein kinase characteristics of the mutant cell. That is, it shows a shift in protein kinase activation with cAMP, it shows the presence of type I kinase and the lack of type II kinase, it shows a lack of in vivo phosphorylation of the $M_\text{r} = 52,000$ protein, and it shows the characteristic lower affinity of the RI subunit for the photoaffinity label 8-N\textsuperscript{32}P\textsuperscript{cAMP}. In addition, we have shown the presence of DNA fragments in the transformant that hybridized at least 50% more to a RI subunit gene fragment from a cDNA clone of bovine testis RI. This last piece of data is also a confirmation that the mutation is linked to the RI subunit, as well as indicating the success of the transfer. These data do not completely rule out the unlikely possibility that the mutation affecting the RI* subunit is closely linked to the RI subunit, but not in the gene encoding this subunit.

Examination of the Southern blots shown in Fig. 6 indicates that CHO DNA cut with both HindIII and BamHI give only one band when hybridized to the 770-base pair PstI fragment of pRI. The simplest explanation for this result is that CHO cells carry only one RI gene, although other explanations are possible. If this were true, then the two forms of RI seen in
Gene Transfer of Mutant RI

A pRI

B pA2

FIG. 6. Hybridization of transformant DNA to cDNA probe of the bovine testes RI subunit gene and to the β-actin gene. DNA was extracted and digested with HindIII (lanes 1-4), EcoRI (lanes 5-8), or BamHI (lanes 9-12), and electrophoresed on agarose gels. After electrophoresis the DNAs were transferred to GeneScreen filters using the procedure of Southern (15). Filters were probed with a 32P nick-translated PstI fragment of the bovine testes RI cDNA clone, pRI (panel A), or with a 32P nick-translated HindIII fragment of β-actin (panel B) (“Materials and Methods”). Autoradiograms of the filters are shown. Panels A and B: lanes 1, 5, 9, are 10001, wild-type DNA; lanes 2, 6, and 10 are 10248, mutant DNA; lanes 3, 7, and 11 are 11564, transformant DNA; lanes 4, 8, and 12 are control DNA (wild-type cells transformed with wild-type DNA).

FIG. 7. Slot blot analysis of DNA from wild-type and transformant cells. The numbers on the left indicate the amount of alkali-treated DNA in micrograms applied to the nitrocellulose with a Schleicher and Schuell slot blot apparatus. Lanes a and c contain DNA from 10001 (wild-type) and lanes b and d contain 11564 (transformant) DNA. Lanes a and b were probed with nick-translated MEP cDNA (24) as a control and lanes c and d were probed with nick-translated RI cDNA.

wild-type and mutant cells (7) could be the products of two different RI alleles. This conclusion is similar to what has been found on the basis of biochemical and genetic analysis in S49 cells (25). Alternative explanations include a single allele with alternate mRNA splicing or a single mRNA which can be translated in two different ways.

The ability to transform CHO cells with DNA from mutant cells will enable us to rescue and identify this and other mutant genes affecting cAMP metabolism by techniques already developed (11, 26). Since many of the proteins involved in cAMP metabolism are present in the cell in small amounts, a method to clone these genes by means other than isolation of mRNA is very advantageous.

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Gene Transfer of Mutant RI

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