MOLECULAR AND CELLULAR BIOLOGY, Feb. 1983, p. 250–256
0270-7306/83/020250-07$02.00/0

Vol. 3, No. 2

Reversion of an S49 Cell Cyclic AMP-Dependent Protein Kinase Structural Gene Mutant Occurs Primarily by Functional Elimination of Mutant Gene Expression

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Received 25 August 1982/Accepted 2 November 1982

The regulatory subunits of cyclic AMP (cAMP)-dependent protein kinase from a dibutyryl cAMP-resistant S49 mouse lymphoma cell mutant, clone U200/65.1, and its revertants were visualized by two-dimensional polyacrylamide gel electrophoresis. Clone U200/65.1 co-expressed electrophoretically distinguishable mutant and wild-type subunits (Steinberg et al., Cell **10**:381–391, 1977). In all 48 clones examined, reversion of the mutant to dibutyryl cAMP sensitivity was accompanied by alterations in regulatory subunit labeling patterns. Some spontaneous (3 of 11) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced (2 of 11) revertants retained mutant subunits, but these were altered in charge, degree of phosphorylation, or both. The charge alterations were consistent with single amino acid substitutions, suggesting that reversion was the result of second-site mutations in the mutant regulatory subunit allele that restored wild-type function, although not wild-type structure, to the gene product. The majority of spontaneous (8 of 11) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced (9 of 11) revertants and all of the revertants induced by ethyl methane sulfonate (14 of 14) and ICR191 (12 of 12) displayed only wild-type subunits. Dibutyryl cAMP-resistant mutants isolated from several of these revertants displayed new mutant but not wild-type subunits, suggesting that the revertant parent expresses only a single, functional regulatory subunit allele. The mutant regulatory subunit allele can, therefore, be modified in two general ways to produce revertant phenotypes: (i) by mutations that restore its wild-type function, and (ii) by mutations that eliminate its function.

The mechanisms leading to phenotypic reversion of structural gene mutations in mammalian tissue culture cells appear generally to be analogous to those described for procaryotic cells. Thus, stable reversion of missense mutations in genes such as those coding for hypoxanthine phosphoribosyltransferase (HPRT) (15), tRNA synthetase (28), RNA polymerase II (11), and dihydrofolate reductase (29) in CHO cells and for thymidylate synthase (2) in mouse FM3A cells occurs as a result of new mutations within the mutant gene which correct or compensate for the original mutation.

The existence of two or more gene copies in animal cells can give rise to revertants by mechanisms not available in haploid cells. For example, HPRT is X-linked in humans (22). HeLa cells are derived from a female patient and, presumably, contain both active and inactive X chromosomes. Milman et al. (20) described a HeLa cell mutant, H23, with a missense mutation in HPRT (19) resulting in an inactive enzyme with an altered isoelectric point. Five independent revertants isolated from H23 were found to synthesize both mutant and wild-type (WT) forms of the protein. HPRT-deficient segregants of the revertants arose at high frequencies and expressed mutant but not WT HPRT. The authors concluded that revertants of H23 must be expressing a newly active but previously silent WT gene.

We describe here a phenomenon in S49 mouse lymphoma cells whereby the presence of a WT gene affects the phenotypic reversion of a mammalian structural gene mutation. These cells contain two active loci that encode the regulatory subunit of cyclic AMP (cAMP)-dependent protein kinase (cA-PK) (27). One class of mutants (k_a mutants), selected for resistance to dibutyryl cAMP (Bt_2cAMP), contain cA-PK activity with an increased apparent activation constant for cAMP (7). This phenotype can general-

ly be attributed to a missense mutation in one of the two regulatory subunit alleles (27). These mutants concomitantly express both the mutant and WT alleles but are of the mutant phenotype, because they contain only low levels of protein kinase capable of activation under the condition of Bt_2cAMP selection. We infer from measurement of the cA-PK activity in cell lysates prepared from multiple independent k_a mutants that this stimulable activity is 25% or less of that present in WT cells (16). That these cells contain kinase with more mutant than WT activity is attributable not to unequal synthesis of the WT regulatory (R) and mutant regulatory (R') subunits, but to preferential assembly of kinase containing R' rather than R subunits (R. A. Steinberg, personal communication). In addition, it is probable that heterodimers in the holoenzyme, which contains two (RR, RR', or R'R') regulatory subunits, have mutant-like activity and that there is a greater rate of degradation of WT than of mutant subunits.

Revertants of one k_a mutant, U200/65.1, are effectively induced by the base substitution mutagens (6) ethyl methane sulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), but also by the frame shift mutagen (1, 8, 18) ICR191 (30). Reversion of U200/65.1 cells always restored the WT levels and activation constant of cA-PK. In addition, the thermal lability of cA-PK activity in all revertants, except some of those induced by MNNG, was restored to that of the wild type. In sum, reversion of U200/65.1 cells apparently leads most often to production of a WT-like enzyme regardless of the type of mutagenesis used to induce revertants.

Since U200/65.1 cells express both mutant and WT R subunit alleles, the simplest interpretation of these results is that reversion occurs occasionally by compensatory mutations that restore WT function to the mutant product, but predominantly by functional elimination of mutant R' expression. Our previous study did not assess the expression of the two individual allelic products in mutant and revertant cells. We therefore could not directly confirm this interpretation.

Many k_a mutants, including most of those induced by MNNG mutagenesis, contain mutant and WT R subunit proteins that are distinguishable in the isofocusing dimension of two-dimensional (O'Farrell) gel electropherograms (27). U200/65.1 cells express R and R' alleles that are so distinguishable. Thus, we could use gel analysis to directly ascertain the effects of reversion on the expression and nature of each product.

In this report we show that only WT R products can be detected in most revertants, that forward mutants selected from these revertants express new mutant but not WT R subunits, and that, therefore, the revertant parents are probably functionally hemizygous for R subunit expression.

MATERIALS AND METHODS

Materials. Media and chemicals came from the following sources: Dulbecco modified Eagle medium (DMEM), horse serum, and fetal calf serum, GIBCO Diagnostics; methionine-free DMEM was obtained as a 10× concentrate from Pacific Biological, and low-methionine medium is methionine-free DMEM containing 10% fetal calf serum; agarose, Seakem; MNNG, Aldrich Chemical Co.; Bt_2cAMP, N^6-(2-aminoethyl)-cAMP–Sepharose, and histone H_{2b} (type V11), Sigma Chemical Co.; [γ-^{32}P]ATP, New England Nuclear Corp.; [^{35}S]methionine, Amersham Corp.; Nonidet P-40, Almega Corp.; ampholines, LKB; acrylamide and methylenebisacrylamide, Bio-Rad Laboratories; sodium dodecyl sulfate, BDH; urea (ultrapure), Schwarz/Mann; X-ray film and photographic chemicals, Eastman Kodak Co.

Cells and culture methods. Cells were grown in suspension culture in DMEM with 3 g of glucose per liter supplemented with 10% heat-inactivated horse serum. Cloning was performed in the same medium but with 4.5 g of glucose per liter and 0.3% agarose over feeder layers of primary mouse embryo fibroblasts (3). All cells used were clonal sublines of mouse lymphoma cell line S49.1 (10). Clone 24.3.2 is a serially subcloned population of S49.1 cells. Clone U200/65.1 is an unselected subclone of U200/65; the latter was selected by plating an MNNG-treated population of 24.3.2 cells in 0.5 mM Bt_2cAMP plus 0.2 mM theophylline (7). Clone T6.2.3 was derived from U200/65.1 by plating an unmutagenized population in 1.0 μM dexamethasone to select for resistance to that drug. Dexamethasone resistance (Dex^r) was employed solely as a means of genetically marking cell lines to rule out cross-cultural contamination among S49 clones (30). Bt_2cAMP-sensitive revertants were isolated from U200/65.1 or T6.2.3 cells after EMS, MNNG, or ICR191 mutagenesis as described in a separate communication (30). Briefly, mutagenized cultures were treated sequentially with Bt_2cAMP to arrest revertant cells and then with bromodeoxyuridine, 33258 Hoechst dye, and white light to kill cycling mutant cells. Clones surviving multiple applications of this counterselection panel were retrieved and screened for sensitivity to Bt_2cAMP. Mutants of the revertants were isolated after mutagenesis with MNNG and plating in 0.5 mM Bt_2cAMP. A list of the clones and their phenotypes and origins is given in Table 1.

Kinase assay. Cells were lysed in 10 mM Tris-hydrochloride–3.0 mM magnesium acetate (pH 7.4), 100,000 × *g* supernatants were prepared, and protein kinase activity was determined by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to histone H_{2b} as previously described (30).

The protein concentration of cell extracts was determined by the method of Lowry et al. (17), with bovine serum albumin used as the standard.

[^{35}S]methionine labeling and extract preparation. Labeling procedures were slightly modified from those of Steinberg (27). Cells (2×10^6) were suspended in 1.0 ml of low-methionine DMEM and incubated for 60 min at 37°C. [^{35}S]methionine (800 to 1,200 Ci/mmol) was

TABLE 1. Clones used

Clone	Phenotype	Origin
24.3.2	WT	Unselected subclone of S49.1
U200/65.1	Bt_2cAMP^r	MNNG-induced mutant of 24.3.2
T6.2.3	Dex^r Bt_2cAMP^r	Dex^r subclone of U200/65.1
T4.2.4, T4.2.5, T4.2.3	Bt_2cAMP^s	Spontaneous revertants of U200/65.1
T4.4.6	Bt_2cAMP^s	EMS-induced revertant of U200/65.1
T10.5.6, T10.5.11, T10.5.14	Dex^r Bt_2cAMP^s	MNNG-induced revertants of T6.2.3
T24.7.1, T24.7.3	Dex^r Bt_2cAMP^s	ICR191-induced revertants of T6.2.3
T30.4.6, T30.4.7, T30.8.3	Dex^r Bt_2cAMP^r	MNNG-induced mutants of T24.7 clones

then added to a final concentration of 100 μCi/ml, and incubation was continued for 3 to 4 h at 37°C. Incorporation was stopped by adding cold phosphate-buffered saline containing 4 mM L-methionine. The cells were washed once and then lysed in 100 μl of buffer containing 10 mM Tris-hydrochloride (pH 7.5), 2 mM dithiothreitol, 2 mM L-methionine, and 0.5% (vol/vol) Nonidet P-40. Extracts were centrifuged for 15 min in a Beckman Microfuge, and the supernatants were used for column chromatography.

Affinity column chromatography. Radiolabeled extracts were subjected to affinity chromatography over 40-μl columns of N^6-(2-aminoethyl)-cAMP–Sepharose as described by Steinberg (27).

Two-dimensional gel electrophoresis. The O'Farrell two-dimensional gel electrophoresis procedure (21) was used with the modifications described by Steinberg (26). Second-dimension sodium dodecyl sulfate gels were brought to 7.5% in polyacrylamide. All gel patterns are shown with the acidic end of the isoelectric focusing dimension at the right and the low-molecular-weight region of the second dimension at the bottom.

RESULTS

Expression of two R subunit alleles in WT and mutant cells. S49 cell R subunits can be visualized by two-dimensional polyacrylamide gel electrophoresis (27). Cells were metabolically labeled with [^{35}S]methionine, cytosols were chromatographed over a cAMP affinity resin, the R subunits were eluted with a strongly denaturing buffer, and the eluates were subjected to electrophoresis. The single-step affinity chromatography yielded a preparation that contained R polypeptide of sufficient relative abundance for ready visualization on gels and a background of other polypeptides. These provided a reference system against which changes in R mobility due to mutation- or phosphorylation-dependent charge modifications could be observed. WT cells contain a single R subunit polypeptide present in both a phosphorylated and nonphosphorylated form. These had nearly identical molecular weights, about 50,000, and distinct isoelectric points, pI 6.0 and 6.3, respectively, in urea gels (Fig. 1a) (27). They were separated in the isofocusing dimension by approximately one charge unit. The more acidic and abundant form is phosphorylated (24, 27).

Many k_a mutants contain four, rather than two, isoelectric forms of R (27). The labeling pattern of the basic shift k_a mutant clone U200/65.1 is shown in Fig. 1b. It contained two R forms that comigrated with those present in WT cells; however, the label now resided primarily in the dephosphorylated form. Two new spots appeared that migrated to the basic side of the WT pattern and were clearly resolved from the others. The more acidic and abundant of the new spots was phosphorylated. Each mutant product was separated from its respective WT form by about two charge units. It was concluded from these and similar data that S49 cells express two R alleles and that k_a mutants generally carry a missense mutation in one of the alleles (27). Among the k_a mutants analyzed in this way, all of the more than 50 that expressed electrophoretically distinguishable products of the mutant allele also expressed the product of the WT allele, i.e., mutant cells were never observed to shut off expression of the WT allele.

Effects of reversion on R subunit labeling patterns. A total of 48 Bt_2cAMP-sensitive revertants of U200/65.1 cells were analyzed. In every case, the labeling pattern of R subunits was altered from that found in the parent mutant (Fig. 1d through g). However, three Bt_2cAMP-resistant clones, adventitious survivors of the selection for sensitive revertants, retained the mutant labeling pattern (Fig. 1c). Alterations in R subunit labeling patterns in the revertants

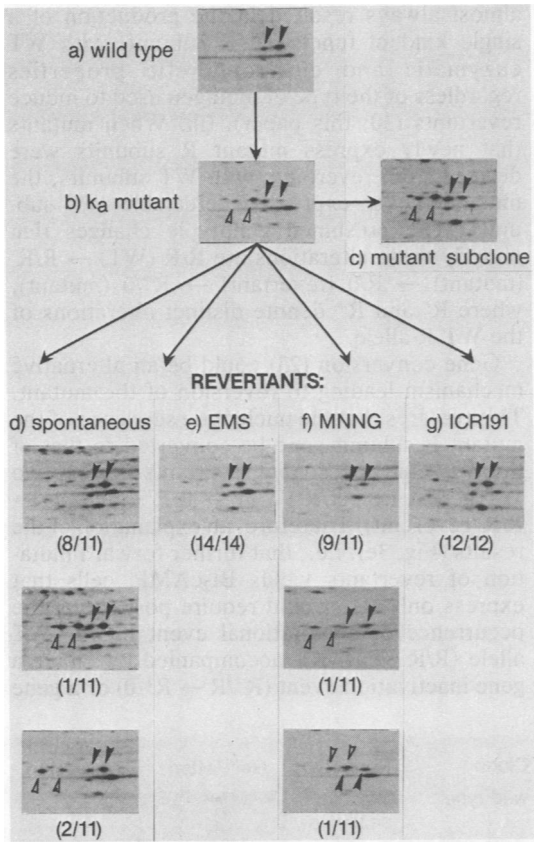

FIG. 1. R subunit expression in S49 WT, mutant, and revertant cells. The relevant portions of autoradiograms obtained after in vivo [^{35}S]methionine labeling of cells, cAMP affinity column chromatography of cell extracts, and two-dimensional gel electrophoresis of the column eluates are shown. The acidic end of the gels is to the right. Solid arrows, Positions to which WT subunits migrated; open arrows, new spots appearing in mutant and revertant patterns. The numbers in parentheses indicate the frequency with which each phenotype was observed among the total number of revertants examined within each class. Clones: (a) 24.3.2; (b) U200/65.1; (c) T24.7.2, a Bt_2cAMP-resistant survivor of the selection for ICR191-induced Bt_2cAMP-sensitive revertants; in each column, the revertants are (from the top): (d) T4.2.4, T4.2.5, and T4.2.3; (e) T4.4.1; (f) T10.5.6, T10.5.11, and T10.5.14; (g) T24.7.1.

therefore were probably not induced by the selection protocol, but instead reflect mutational events associated with reversion of the phenotype.

A number of types of changes from the mutant labeling pattern were observed among the revertants. In a few rare cases, among spontaneous (Fig. 1d) and MNNG-induced (Fig. 1f) revertants, mutant subunits were retained but changed in charge, degree of phosphorylation, or both. The mutant spots in the basic shift spontaneous revertant clone (T4.2.3) and the acidic shift MNNG-induced revertant clone (T10.5.14) were altered by a single unit of charge or less. In addition, the MNNG-induced revertant clone (T10.5.11), displaying a change only in the degree of phosphorylation of the mutant and WT subunits, was previously shown to contain cA-PK activity, with a half-life for thermal denaturation more nearly like that of the mutant than of the WT cA-PK (30). These observations are consistent with the interpretation that the mutant R′ subunit genes in these revertants experienced second-site mutations that restored their WT function, but not the WT structure.

The most striking result was that the large majority of revertants had R subunit labeling patterns indistinguishable from that of the WT (Fig. 1d through g). Most of the spontaneous (8 of 11) and MNNG-induced (9 of 11) and all of the EMS- (14 of 14) and ICR191-induced revertants (12 of 12) appeared to have a gel pattern identical to that of the WT cells.

Two alternative hypotheses can explain this result. (i) The mutant gene is particularly susceptible to mutagenesis at a specific site(s), i.e., has a hot spot, which neutralizes both the phenotypic effects and charge change of the original mutation, thus leading to a WT-like R product, phenotype, and R subunit labeling pattern; or (ii) the expression of mutant R′ is eliminated either by mutations within the structural gene that lead to nonfunctional products not detected on the gels or by repression of mutant R′ synthesis. The labeling patterns in the second case thus reflect the expression of the single remaining WT allele.

These hypotheses make a powerful and testable prediction. Suppose that revertants express two R subunit alleles whose products exactly comigrate electrophoretically; then MNNG-induced k_a mutants selected from the revertants that newly express a mutant R should also express WT R. However, if revertants express only a single R allele, then such derived mutants should express no WT subunits, a result never observed when k_a mutants selected from WT Bt_2cAMP^s rather than revertant Bt_2cAMP^s cells were so analyzed.

Functionally hemizygous expression of R subunits. Three revertants, one induced by EMS (T4.4.1) and two induced by ICR191 (T24.7.1 and T24.7.3), were mutagenized with MNNG, allowed a 3- to 6-day expression period, and plated in the presence of Bt_2cAMP. Resistant mutants arose at frequencies of about 10^{-4}. Several Bt_2cAMP^r clones contained cA-PK activity with increased K_a for cAMP (Fig. 2) and therefore could possibly carry R proteins with discernible charge alterations.

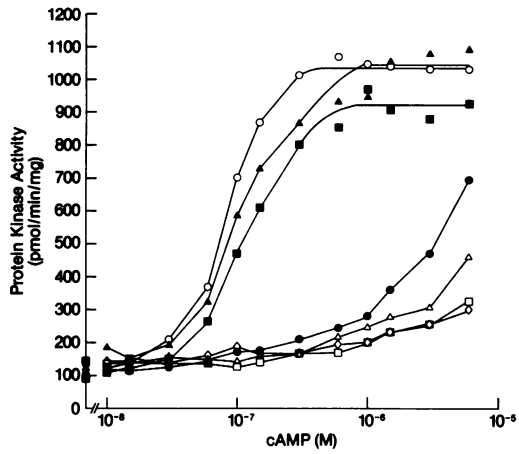

FIG. 2. cA-PK activity in cell extracts. Cells: 24.3.2 (○); U200/65.1 (●); ICR191-induced revertants T24.7.1 (▲) and T24.7.3. (■); Bt_2cAMP^r segregants of ICR191-induced revertants T30.4.6 (△), T30.4.7 (□), and T30.8.3 (◇).

Figure 3 shows the genealogy of clones leading to the Bt_2cAMP^r mutant of one revertant, their respective R subunit electrophoretic patterns, and our interpretation of them in terms of the nature and number of R alleles expressed. Of the mutants isolated from the revertants, two from T24.7.1, one from T24.7.3, and two from T4.4.1 displayed R subunits with charge alterations in the acidic direction varying in magnitude from 1 to 2 charge units. Figure 3d shows the electropherogram of one such mutant isolated from T24.7.1. The charge shift in R was associated with an absence of detectable label in the location to which WT R subunits migrated (Fig. 3d). Similar results were obtained with the other four mutants analyzed in this way (data not shown). These data are consistent with the hypothesis that reversion of U200/65.1 cells can occur as the result of events that eliminate functional expression of the mutant R′ allele.

DISCUSSION

The presence of two active genes encoding the R subunit of cA-PK and the dominant effect of mutant R′ on the cellular phenotype make possible a novel mechanism for the reversion of a mammalian structural gene mutant. Phenotypic reversion of this mutant, which coexpresses the products of mutant and WT genes, can occur by functional elimination of mutant R subunit expression. The following observations support this. (i) ICR191, a frame shift mutagen in bacterial (1) and apparently in mammalian systems (8, 18), very effectively increased the frequency of revertants (spontaneous, $<3 \times 10^{-7}$; ICR191, 1.5×10^{-5}) (30). (ii) Reversion of phenotype almost always resulted in the production of a single kind of functional R subunit, with WT enzymatic and electrophoretic properties regardless of the type of mutagen used to induce revertants (30; this paper). (iii) When mutants that newly express mutant R subunits were derived from revertants with WT subunits, the mutants never expressed detectable WT subunits. The presumed genotypic changes that underly these alterations are R/R (WT) → R/R′ (mutant) → R/0 (revertant) → R*/0 (mutant), where R′ and R* denote distinct alterations of the WT R allele.

Gene conversion (23) could be an alternative mechanism leading to reversion of the mutant. This requires that the nucleotide sequence of the mutant R subunit gene be converted to that of the WT sequence, so that revertants express two WT genes, i.e., R/R (WT) → R/R′ (mutant) → R/R (revertant). Therefore, an explanation of the results (Fig. 3e), i.e., that further forward mutation of revertants yields Bt_2cAMP^r cells that express only R*, would require postulating the occurrence of a mutational event in one WT allele (R/R → R*/R), accompanied by either a gene inactivation event (R*/R → R*/0) or a gene

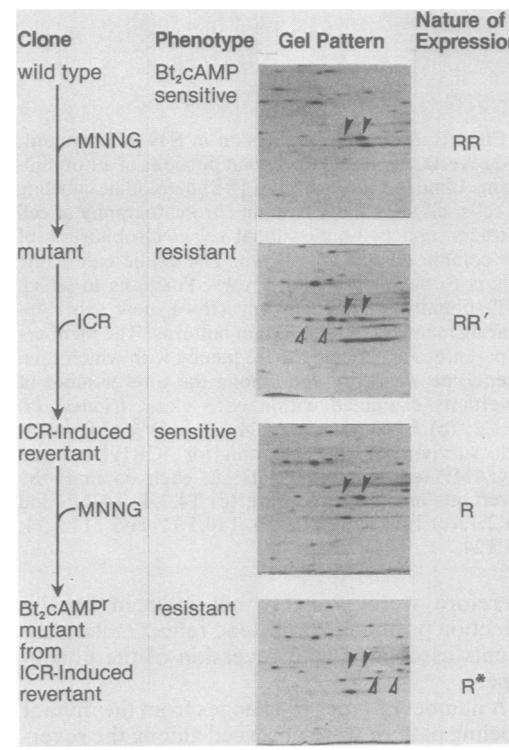

FIG. 3. Hemizygous expression of R subunits in ICR191-induced revertants. Solid arrows, Positions to which WT subunits migrated; open arrows, new spots appearing in mutant and revertant patterns. Cells: (a) 24.3.2; (b) U200/65.1; (c) T24.7.1; (d) T30.4.6.

conversion (R*/R → R*/R*) of the remaining WT allele. We believe the data are more simply explained by the functional elimination of mutant R' than by gene conversion.

Functional elimination of mutant allele expression is likely to account for most revertants, because mutagens, especially ICR191, are more likely to mutate genes encoding R' to produce an inactive product than a product with WT activity. Indeed, most (43 of 48) of the revertants we obtained had phenotypes and labeling patterns consistent with this notion. Furthermore, three randomly selected revertants among this predominant class all appeared to be functionally hemizygous for R subunit expression.

Functional elimination of the products of one R subunit allele may require enhanced expression of the remaining allele to maintain cell viability and the WT phenotype. Dissociation of the inactive cA-PK tetramer by cAMP results in the release and activation of catalytic (C) subunits (13, 14), an event that is ultimately lethal for WT (4, 5) and revertant (30) S49 cells. In the unstimulated state, R subunit expression must be at least equal to C subunit expression to maintain C subunits in the inactive holoenzyme complex. WT cells contain approximately equal levels of R and C subunits (R. A. Steinberg and T. R. van Daalen Wetters, unpublished data), so that loss of significant amounts of R expression or activity by mutation, without concomitant reduction in C expression, would seriously compromise cell viability. The ICR191-induced revertants had WT levels of CA-PK activity and, in addition, showed no appreciable activity above WT levels in the unstimulated state (Fig. 2). The stoichiometry of the dissociation reaction for cA-PK,

$$4\ \mathrm{cAMP} + \underset{\text{(catalytically inactive)}}{\mathrm{R_2C_2}} \rightleftharpoons \mathrm{R_2\,cAMP_4} + \underset{\text{(catalytically active)}}{2\ \mathrm{C}}$$

implies that the revertants must, therefore, contain WT levels of R as well as C subunits. The steady-state level of the R product in a cell with a single WT allele could equal that in a cell with two such alleles as the result of compensatory mechanisms that increase synthesis or decrease turnover of the polypeptide, mechanisms that are available to affect levels of R subunit expression in WT cells (24, 25). Studies on R subunit metabolism in these revertants are required to resolve this question.

It is possible that most ICR191-induced revertants express truncated R subunit polypeptides whose synthesis is directed by the mutant allele as a result of nonsense or deletion mutations. These products were probably not detected on the gels because they are too small or because they have lost the ability to bind cAMP and are therefore lost in the affinity chromatography step before electrophoresis. The identification of such fragments, as by immunoprecipitation, would constitute powerful evidence in support of our model for the predominant mechanism of reversion of U200/65.1 cells.

Some spontaneous and MNNG-induced revertants have been identified as potential second-site revertants. Reversion of phenotype in these cells occurred because a second mutational event within the mutant R subunit gene restored WT function to its product. These revertants expressed products from the mutant allele that were altered in charge, degree of phosphorylation, or both (Fig. 1). Two revertants displaying charge changes, T4.2.3 and T10.5.14, contained R subunits that, after proteolytic digestion, showed electrophoretic patterns differing from those in WT or mutant cells (R. A. Steinberg, unpublished data). This result provides strong evidence that these revertants indeed result from second-site genetic alterations in the mutant allele.

Lastly, the conclusion that K_a mutants carry true structural gene mutations in R subunit genes and that these mutations result in the mutant phenotype was based on the regular association of alterations in R subunit polypeptide function (9, 12) and charge (27) with the altered phenotype. Because, as reported here, all of the 48 phenotypic revertants of one k_a mutant examined by gel analysis carried new alterations in the mutant R subunit polypeptide, we confirm that conclusion.

ACKNOWLEDGMENTS

We thank R. A. Steinberg and V. E. Groppi, Jr., for their generous assistance in leading us through the nuances of the two-dimensional gel electrophoresis procedure.

This work was supported by National Science Foundation grant PCM-7807382. T. van D.W. was supported by Public Health Service training grant 2 T32 CA09270-06 and P.C. is the recipient of a Research Career Development Award from the National Institutes of Health.

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