Association of a 68,000-dalton Protein with Adrenocorticotropic-sensitive Adenylate Cyclase Activity in Y1 Adrenocortical Tumor Cells*

(Received for publication, February 25, 1981, and in revised form, June 29, 1981)

Valerie M. Watt‡ and Bernard P. Schimmer
From the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada MSG 1L6

This report explores the biochemical basis for clonal variation in adrenocorticotropic (ACTH)-sensitive adenylate cyclase activity in the Y1 mouse adrenocortical tumor cell line. We demonstrate that the level of a specific protein, designated p68, is significantly correlated with the ability of adrenocorticotropic to stimulate adenylate cyclase activity among Y1 subclones (p = 0.004; r = 0.65). p68 was characterized by its molecular weight in sodium dodecyl sulfate polyacrylamide gels (Mr = 68,000) and by its isoelectric point as determined by two-dimensional gel electrophoresis (pI = 7.2). On two-dimensional gels, the protein migrated as a major spot with satellite spots 0.1 pH unit on either side. Homogenates and plasma membrane fractions from clones highly responsive to ACTH had large amounts of p68. In homogenates from highly responsive clones, p68 represented 10 to 12% of the total protein. Homogenates and plasma membrane fractions from clones insensitive to ACTH were deficient in p68. In homogenates from the insensitive clones Y6 and OS3, p68 represented <0.8% of the total protein. A somatic cell hybrid, formed by fusion of these two ACTH-insensitive clones recovered ACTH-sensitive adenylate cyclase activity and concomitantly expressed appreciable levels of p68. It is suggested that p68 may regulate the transfer of information from the occupied ACTH receptor to the catalytic subunit of adenylate cyclase.

The action of ACTH on adenylate cyclase appears to be similar to the actions of other polypeptide hormones and neurotransmitters on the enzyme (Glynn et al., 1979) and obligatory for many of the hormone's effects on the adrenal cortex (Halkerston, 1975; Schimmer, 1980). Components of the ACTH-stimulated adenylate cyclase system in the adrenal cortex include a specific hormone receptor on the cell surface, guanyl nucleotides and guanyl nucleotide-binding proteins, divalent cations, and a catalytic subunit which faces intracellularly (for review, see Glynn et al., 1979). Nevertheless, the sites and mechanism of ACTH action on the adenylate cyclase system are not well defined.

We have analyzed the mechanism of hormonal regulation of adenylate cyclase activity in the ACTH-responsive adrenocortical tumor cell line, Y1, using a genetic approach (for review see, Schimmer, 1980). In previous reports, we isolated and described several variant subclones of the Y1 cell line in which the adenylate cyclase system was unresponsive to ACTH (Schimmer, 1969, 1972; Schimmer et al., 1979; Rae et al., 1979a). Adenylate cyclase activity in these variants was stimulated by fluoride, guanyl nucleotides, and cholera toxin (Rae et al., 1979b; Schimmer, 1972), suggesting that the adenylate cyclase system was partially intact. Furthermore, studies with synthetic fragments of ACTH suggested that the lesions in these clones were not restricted to the tight binding region of the ACTH receptor (Rae et al., 1979b). In this study, we have explored the molecular basis for the loss of ACTH-sensitive adenylate cyclase activity in variant Y1 cells, by comparing the profiles of proteins in ACTH-responsive and insensitive clones. We demonstrate that the presence of one protein (Mr = 68,000; pI = 7.2) correlates significantly with the integrity of the ACTH-sensitive adenylate cyclase system. We suggest that this protein may play a role in determining the sensitivity of the adenylate cyclase system to ACTH.

EXPERIMENTAL PROCEDURES

Materials—Nutrient mixture F10 was purchased from Connaught Laboratories; sera were from Gibco (2-[3H]ATP (16 to 27 Ci/mmol) and PCS scintillation fluid mixture were from Amersham/Searle; l-[35S]methionine (500 to 980 Ci/mmol) and 2,5-diphenyloxazole from New England Nuclear; SB-5 film from Kodak. Ammonium persulfate, Dowex 50 (AG 50W-X8), SDS, and urea were obtained from Bio-Rad; RNA polymerase from Boehringer Mannheim; N,N-dimethylbenzenesulfonyl chloride from Eastman; Ampholines from LKB; dimethylsulfoxide from Matheson, Coleman and Bell; Nonidet P-40 from Particle Data Laboratories. GPP(NH)p was purchased from ICN Pharmaceuticals; theophylline from Schwarz-Mann; bovine serum albumin, catalase, and Trizma (2-amino-2-hydroxymethyl-1,3-propanediol) base from Sigma; ACTH1-24 (bovine) from Nutritional Biochemicals and ACTH1-24 (porcine) from Sigma. ACTH1-24 (Organon) was a gift from Dr. H. Strade (Organon, Inc., West Orange, NJ).

Cells and Cell Culture—All cell lines were derived from the mouse adrenocortical tumor cell line, Y1 (Yasumura et al., 1969). This clone is available currently from the American Type Culture Collection (No. CCL 79), and is designated “Y1-ATCC” in this paper. Several stable, ACTH-responsive subclones were isolated from the Y1-ATCC line by single cell-plating techniques and include: Y1-BS1 (Schimmer, 1979), Y1-BS8, and Y1-BS11. Other clones used in this study were two spontaneous, ACTH-insensitive variants, Y6 and OS3 (Schimmer, 1969, 1972); two cell lines deficient in hypoxanthine-guanine phosphoribosyltransferase activity, Y1-HGPRT- and OS3-HGPRT; (Schimmer et al., 1977); and Kin and Cyc mutants selected for resistance to 8-bromoadenosine 3',5'-monophosphate (Rae et al., 1979; Schimmer et al., 1979). All cell lines were grown as monolayers on plastic tissue culture dishes at 37 °C. Cells were cultured in Nutrient F10 medium supplemented with 15% heat-inactivated horse serum, 2.5% heat-inactivated fetal calf serum, and antibiotics. The methods of cell culture have been detailed elsewhere (Schimmer, 1979). Cell Fusion—Y6 × OS3 hybrids were prepared using the half-
glycerol was added to a final concentration of 10% (w/v). For two-
adenylate cyclase activity, the stimulatory effect of ACTH$_{1-24}$ expressed as picomoles of cAMP formed per min per mg of protein.

Preparation of Plasma Membrane Fractions—Fractions enriched in plasma membranes were prepared from monolayers of adrenocortical tumor cells by differential centrifugation of cell homogenates followed by centrifugation in a discontinuous sucrose gradient (Schimmer et al., 1979). The specific activity of (Na + K)-ATPase in these fractions was increased at least 20-fold.

Adenylate Cyclase Activity—Cell monolayers at saturation density were rinsed with 20 mM Tris-HCl (pH 7.7), 1 mM MgCl$_2$, and 250 mM sucrose, scraped in 20 mM Tris-HCl (pH 7.7), 1 mM MgCl$_2$, and 1 mM dithiothreitol, and homogenized at 0°C in a Dounce homogenizer. ACTH$_{1-24}$ was determined by measuring the conversion of [3H]ATP to [3H]cAMP as described by Schimmer (1972). The reaction mixture contained [3H]ATP (approximately 1.3 × 10$^6$ cpm), 1 mM disodium ATP, 2 mM MgCl$_2$, 13 mM Tris-HCl (pH 7.7), 6 mM theophylline, and 50 μg of albumin in a total volume of 80 μl. ACTH, NaF, and GPP(NH)p were included at concentrations indicated. The reaction was carried out for 10 min at 37°C and was stopped by adding 0.1 ml of a recovery mixture (40 mM disodium ATP, 12.5 mM cAMP) and then by boiling for 3 min. Labeled cAMP was separated from other labeled compounds by chromatography on columns of Dowex 50 and treatment with BaSO$_4$. Adenylate cyclase activity was expressed as picomoles of cAMP formed per min per mg of protein.

Polyacrylamide Gel Electrophoresis—Cell monolayers were incubated with [35S]methionine (4.55 μCi/ml) in medium for 42 h. The monolayers at saturation density then were rinsed with 200 mM sucrose, scraped in 20 mM Tris-HCl (pH 6.5), and homogenized in a Dounce homogenizer. Samples were mixed with 480 mM β-mercaptoethanol and SDS, at a ratio of protein of at least 5:1 (w/w), and were boiled for 10 min. For analysis on SDS-polyacrylamide gels, the samples were incubated with [3S]methionine, plasma membrane fractions were prepared, and the proteins in total homogenates from these clones. Y1-BSI, Y6, and OS3 cells were incubated with [35S]methionine, plasma membrane fractions were prepared, and the proteins in total homogenates and in the plasma membrane fractions were analyzed following electrophoresis in SDS-polyacrylamide gels. In the plasma membrane fraction from Y1-BSI cells, at least six major bands, ranging in molecular weight from 30,000 to 108,000, were visible in stained gels and in fluorographs (Fig. 1a).

Gel Electrophoresis of Proteins from ACTH-responsive and-insensitive Clones—The ability of ACTH to stimulate adenylate cyclase activity in Y1-BSI cells, but not in Y6 and OS3 cells, prompted us to look for differences in protein composition in the plasma membrane fractions and in total homogenates from these clones. Y1-BSI, Y6, and OS3 cells were incubated with [35S]methionine, plasma membrane fractions were prepared, and the proteins in total homogenates and in the plasma membrane fractions were analyzed following electrophoresis in SDS-polyacrylamide gels. In the plasma membrane fraction from Y1-BSI cells, at least six major bands, ranging in molecular weight from 30,000 to 108,000, were visible in stained gels and in fluorographs (Fig. 1a). In the plasma membrane fractions from Y1-BSI, Y6, and OS3 cells, adenylate cyclase activity was markedly increased by fluoride but was stimulated less than 2-fold by 20 μM ACTH$_{1-24}$ (Table I). The small stimulation of adenylate cyclase activity seen in the Y6 clone with high concentrations of ACTH$_{1-24}$ appeared to result from a nonspecific, polyacationic effect of the hormone similar to that observed by Wolff and Cook (1977).

Adenylate cyclase activity was measured in the presence of 10 μM GPP(NH)p, ACTH$_{1-24}$ (20 μM) and NaF (15 mM) were added as indicated. Values shown are averages of duplicate or triplicate samples from representative experiments and are expressed as picomoles of cAMP formed per min per mg of protein.

| Clone      | Fraction        | ACTH$_{1-24}$ Fluoride |
|------------|-----------------|------------------------|
| Y1-BSI     | Cell homogenate | 16                     |
| Y6         | Plasma membrane | 150                    |
| Y6         | Cell homogenate | 11                     |
| OS3        | Plasma membrane | 88                     |
| OS3        | Cell homogenate | 7                      |
| Plasma membrane | 49             | 50                     |

Adenylate cyclase activity in ACTH-responsive and-insensitive Y1-adrenal clones was 1.7-fold greater than the effect of NaF on enzyme activity (Table I). In plasma membrane fractions from Y1-BSI cells, adenylate cyclase activity also was stimulated by ACTH$_{1-24}$ to levels greater than the levels achieved with fluoride (Table I). In the plasma membrane fraction, but not in total homogenates, GPP(NH)p was required for the stimulatory effect of ACTH. In homogenates and in plasma membrane fractions from Y6 and OS3 cells, adenylate cyclase activity was markedly increased by fluoride but was stimulated less than 2-fold by 20 μM ACTH$_{1-24}$ (Table I). The small stimulation of adenylate cyclase activity seen in the Y6 clone with high concentrations of ACTH$_{1-24}$ appeared to result from a nonspecific, polyacationic effect of the hormone similar to that observed by Wolff and Cook (1977).

Protein Determination—Proteins were measured by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

RESULTS

Adenylate Cyclase Activity in ACTH-responsive and-insensitive Adrenal Clones—Homogenates and fractions enriched in plasma membranes were prepared from clones Y1-BSI, Y6, and OS3. In homogenates prepared from Y1-BSI cells, ACTH$_{1-24}$ stimulated adenylate cyclase activity 20-fold over basal levels (Table I). Although NaF also stimulated adenylate cyclase activity, the stimulatory effect of ACTH$_{1-24}$ was 1.7-fold greater than the effect of NaF on enzyme activity (Table I). In plasma membrane fractions from Y1-BSI cells, adenylate cyclase activity also was stimulated by ACTH$_{1-24}$ to levels greater than the levels achieved with fluoride (Table I). In the plasma membrane fraction, but not in total homogenates, GPP(NH)p was required for the stimulatory effect of ACTH. In homogenates and in plasma membrane fractions from Y6 and OS3 cells, adenylate cyclase activity was markedly increased by fluoride but was stimulated less than 2-fold by 20 μM ACTH$_{1-24}$ (Table I). The small stimulation of adenylate cyclase activity seen in the Y6 clone with high concentrations of ACTH$_{1-24}$ appeared to result from a nonspecific, polyacationic effect of the hormone similar to that observed by Wolff and Cook (1977).

Gel Electrophoresis of Proteins from ACTH-responsive and-insensitive Clones—The ability of ACTH to stimulate adenylate cyclase activity in Y1-BSI cells, but not in Y6 and OS3 cells, prompted us to look for differences in protein composition in the plasma membrane fractions and in total homogenates from these clones. Y1-BSI, Y6, and OS3 cells were incubated with [35S]methionine, plasma membrane fractions were prepared, and the proteins in total homogenates and in the plasma membrane fractions were analyzed following electrophoresis in SDS-polyacrylamide gels. In the plasma membrane fraction from Y1-BSI cells, at least six major bands, ranging in molecular weight from 30,000 to 108,000, were visible in stained gels and in fluorographs (Fig. 1a). In the plasma membrane fractions from Y1-BSI, Y6, and OS3 cells, there was a striking reduction in the stained and labeled band at 68,000 daltons (Fig. 1a). Although the protein profiles of these clones were similar, differences in the amounts of other bands also were observed (e.g. $M_r = 30,000$; Fig. 1a). We have concentrated on the 68,000-dalton protein since the change in the amount of this protein was dramatic, and was observed in both stained and labeled preparations and in both variants. The difference in the amount of the 68,000-dalton protein present in the ACTH-responsive Y1-BSI clone and the insensitive variants also was visible in stained gels and fluorographs of total cell proteins (Fig. 1b). This difference also was observed after labeling cells with [35S]methionine for 1 h or with [3H]leucine for 24 h. Quantitative analysis of fluorographs indicated that in Y1-BSI cells, the band at 68,000 daltons represented 10.6% ± 0.7% (n = 7).

2 The polycationic nature of ACTH$_{1-24}$ was demonstrated previously (Rae and Schimmer, 1974). ACTH$_{1-24}$ stimulated adenylate cyclase in Y6 cells only at high concentrations of the peptide (2 to 20 μM). ACTH$_{1-24}$ at these concentrations was without effect (e.g. Table III). Two other polycations, polylysine and polyarginine, also stimulated adenylate cyclase in Y6 cells over the same concentration range (J. Tsao and B. P. Schimmer, unpublished observations).
of total radioactive protein. In Y6 and OS3 cells, the band at 68,000 daltons represented ≤0.8% of the labeled protein. Proteins from total homogenates of Y1-BS1, Y6, and OS3 cells also were analyzed by two-dimensional gel electrophoresis. In homogenates from Y1-BS1 cells, 83% of the radioactively labeled 68,000-dalton protein focused at a pI of 7.2 ± 0.1 (n = 4), with satellite spots displaced 0.1 pH unit on either side (Fig. 2). This protein is designated p68. The 68,000-dalton protein associated with the plasma membrane fraction of Y1-BS1 cells had the same isoelectric point (Schimmer et al., 1979). In both variant adrenal clones, Y6 and OS3, the amount of p68 in plasma membrane fractions and in total cell homogenates, as analyzed by two-dimensional gel electrophoresis, was less than in Y1-BS1. Quantitative analysis of two-dimensional gels of total Y6 homogenates gave the same result as analysis of one-dimensional tracks, i.e. the amount of p68 in Y6 cells was approximately 3% of that in Y1-BS1 cells.

Distribution of p68 between Cytosol and Membranes—The abundance of p68 in Y1-BS1 cells precluded it from being exclusively localized in the plasma membrane fraction, an organelle which comprises a small fraction (approximately 2.5%) of total cell protein. Therefore, the distribution of p68 between the cytosol and particulate fractions of Y1-BS1 cells was determined. Fluorographs of radioactively labeled proteins from total homogenates and cytosolic and particulate fractions separated on SDS-polyacrylamide gels are shown in Fig. 3. Although a significant amount of p68 (30%) was associated with the particulate fraction (track B), 70% of p68 was present in the soluble fraction (track C). To determine whether the soluble p68 could associate with plasma membranes during fractionation, a soluble fraction was prepared from Y1-BS1 cells labeled with L-[35S]methionine and mixed with a total homogenate of unlabeled Y1-BS1 cells. The plasma membrane fraction was isolated from this mixture, solubilized, and electrophoresed. A 68,000-dalton band from the labeled soluble fraction did associate with the plasma membranes during purification (Fig. 3, track D). A comparison of the specific activities of p68 in each fraction, however, suggested that not more than 50% of p68 in the plasma membrane fraction resulted from co-fractionation of the cytosolic p68. Attempts to reconstitute ACTH sensitivity in Y6 cells or modify ACTH-sensitive adenylate cyclase activity in Y1-BS1 cells by adding back soluble fractions containing p68 were unsuccessful (data not shown).

Adenylate Cyclase Activity and Levels of p68 in Y6 × OS3 Hybrids—Y6 cells in limiting numbers and OS3-HGPRT- cells were fused with Sendai virus and two colonies were isolated following selective growth in amethopterin. Whereas both Y6 and OS3-HGPRT- cells had nearly diploid chromosome numbers, the two clones isolated after fusion and selection had polyploid karyotypes, indicating their hybrid nature (Table II). Inasmuch as this fusion and selection procedure favors the isolation of heterologous hybrids (Davidson and Ephrussi, 1965), these clones are tentatively designated Y6 × OS3-HGPRT- hybrids. The two parental clones and the Y6 × OS3-HGPRT- hybrid, clone 2, had adenylate cyclase activities which were stimulated by fluoride but which were insensitive to ACTH₁-₂₄ (Table II). In one hybrid, Y6 × OS3-
and ACTH-sensitive Adenylate Cyclase Activity

**FIG. 2.** Two-dimensional gel electrophoresis of proteins from Y1-BS1 cells. A fluorograph from cells labeled with L-[35S]methionine is shown. The outlined area indicates the position of the major protein with $M_r = 68,000$.

### TABLE II

**Adenylate cyclase activity and karyotype analysis of Y6 × OS3-HGPRT− hybrids**

Y6 and OS3-HGPRT− cells were fused with Sendai virus and two colonies were isolated following selective growth in amethopterin. Chromosome numbers for parental lines are modal numbers based on scores from at least 16 spreads. Values for hybrids are averages with the range for more than 85% of the spreads counted shown in parentheses. Cell homogenates were assayed for adenylate cyclase activity with no additions, ACTH1-24 (20 pm) or NaF (15 mm). Activities are expressed as picomoles of cAMP formed per min per mg of protein and in hybrid clones are averages of triplicate samples.

| Clone     | Chromosome no. | Adenylate cyclase activity |
|-----------|----------------|---------------------------|
|           |                | Basal | ACTH1-24 | Fluoride |
| Y6        | 39             | 8     | 21       | 220      |
| OS3-HGPRT−| 42             | 4     | 7        | 200      |
| Y6 × OS3-HGPRT− | 70 (54-75) | <1 | 240 | 240 |
| Clone 1   | 73 (62-81)    | 2     | 10       | 190      |

HGPRT− clone 1, adenylate cyclase was activated by ACTH1-24 to the same level achieved with fluoride (Table II). Associated with the recovery of ACTH-sensitive adenylate cyclase activity in the hybrid, Y6 × OS3-HGPRT− clone 1, was an increased level of p68 (Fig. 4). The level of p68 in the ACTH-responsive hybrid, clone 1, was increased at least 16-fold over the level of p68 in the two-unresponsive parental clones. The ACTH-insensitive hybrid, clone 2, had a level of p68 similar to that found in the unresponsive parents (Fig. 4).

**Clonal Variations in ACTH-sensitive Adenylate Cyclase Activity and the Levels of p68—**The effects of ACTH1-24 on adenylate cyclase activity were determined in 18 independent subclones of the Y1 line and compared with the level of p68 in each clone in order to further explore the relationship between these two variables. Adenylate cyclase activity was assayed in cell homogenates with no additions or with maximally effective concentrations of ACTH1-24 or NaF (Table III). Basal levels of adenylate cyclase activity in all clones were low and ranged from 2 to 12 pmol of cAMP formed/min/mg of protein. Fluoride-stimulated adenylate cyclase activity ranged from 160 to 340 pmol of cAMP formed/min/mg of protein and, in all clones, reflected a substantial increase in enzyme activity. The relative effectiveness of ACTH1-24 on adenylate activity among the different clones varied considerably (Table III). Clonal variations in responsiveness to ACTH were apparent in the absolute levels of ACTH-stimulated adenylate cyclase activity, in the fold increase over basal levels and in the ratio of activities produced by ACTH1-24 and fluoride (ACTH/F− activity ratio; Table III). The expression of the effectiveness of ACTH1-24 on adenylate cyclase activity as an ACTH/F− activity ratio was used as an estimate of the relative sensitivity of adenylate cyclase to ACTH. The Y1

**FIG. 3.** Gel electrophoresis of proteins in subcellular fractions from Y1-BS1 cells. Y1-BS1 cells, labeled with L-[35S]methionine, were homogenized in 20 mM Tris-HCl (pH 6.8) containing 0.1 mM phenylmethylsulfonyl fluoride and centrifuged at 100,000 × g for 60 min. The pellet was washed once with the same Tris-HCl buffer and the resultant supernatant fractions were combined. The total homogenate (track A), 100,000 × g pellet (track B), and the combined supernatant fractions (track C) were electrophoresed on 7.5% polyacrylamide gels and analyzed by autoradiography. In a separate experiment, the soluble fraction from radioactively labeled Y1-BS1 cells was mixed with 10 times the number of unlabeled Y1-BS1 cells and the plasma membrane fraction was isolated and analyzed by autoradiography following gel electrophoresis. Track D shows the labeled soluble proteins which were recovered in the plasma membrane fraction.
p68 and ACTH-sensitive Adenylate Cyclase Activity

Clones were ranked in order of decreasing responsiveness to ACTH as determined by the ACTH/F- activity ratio (Table III). Clones such as Y1-BSI and several of the Kin mutants (Kin 7, Kin 8, and Kin 9) had ACTH-stimulated adenylate cyclase activities which were greater than or equal to the activity measured in the presence of fluoride. Clones such as Cyc-101, Y6, OS3, and Y6 × OS3 HGPRT- clone 2 were insensitive to ACTH. The other clones listed showed intermediate levels of response to ACTH. Adenylate cyclase activity in the parent Y1 clone, Y1-ATCC, was stimulated approximately 8-fold by ACTH; however, this response was less than 20% of the catalytic activity seen in the presence of fluoride (Table III). In previous studies, similar ACTH/F- activity ratios were observed in homogenates of Y1-ATCC cells (Tautton et al., 1969; Schimmer, 1972). Each Y1 subclone in monolayer culture was labeled with L-[35S]methionine for 42 h, and the amount of p68 was quantitated following SDS-polyacrylamide gel electrophoresis of total homogenates and autoradiography. The amount of p68, like ACTH-sensitive adenylate cyclase activity, varied among the subclones of the Y1 line (Table III). The addition of 0.1 mM phenylmethylsulfonyl fluoride to the homogenizing buffer in order to inhibit protein degradation in many of the subclones did not alter the level of p68. In clones highly responsive to ACTH such as Y1-BSI, Kin-7, and Kin-8, p68 represented approximately 10 to 12% of the total labeled protein. In clones which were much less sensitive or insensitive to ACTH, such as Y1-ATCC, Y6, Y6 × OS3-HGPRT- clone 2, Cyc-101 and OS3, the amount of p68 ranged from 0.3 to 2.5% of total labeled protein. The greatest variability in the correlations between levels of p68 and responsiveness to ACTH were observed in clones with inter-

Fig. 4. Gel electrophoresis of Y6 × OS3-HGPRT- hybrids. Cells were labeled with L-[35S]methionine, and homogenates were electrophoresed on 7.5% polyacrylamide gels and analyzed by autoradiography. OS3-HGPRT- (track A); Y6 × OS3-HGPRT- clone 1 (track B); Y6 × OS3-HGPRT- clone 2 (track C). The profile of proteins obtained from homogenates of the Y6 parent is shown in Fig. 1b.

| Clone | Adenylate cyclase activity | ACTH/F- | p68 |
|-------|----------------------------|---------|-----|
|       | Basal                       | ACTH    | Fluoride |     |     |
| Y1-BSI| 8 ± 1                       | 250 ± 10| 220 ± 10| 1.22 ± 0.07 (24) | 10.6 ± 0.7 (7) |
| Kin 7 | 8 ± 2                       | 240 ± 10| 220 ± 30| 1.13 ± 0.18 (4)  | 9.8 (2)    |
| Kin 9 | 2                           | 180     | 170     | 1.08 (1)         | 11.4 (1)   |
| Kin 8 | 7 ± 1                       | 290 ± 20| 310 ± 25| 0.96 ± 0.08 (4)  | 9.6 ± 0.8 (3) |
| Y6 × OS3-HGPRT- Clone 1 | 7 ± 2 | 140 ± 10 | 190 ± 15 | 0.71 ± 0.07 (4) | 12.1 ± 0.5 (3) |
| Kin 4 | 2                           | 130     | 200     | 0.63 (1)         | 12.1 (1)   |
| Y1-HGPRT- Clone 1 | 6 ± 1 | 140 ± 15 | 250 ± 20 | 0.53 ± 0.04 (14) | 0.8 ± 0.1 (4) |
| Y1-BS8| 7 ± 1                       | 120 ± 6 | 260 ± 10| 0.45 ± 0.02 (30) | 0.8 ± 0.1 (3) |
| Cyc 102 | 12                      | 120     | 260     | 0.43 (1)         | 12.4 ± 1.1 (3) |
| Kin 1 | 6 ± 1                       | 85 ± 15 | 220 ± 20| 0.41 ± 0.08 (10)| 3.9 ± 0.3 (4) |
| Y1-BS11| 6 ± 1                      | 130 ± 20| 300 ± 50| 0.40 ± 0.07  | 1.2 (1)    |
| Cyc 103 | 4                        | 105     | 280     | 0.36 (1)         | 11.0 (2)   |
| Kin 2 | 5 ± 1                       | 60 ± 10 | 230 ± 25| 0.25 ± 0.04 (8) | 10.5 ± 0.7 (4) |
| Y1-ATCC| 8 ± 2                      | 65 ± 10 | 320 ± 30| 0.17 ± 0.02 (10) | 1.7 ± 0.1 (4) |
| Y6 | 6 ± 1                       | 16 ± 6  | 260 ± 70| 0.03 ± 0.01 (3)  | 0.3 (2)    |
| Y6 × OS3-HGPRT- Clone 2 | 3 ± 1 | 7 ± 1 | 160     | 0.02 (1)         | 0.4 (2)    |
| Cyc 101 | 7                        | 12      | 340     | 0.02 (2)         | 2.5 (2)    |
| OS3 | 3 ± 1                       | 4 ± 1   | 220 ± 45| <0.01 (3)        | 0.8 ± 0.1 (3) |

Cell homogenates were assayed for adenylate cyclase activity with no additions, NaF (16 mM), or with maximally effective concentrations of ACTH (2 µM for ACTH from Nutritional Biochemicals; 20 µM for ACTH from Sigma). Adenylate cyclase activity is expressed as picomoles of cAMP formed per min per mg of protein. The level of p68 was determined by quantitation of autoradiographs from SDS-polyacrylamide gels and is expressed as the percentage of total cell protein.

Table III

Clonal variations in ACTH-sensitive adenylate cyclase activity and in the levels of p68
mediate ACTH/F- activity ratios (Table III). Clones such as Cyc-103 and Kin-2 had low ACTH/F- activity ratios but had levels of p68 equivalent to the levels found in highly responsive clones. In contrast, the ACTH-responsive clones Y1-HGPRT and Y1-BS8 had levels of p68 comparable to the levels found in ACTH-resistant clones (Table III). In all 18 clones of the Y1 line, the relative amount of p68 correlated significantly with hormone-sensitive adenylate cyclase activity expressed as the ACTH/F- activity ratio ($p = 0.004$; $r = 0.65$). The correlation coefficient was determined using product-moment correlation analysis (Ostle and Mensing, 1975). Significant correlations also were observed when the relative amount of p68 was compared with the absolute level of ACTH-sensitive adenylate cyclase activity ($p = 0.01$) or with the fold stimulation by ACTH over the basal level ($p = 0.01$).

**DISCUSSION**

We have found a significant correlation between the ability of ACTH to stimulate adenylate cyclase activity and the amount of a specific protein, p68, among subclones of the Y1 adrenocortical tumor cell line. Clones highly stimulated by ACTH have large amounts of p68; clones lacking ACTH-sensitive adenylate cyclase activity have little p68; deviations from the correlation are found in clones with intermediate sensitivity to ACTH (Table III). An association of p68 with ACTH-sensitive adenylate cyclase activity also was observed in hybrids formed between the two ACTH-insensitive adrenal clones, Y6 and OS3. One hybrid, clone 1, recovered ACTH-sensitive adenylate cyclase activity and, concomitantly, acquired appreciable levels of p68. In contrast, the hybrid clone 2 remained insensitive to ACTH and retained the low levels of p68 associated with unresponsive clones (Table II). The mechanism responsible for the reconstitution of ACTH-sensitive adenylate cyclase activity in the hybrid clone 1 is unknown; however, preliminary evidence suggests that restoration of ACTH sensitivity through cell fusion is an infrequent event and, therefore, probably not due to a simple complementation of two different mutations.

The co-expression of ACTH-sensitive adenylate cyclase activity and p68 may be the result of their coordinate regulation and need not imply a role for p68 in the modulation of ACTH-sensitive adenylate cyclase activity. The factors influencing the differential expression of p68 among Y1 clones are themselves of interest. Our working hypothesis, however, envisages a causal relationship between p68 and the sensitivity of adenylate cyclase to ACTH. Although the correlation between the amount of p68 and sensitivity of adenylate cyclase to ACTH is significant ($p = 0.004$), the value of the correlation coefficient ($r = 0.65$) does not indicate a perfect association between these two variables. Within the context of our working hypothesis, the low correlation coefficient can be rationalized at least in two ways. First, the hormone-sensitive adenylate cyclase system is complex, and its activity is determined by several membrane proteins including hormone receptors, a guanyl nucleotide-binding protein, and a catalytic subunit. In addition, other factors including divalent cations, soluble factors, guanyl nucleotides, and the lipid bilayer itself have been shown to influence the effects of hormones on adenylate cyclase activity (for review, see Ross and Gilman, 1980). In the experiments described here, adenylate cyclase activity was assayed in all the subclones under uniform conditions which were established previously (Taunton et al., 1969; Schimmer, 1972). No attempt was made to optimize assay conditions for each of the 18 clones in order to reduce variability in hormone responsiveness unrelated to the level of p68. Accordingly, the low correlation coefficient between ACTH-sensitive adenylate cyclase activity and the level of p68 may have resulted from variations in one or more of the other components of the system. In some hepatoma and lymphoma clones, for example, lack of hormone-responsive adenylate cyclase activity resulted from alterations in hormone receptors, guanyl nucleotide-binding proteins, and catalytic subunits (Insel et al., 1976; Haga et al., 1977; Wicks et al., 1978). Second, this study focused on the level of p68 in relation to ACTH-sensitive adenylate cyclase activity. The precise function of p68, however, remains undefined, and it is conceivable that in some clones the activity of p68 has been altered. Therefore, the putative activity of p68 rather than the absolute level of protein may be more relevant to ACTH-sensitive adenylate cyclase activity.

Little or no p68 was detected when total proteins from normal mouse tissues (liver, skeletal muscle, fat, testis, brain, kidney, lungs, heart, stomach, and adrenal) were analyzed by two-dimensional gel electrophoresis. Therefore, we consider the high levels of p68 found in some Y1 subclones (Table III) to be atypical and to have resulted from overproduction by some clones. The overproduction of p68 may be analogous to the overproduction of receptors for epidermal growth factor and nerve growth factor in human epithelial carcinoma and human melanoma cell lines (Fabricant et al., 1977). In clones which contain high levels of p68, the protein was found mainly in the soluble fraction. Nevertheless, the avidity of p68 for plasma membrane fractions (Fig. 3) is consistent with a role for p68 in regulating adenylate cyclase activity. In other systems, soluble factors have been reported to influence the responsiveness of adenylate cyclase to hormone (e.g. MacNeil et al., 1980). Although the relationship between these soluble factors and p68 is unknown, p68, unlike the soluble factors, does not influence adenylate cyclase activity when added to plasma membranes. Based on our earlier studies on the ACTH-insensitive clones Y6 and OS3 (Schimmer, 1969, 1972; Rae et al., 1979b; Schimmer et al., 1979), we suggest that p68 has a role in the transduction of the signal of ACTH binding to activation of adenylate cyclase.

Acknowledgments—We thank Jennivine Tao and Sheila Power for their excellent technical assistance; Drs. C. J. Ingles, A. Kukesia, B. G. Louis, and C. C. Yip for their helpful discussion and advice; and Drs. D. H. MacLennan and J. Logothetopoulos for their critical reviews of the manuscript.

**REFERENCES**

Alton, T. H., and Lodish, H. F. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 2016-2020

Bonner, W. M., and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83-88

Fabricant, R. N., DeLarco, J. E., and Todaro, G. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 565-569

Glynn, T., Cooper, D. M. F., and Schulster, D. (1979) Mol. Cell. Endocrinol. 12, 99-121

Haga, T., Ross, E. M., Anderson, H. J., and Gilman, A. G. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 2016-2020

Hakomori, S. I., and Kornfeld, S. (1977) Adv. Cyclic Nucleotide Res. 6, 99-136

Insel, P. A., Maguire, M. E., Gilman, A. G., Bourne, H. R., Coffino, P., and Melman, K. L. (1976) Mol. Pharmacol. 12, 1062-1069

Klaassen, U. K. (1970) Nature 227, 680-685

Laskey, R. A., and Mills, A. D. (1975) Eur. J. Biochem. 56, 335-341

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1961) J. Biol. Chem. 193, 265-275

MacNeil, S., Crawford, A., Amirrasooli, H., Johnson, C., and Cook, V. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1968-1972

MacNeil, S., Crawford, A., Amirrasooli, H., Johnson, C., Pollock, A., Ollis, C., and Tomlinson, S. (1980) Biochem. J. 188, 393-400

Ostle, B., and Mensing, R. W. (1975) J. Biol. Chem. 249, 5649-5653

Rose, B. L., Gutmann, N. S., Tsao, J., and Schimmer, B. P. (1979a) Proc. Natl. Acad. Sci. U. S. A. 79, 1990-1994

Rae, P. A., and Schimmer, B. P. (1974) J. Biol. Chem. 249, 5649-5653

Rae, P. A., Tsao, J., and Schimmer, B. P. (1979b) Can. J. Biochem. 57, 509-516
p68 and ACTH-sensitive Adenylate Cyclase Activity

Ross, E. M., and Gilman, A. G. (1980) *Annu. Rev. Biochem.* 49, 533–564

Rothfels, K. H., and Siminovitch, L. (1958) *Stain Technol.* 33, 73–77

Schimmer, B. P. (1969) *J. Cell. Physiol.* 74, 115–122

Schimmer, B. P. (1972) *J. Biol. Chem.* 247, 3134–3138

Schimmer, B. P. (1979) *Methods Enzymol.* 58, 570–574

Schimmer, B. P. (1980) *Adv. Cyclic Nucleotide Res.* 13, 181–214

Schimmer, B. P., Rae, P. A., Gutmann, N. S., Watt, V. M., and Tsao, J. (1979) *Cold Spring Harbor Conf. Cell Proliferation* 6, Book A, 281–297

Schimmer, B. P., Tsao, J., and Cheung, N. H. (1977) *Nature* 269, 162–163

Taunton, O. D., Roth, J., and Pastan, I. (1969) *J. Biol. Chem.* 244, 247–253

Wicks, W. D., Leichtling, B. H., Wimalasena, J., Wolfe, B. B., Harden, T. K., and Su, Y.-F. (1978) in *Molecular Biology and Pharmacology of Cyclic Nucleotides, Proceedings of the NATO Advanced Study Institute on Cyclic Nucleotides* (Polco, G., and Paoletti, R., eds) pp. 13–16, Elsevier, North Holland Biomedical Press, Amsterdam

Wolff, J., and Cook, G. H. (1977) *Endocrinology* 101, 1767–1775

Yasumura, Y., Buonassisi, V., and Sato, G. (1966) *Cancer Res.* 26, 529–535