Metabolically Labeled Cell Membrane Proteins in Spontaneously and in SV40 Virus Transformed Mouse Fibroblasts†

Javier M. Coll,‡ Samuel W. Luborsky, and Peter T. Mora*

ABSTRACT: A family of mouse fibroblast cell lines in exponential phase of growth were compared in protein constitution of their cell membranes. In preparations from these cells enriched in cell-surface membrane we observed one protein component (apparent molecular weight about 250 000) consistently to be reduced or absent in an SV40 virus transformed cell line, when compared with the normal cell line. No such compositional difference was observed in a spontaneously transformed tumorigenic clonal derivative cell line, or in subclones of such a derivative cell line, with or without SV40 virus infection. However, in metabolic labeling experiments with ^{14}C-labeled mixed amino acids, a consistent decrease also was demonstrated in the biosynthesis of the same protein in the SV40 virus infected subclone, as compared to an uninfected sister subclone, during exponential growth. This specific difference in biosynthesis is apparently related to the presence and functioning of the SV40 gene, and correlates with the ability of these cells to grow in viscous medium, but not with cellular tumorigenicity.

The proliferation of fibroblasts in tissue culture and the nature of the cell surface have been under intense investigation in recent years (Pardee, 1975). Altered chemical composition and organization of various membrane components have been observed and are pertinent to the growth of virus transformed cells in attachment to a substratum in culture (see review by Hynes, 1976). For example, differences were noted in a large, external, transformation-sensitive (LETS)[1] glycoprotein of high molecular weight (ca. 250 000). In virally transformed cells the LETS protein is absent, or markedly reduced at any cell density. In normal cells the LETS protein is strongly dependent on cell growth rate: it is present in especially high amount on the surfaces of cells which are arrested in G_1 (G_0) by density or by serum starvation, but in much lower amount in exponentially growing cells (Hynes and Bye, 1974; Hynes, 1974). The following study was undertaken to seek changes that may be observable by metabolic labeling in surface-membrane proteins when various cell types are compared in the optimal, exponential phase of growth.

Cell "transformation" is being used inconsistently in the literature. It often refers to virus-induced differences in cell morphology, or to cell growth individually or in large numbers, both when the cells are attached to substratum or when suspended in viscous medium. It sometimes refers to a cell which contains or expresses virus gene(s). Seldom have studies related cell-membrane changes directly to tumorigenicity in the syngeneic host (Mora, 1976). It is well known that tumorigenic transformation of cells in culture often occurs "spontaneously", and this may or may not coincide with various changes in cell-growth properties induced by viruses (Sanford et al., 1972).

In our earlier work we compared growth in culture, and also growth as tumors in the syngeneic mouse, of cells transformed either by SV40 virus, or spontaneously. It became clear that the correlation of these growth properties is complex, that certain spontaneously transformed mouse cell lines are much more tumorigenic than their SV40-transformed counterparts (McFarland et al., 1975), and that rejection of SV40-transformed cells is probably a result of new SV40-specific surface antigens which they have acquired (Smith et al., 1970; Pancake and Mora, 1976; Luborsky et al., 1976; Chang et al., 1977).

The present publication relates cell-membrane protein composition to cell growth properties in normal, nontumorigenic cells and in derivative SV40 or spontaneously transformed cells. It includes observations on a derivative spontaneously transformed cloned tumorigenic cell line, and on sister subclones with or without SV40 antigens which were obtained by a cloning procedure which was not biased by differences in growth properties in culture. These related cell lines (see Figure 1) were chosen to sort out the effects of various types of "transformations" on cell-membrane proteins, and to relate possible changes to tumorigenicity.

Experimental Section

Cell Lines. The N AL/N cells are newly established normal AL/N mouse embryo cells: 10^7 cells do not produce tumors following im injection into the syngeneic AL/N mouse (McFarland et al., 1975). They have low saturation density in monolayers when grown attached to substratum (Falcon plastic dish, 0.6×10^5 cells/cm^2) and poor growth in viscous Methocel suspension (<0.0001% colony formation) (McFarland et al., 1975).

The SV AL/N cells are AL/N mouse embryo cells transformed by SV40 virus in an early tissue-culture transfer (Takemoto et al., 1968a). The cells are nonvirus producers, are strongly positive for SV40 T antigen (T Ag) and SV40-specific surface antigen(s) (Smith and Mora, 1972; Pancake and Mora, 1974, 1976; Luborsky et al., 1976), and carry the whole SV40 virus genome, as was shown by virus rescue experiments (Takemoto et al., 1968b). The SV AL/N cells do not produce tumors at 10^8 cells/mouse, apparently due to the presence of strong SV40-specific tumor transplant antigen(s) (McFarland et al., 1975; Smith et al., 1970). The cells have high saturation density in monolayers (5.3×10^5 cells/cm^2) and good growth in viscous Methocel suspension (10% colony formation) (McFarland et al., 1975).

† From the Macromolecular Biology Section, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received July 15, 1976.*

‡ Current address: Instituto Nacional De Prevision, Clinica Puerta De Hierro, Bioquimica Experimental, San Martin de Porres, 4, Madrid-35 Spain.

[1] Abbreviations used are: LETS, large, external, transformation sensitive; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

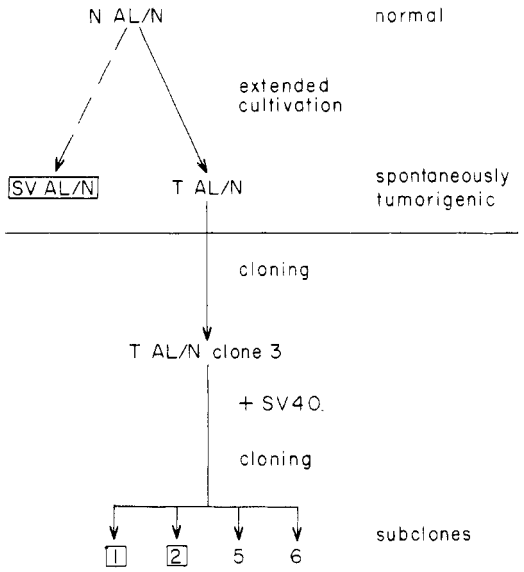

FIGURE 1: Summary of genealogic relationships and some of the properties of the cell lines. Above the horizontal bar are the mass cell lines; below are the clonal lines. Boxes indicate SV40 antigen positive cells and also ability to grow well in suspension in viscous medium.

The T AL/N cells are established cells which became tumorigenic "spontaneously" after 24 tissue-culture transfers (Mora, et al., 1969) of the same cell line from which, at an earlier tissue-culture transfer, the SV AL/N cell line was derived (Takemoto et al., 1968a). The T AL/N cells grow to a high saturation density (5.5×10^5 cells/cm^2) in monolayers, but grow only moderately well in viscous Methocel (2% colony formation) (McFarland et al., 1975). Cloning of T AL/N cells was at the 68th tissue-culture transfer, and derivatives of a single cell, designated T AL/N clone 3, were chosen for all subsequent studies. The T AL/N clone 3 cells are also strongly tumorigenic (at $\sim 10^2$ cells/mouse) and exhibit high saturation density in monolayers (3×10^5 cells/cm^2). However, colony formation in viscous Methocel suspension is low ($\lesssim 1\%$, cf. Table III). Various biochemical properties of SV AL/N and T AL/N cells, such as ganglioside composition and biosynthesis, have been published (Mora et al., 1969; Mora, 1973).

The T AL/N clone 3 cells were infected with SV40, at a multiplicity of infection (virus/cell = 300) which was expected to infect somewhat less than half of the cells, and then subclones clearly originating from single cells were obtained by "unbiased" cloning in respect to growth properties in tissue culture, employing Falcon microtest plates, and placing on the average <0.4 cells/well into each of the 96 wells. The wells were surveyed daily and clones were picked only from wells in which there was only one cell at the onset, and the cell growth was from one focus. Two SV40 T antigen positive subclones (1 and 2) were studied. The subclones 1 and 2 have a low adherence to the Falcon dish after reaching confluency, and give saturation density values of 1.0×10^5 and 0.6×10^5 cells/cm^2, respectively. Their growth in viscous Methocel is high: 20 and 10% colony formation, respectively. Both of these T antigen positive subclones are moderately tumorigenic at 10^4 cells/mouse. They possess SV40-specific surface antigens and transplantation antigens, and carry a whole SV40 genome which is rescuable by cocultivation with a permissive (CV1) cell. Two T antigen negative sister subclones (5 and 6) were also studied. These later cells have high tumorigenicity ($10^{1.5}$ and $<10^1$ cells/mouse, respectively) and high saturation density (2.4×10^5 and 3.5×10^5 cells/cm^2), but low colony formation in viscous Methocel suspension ($\ll 1\%$). These subclones 5 and 6 also showed no sign of SV40-specific surface or transplantation antigens (Mora et al., in preparation). These T antigen negative subclones thus can be considered as apparent reclones of T AL/N clone 3 cells. Figure 1 indicates the genealogic relationships of the cell lines.

All cells were grown at 37 °C either in Falcon plastic petri dishes in a humidified CO_2 incubator, or in Corning plastic roller bottles (490 cm^2 growth surface), in reinforced Eagles medium containing twice the usual amounts of amino acids and vitamins, added antibiotics, and 10% fetal calf serum, as described previously (Mora et al., 1969).

Metabolic Labeling of Cells. Cells were labeled during exponential growth at a stage when they covered less than half of the available substratum. Cells in roller bottles were washed with reinforced Eagles medium and overlayed with 20 mL of labeling medium (regular Eagles medium diluted 20-fold with "amino acid deficient" regular Eagles medium, with added glutamine and 10% fetal calf serum) which contained 5 μCi/mL of ^{14}C-labeled mixed amino acids (New England Nuclear), and were grown for 7 h at 37 °C. Labeling with 2 μCi/mL [^{3}H]glucosamine was carried out for 16 h in reinforced Eagles medium and 10% fetal calf serum, in Falcon plastic petri dishes. At the end of the labeling period, in both cases, cells were still preconfluent (for growth rates, see McFarland et al., 1975).

Cell Disruption. All further manipulations were carried out in the cold. Cells ($0.5-1 \times 10^8$) were scraped from the substratum after washing two times with TD (TD is Tris-buffered saline containing, per liter, NaCl, 8 g; KCl, 0.38 g; Na_2HPO_4, 0.1g; dextrose, 1 g; Sigma 7-9, 3.0 g; adjusted to pH 7.4 with HCl). The resulting cell pellet (0.1->0.3 mL) was resuspended (Vortex mixer) in 5 mL of hypotonic buffer (10 mM Tris-HCl, pH 8, 10 mM sodium iodoacetate (Atkinson and Summers, 1971), and 10^{-3} M phenylmethanesulfonyl fluoride), immediately centrifuged at 230*g* for 3 min, and again resuspended in the hypotonic buffer, to ensure the low (<50 mM) salt concentration necessary for complete cell disruption (cf. Atkinson and Summers, 1971). This cell suspension was sampled for radioactivity and for protein determinations, and was kept in hypotonic buffer for 40-60 min. Cell swelling was periodically monitored by phase microscopy. The swollen cells were homogenized with two to four strokes in a tight-fitting glass Dounce homogenizer (Type B pestle, Kontes Glass Co.). Extent of rupture produced was also monitored by phase microscopy.

Preparation of Membrane Fraction. To stabilize nuclei, 0.1 volume of 30 mM $MgCl_2$, 100 mM NaCl solution was added to the homogenate (producing "Mg hypotonic" buffer), and the suspension was centrifuged at 230*g* for 90 s. The supernatant solution ($\sim$5 mL), free of nuclei, was layered in an SW41 tube onto a discontinuous sucrose gradient composed of 3.5 mL of 30% (w/w) sucrose over 2.5 mL of 45% (w/w) sucrose (both prepared in Mg hypotonic buffer). The tubes were centrifuged at 7000*g* for 40 min. The band at the lower interface was carefully siphoned into a syringe (1.5-2 mL), diluted to 5 mL with Mg hypotonic buffer, layered onto another identical discontinuous sucrose gradient, and centrifuged as before, for 20 min. The band at the lower interface was collected, diluted to 5 mL with Mg hypotonic buffer, and centrifuged in an SW 50.1 tube at 10 000*g* for 20 min. The resulting membrane pellet (Atkinson and Summers, 1971) was taken up in the "final sample buffer" containing 2% sodium dodecyl sulfate and 5% β-mercaptoethanol (Laemmli, 1970),

TABLE I: Examples of Distribution of Protein and Glycoprotein into Subcellular Fractions.

	Subclone 1		Subclone 5		HeLa Cells		
Cell number × 10^7:	3.8		5.2				
Wet cell pack (mL):	0.3		0.35				
Cell protein[a] (mg):	6.3		5.8				
	Protein[a] (% mg)	Glycoprotein[b] (% cpm[c,d])	Protein[a] (% mg)	Glycoprotein[b] (% cpm[c,e])	Protein[a,h] (% mg)		Glycoprotein[b,h] (% cpm[g])
Samples from:							
Cell suspension	100	100	100	100	100[i]	100[j]	100[i]
Homogenate	88	86	73				
230*g* supernatant	71	79	68	41			54
Lower sucrose interphase							
First centrifugation	24	31	23	17		13	15
Second centrifugation	19	17	16	7	7	6	10
Membrane pellet	15	(8)[f]	10	(5)[f]	5[i]		5[i]

[a] Lowry technique, subclones 1 and 5; Cl_3AcOH precipitation of incorporated amino acid counts, HeLa cells (Atkinson and Summers, 1971). [b] Uptake of [^{3}H]GlcNH_2, described under Experimental Section, subclones 1 and 5; uptake of [^{3}H]fucose, HeLa cells (Atkinson and Summers, 1971). [c] % of total counts incorporated at each step of purification. Aliquot of suspension was dissolved in "final buffer" (Laemmli, 1970), dialyzed overnight, 5 °C, against three changes of 200 volumes 1/10 diluted "final buffer". [d] Total ^{3}H uptake: 2.38 × 10^6 cpm. [e] Total ^{3}H uptake: 8.08 × 10^6 cpm. [f] Estimated by assuming 80% nondialyzable counts, as observed in preceding three purification steps for both subclones 1 and 5. [g] Total Cl_3AcOH precipitated counts (Atkinson and Summers, 1971). [h] To sediment nuclei, centrifugal force was 31% higher than employed by us. [i] Column, from Table I, Atkinson and Summers, 1971; preparation includes extra washings of membrane pellet. [j] Column, from Table III, Atkinson, 1975, after correction for recovery of plasma membrane.

diluted appropriately with this buffer (see below) to be used for gel electrophoresis, boiled for 2-3 min, and stored at −70 °C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Slab Electrophoresis. Vertical gel slab electrophoresis was performed in a Pharmacia GE-4 gel-electrophoresis apparatus. Preparation of gel slabs and conditions of electrophoresis were as described by Laemmli (1970), with minor modifications. A 4% acrylamide stacking gel (0.5-cm long) was cast simultaneously at the top of the 8% acrylamide separating gel. Both gels contained 0.1% sodium dodecyl sulfate. The 30-μL sample solutions of membrane preparations, which contained 60-65 μg of protein, and ~10 000 cpm when radioactive (^{14}C), were mixed with ^{3}H-labeled marker proteins (see below) and placed in sample spacing slots. Electrophoresis was carried out with a current of 40 mA/gel for 2 h, sufficient for the bromphenol blue tracking dye to migrate nearly to the bottom of the gel. Gels were fixed and stained with Coomassie blue dye using the procedures of Fairbanks et al. (1971).

The protein molecular weight standards used were: skeletal muscle myosin (200 000, gift of Dr. S. Mulhern), β-galactosidase (130 000, Worthington), bovine serum albumin (67 000, Schwarz/Mann), and ovalbumin (45 000, Pharmacia). Labeling of these protein standards was carried out with [^{3}H]-dimethyl sulfate (New England Nuclear) by the general method (Smith et al., 1967) adopted for proteins (Kiehn and Holland, 1970). About 700 cpm of each ^{3}H-labeled protein was mixed into each ^{14}C-labeled cell membrane preparation before electrophoresis.

For separation of radioactive membrane components, only four positions (2, 5, 8, 11) were used in the GE-4 sample applicator guide. After electrophoresis, gel slabs were frozen in dry ice-alcohol and four wide vertical strips were cut half way between areas where the samples were applied. These frozen strips were sliced into 1-mm sections with a Bio-Rad gel slicer. To count radioactivity, each slice was treated essentially as given before (Grower and Bransome, 1970), with the following modifications. To each slice in a glass screw-top scintillation vial, a mixture of 0.6 mL of diluted tissue solubilizer (Amersham/Searle), NCS-H_2O (9:1, v/v), was added, incubated at 55 °C overnight, and cooled to room temperature. Into this solution, 15 mL of Aquasol (New England Nuclear) counting cocktail containing 0.12% acetic acid was added. Vials were stored at 4 °C in the dark for at least 24 h. Radioactivity was determined in a Packard liquid scintillation spectrophotometer. Channels were adjusted so that no ^{3}H counts were detected in the ^{14}C channel; the extent of ^{14}C count overlap in the ^{3}H channel was 15%. Samples were checked for quenching using external channel ratios; correction for background counts (~13 cpm) was made from measurements on similarly treated gel slices not containing radioactive materials.

Gel Slab Autoradiography. For these experiments, each sample put on the gel slab contained about 75 000 cpm of the membrane preparation, ^{14}C-labeled with mixed amino acids. After electrophoresis as given above, gel slabs were dried by the method of Maizel (1971) and exposed to Kodak (R. P. Royal) medical x-ray film for 7-10 days. For molecular weight markers, standard proteins were used (gifts of S. Pancake): ovalbumin, bovine serum albumin, and myoglobin (~17 000 mol wt), labeled with [^{14}C]formaldehyde (Rice and Means, 1971).

Mycoplasma Contamination. At some unknown time during the course of this work the cell lines became contaminated with *Mycoplasma hyorhinis*. Various treatment schedules with antibiotics (cf. Barile, 1973) were unsuccessful to eradicate mycoplasma completely. However, a significant reduction in mycoplasma contamination, as detected by orcein staining (Fogh and Fogh, 1969), was achieved by treatment for 1 week with 500 μg/mL Kanamycin and for 3 successive weeks with 100 μg/mL chlorotetracycline. Cell-membrane preparations from such cells, and also from independently obtained, mycoplasma-free, mouse cell lines were used for comparison in slab gel electrophoresis analyses. These included an SV40 transformed, cloned NIH Swiss 3T3 clone 5 (gift of G. Todaro) cell line.

Results

Cell Disruption and Membrane Preparation. Each cell type

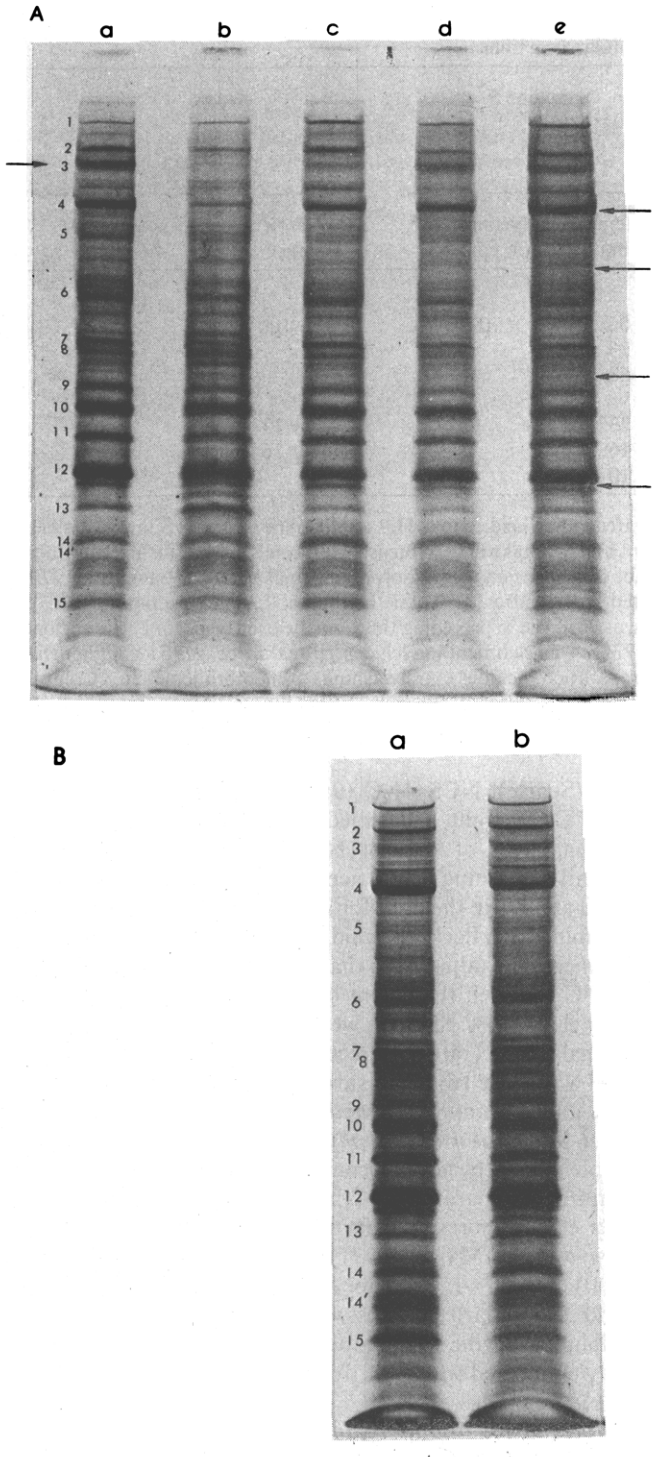

FIGURE 2: Separation of protein components of membrane preparation in sodium dodecyl sulfate–8% acrylamide gel slab electrophoresis. See Experimental Section for details. Migration from top to bottom. Coomassie blue staining. (A) Gel slab with 60 μg of membrane protein preparations from each: (a) N AL/N, (b) SV AL/N, (c) T AL/N clone 3, (d) subclone 5 (T Ag −), (e) subclone 1 (T Ag +) cells. The protein markers migrated as shown by arrows on the right side (from top to bottom): myosin (200 × 10^3), β-galactosidase (130 × 10^3), bovine serum albumin (67 × 10^3), and ovalbumin (45 × 10^3). (B) (Conditions as A): (a) subclone 2 (T Ag +), (b) subclone 6 (T Ag −) cells. Band 3 coincides with the band marked by an arrow in A, and also is in line with the position of the peak b in the radioactive preparations (see Figure 4 and Table II below).

required somewhat different conditions for swelling and homogenization. These established mouse cell lines needed extended time (40–60 min) in hypotonic buffer for sufficient swelling to occur to allow near complete (>90%) disruption of cells with the least number of strokes (2–4 strokes) in the Dounce homogenizer. If swelling time was shorter (such as 5–10 min, as in Atkinson and Summers (1971) for HeLa cells), more strokes were required, which produced undesirable extensive disruption of the membranes. Membrane fragments were recognizable by phase microscopy; large cell ghosts were observed only seldom.

The disruption of proteins into subcellular fractions was generally similar to that reported when similar surface membrane pellets were obtained from HeLa cells (Atkinson and Summers, 1971; Atkinson, 1975). The distribution into the subcellular fractions of nondialyzable label (in glycoproteins) from a carbohydrate precursor, [^{3}H]glucosamine, was also similar to that reported before (Atkinson and Summers, 1971; Atkinson, 1975) when labeling was with [^{3}H]fucose (Table I).

Pattern of Protein Components in the Membrane Pellets. Membrane pellets were prepared, solubilized in 2% sodium dodecyl sulfate and 5% mercaptoethanol, subjected to vertical gel slab electrophoresis in the sodium dodecyl sulfate–8% acrylamide gel, and the electropherograms were stained with Coomassie blue. At least 15 major bands were present which were numbered in order of migration according to molecular weight, and which covered the apparent molecular weight range of approximately 20–300 × 10^3. Many other less prominent bands were also clearly present (Figure 2). Protein patterns (position and staining intensity of the numerous bands) were generally similar in the various cell membrane preparations.

There was no reproducible difference discernible in the apparent molecular weight region below 150 × 10^3. However, minor differences appeared reproducibly in the high-molecular-weight region. In membrane preparations from SV AL/N cells, a protein band (3 in Figure 2) was much reduced (or absent), as compared to the corresponding band 3 from N AL/N cells. This protein band was detectable in T AL/N clone 3 cells, in both T antigen positive subclones 1 and 2 and negative subclones 5 and 6, but in all of these cells, in a somewhat lower amount than in N AL/N cells. Moreover, in SV AL/N cell band 3 was much reduced as compared to bands 1 and 2. An apparent molecular weight of 243 000 was estimated for the band 3 component from the best linear fit of a calibration curve employing the protein markers (not shown). Band 3 is in the imprecise region of the gel; would the upward curvature be taken into account, an even higher (>250 000) molecular weight may be estimated. A somewhat lower molecular weight component, band 4, was also present in SV AL/N cells, but in lower amount than in all the other cell lines. Although there were changes in other membrane components in the different membrane preparations, none was as reproducible as the changes in the band 3 component. This component is a glycoprotein, since it was the only one clearly stained by periodic acid–Schiff reagent treatment of the gel (data not shown).

Distribution of Metabolically Labeled Protein Components. During 7-h growth at 37 °C, 60–70% of the total added ^{14}C-labeled amino acid radioactivity was taken up by the whole (washed) cells. About 2–3% of this radioactivity was collected in the membrane pellet, generally giving a specific activity of about 10^6 cpm/mg of protein. With these membrane preparations about 10 000 cpm input was found to give the most reproducible resolution upon electrophoresis in the sodium dodecyl sulfate–8% acrylamide gel slab, after slicing and counting. The radioactivity profiles were fundamentally similar from the various cell lines (Figure 3). All the major radioactive

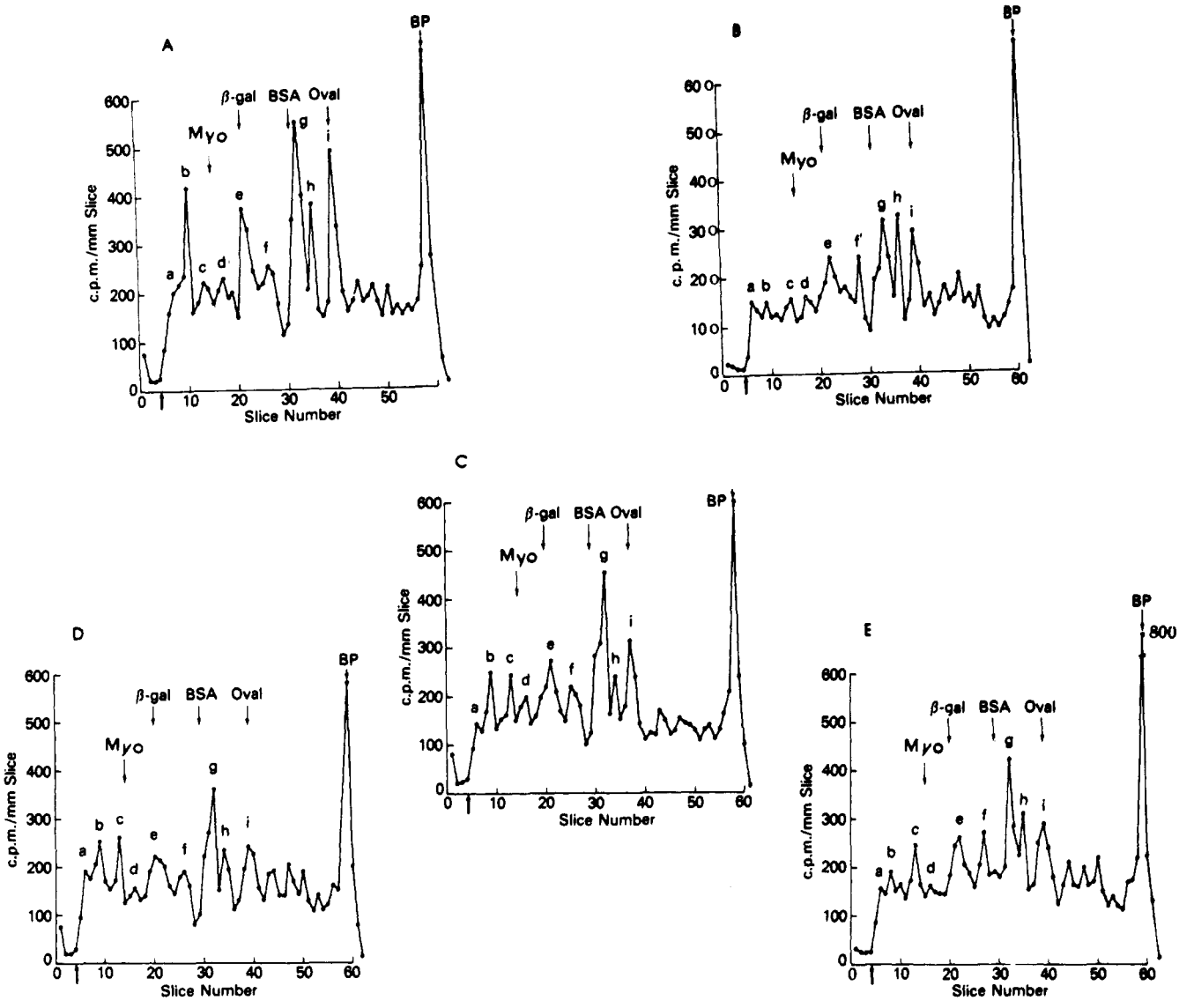

FIGURE 3: Examples of distribution of labeled membrane protein components from various cell lines after separation by sodium dodecyl sulfate-acrylamide gel slab electrophoresis. See Experimental Section for ^{14}C labeling of cells with mixed amino acids, for separation of cell membranes, for electrophoresis, and for counting of radioactivity in 1-mm gel slices. (A) N AL/N, (B) SV AL/N, (C) T AL/N clone 3, (D) subclone 5 (T Ag−), (E) subclone 1 (T Ag +) cells. Letters identify major radioactive peaks. The N AL/N cell membrane preparation is from a separate slab with high input of radioactivity (11 000 cpm); all the other membrane preparations were analyzed on a single slab with input of ~9000 cpm for each. The positions of ^{3}H-labeled marker proteins, β-galactosidase (β-gal, 130 000 daltons), bovine serum albumin (BSA, 67 000 daltons), and ovalbumin (oval, 45 000 daltons), are indicated by arrows. The single arrow below the abscissa shows the end of stacking and the beginning of separating gel. Electrophoresis and migration were from left to right. BP, bromophenol blue tracking dye. (For quantitation of separated components several such gel slab profiles were used; see Table II.)

peaks in each cell membrane preparation appeared in virtually the same slice number. These peaks were designated alphabetically in Figure 3 as judged by their relative mobility to each other and to the marker labeled proteins. They corresponded to the major protein bands detected by Coomassie blue staining and designated in numerical order in Figure 2. In Figure 3, radioactive peaks with shoulders apparently represent multiple or broad protein bands unresolved by slicing, which appeared resolved when stained. Radioactivity peaks with shoulders as well as sharp peaks without shoulders from the various cell lines generally appeared reproducibly in the same gel slice.

A single peak marked b was much reduced (or absent) in the membrane preparation from SV AL/N cells, as compared both to the other components in the same preparation and to N AL/N cells (Figure 3). The position of this peak b, which by its relative mobility represented an approximately 250 000 apparent molecular weight protein, corresponded to the position of band 3 in Figure 2. This radioactive peak b was clearly also present in membrane preparations from the parent T AL/N clone 3 cells and in T antigen negative subclone 5 cells; however, it was reduced (or absent) in preparations from T antigen positive subclone 1 cells. Other peaks at first sight also may appear to be different when comparing single radioactive profiles from different cell lines (cf. peaks g and h in Figure 3). Any difference other than in peak b, however, was found to be not reproducible (see below). No sharp peaks were resolved below about 45 000 apparent molecular weight.

To compare quantitatively radioactive components in the various peaks, independently labeled cell membrane preparations were obtained repeatedly from the various cell lines and were analyzed by procedures exemplified in Figure 3. The various peaks with shoulders as well as the sharp peaks without shoulders appeared reproducibly in the same positions. From such experiments the average percent of radioactivity and percent deviation from the mean was calculated for each peak, and is given in Table II. Comparison of results obtained re-

TABLE II: Quantitation of Radioactivity Distribution in Major Protein Peaks (% cpm Recovered[a]).

Cell	a	b	c	d[b]	e	f	f′	g	h	i
N AL/N	2.8	7.4	5.5	4.3	10.8	7.1		12.4	5.5	9.6
	(0.2)	(0.35)	(0.3)		(2.0)	(0.3)		(0.15)	(0.25)	(0.7)
T AL/N clone 3	3.3	5.2	5.4	4.9	11.4	7.1		12.6	3.7	8.4
	(0.15)	(0.1)	(1.3)		(1.2)	(0.5)		(0.5)	(0.05)	(1.1)
Subclone 5	5.0	5.6	6.0	4.2	9.2	6.4		9.9	4.9	8.3
	(0.7)	(0.4)	(0.5)		(1.6)	(0.5)		(0.6)	(0.1)	(1.0)
Subclone 1	3.7	2.8	6.0	4.1	9.5	5.8		9.2	6.0	9.2
	(0.4)	(0.1)	(0.4)		(1.0)	(0.6)		(1.3)	(0.2)	(1.1)
SV AL/N	2.6	2.3	3.4	4.9	8.5		5.0	10.5	4.4	10.5
	(0.5)	(0.3)	(0.6)		(1.3)		(0.6)	(1.1)	(0.1)	(2.1)

[a] Average calculated from three independent experiments, similar to examples in Figure 3. The numbers represent the sum of cpm within a peak expressed as % of total cpm recovered through the whole gel, including the cpm in the large peak comigrating with bromophenol blue. Numbers in parentheses represent ● percent deviation from the mean. [b] From a single experiment.

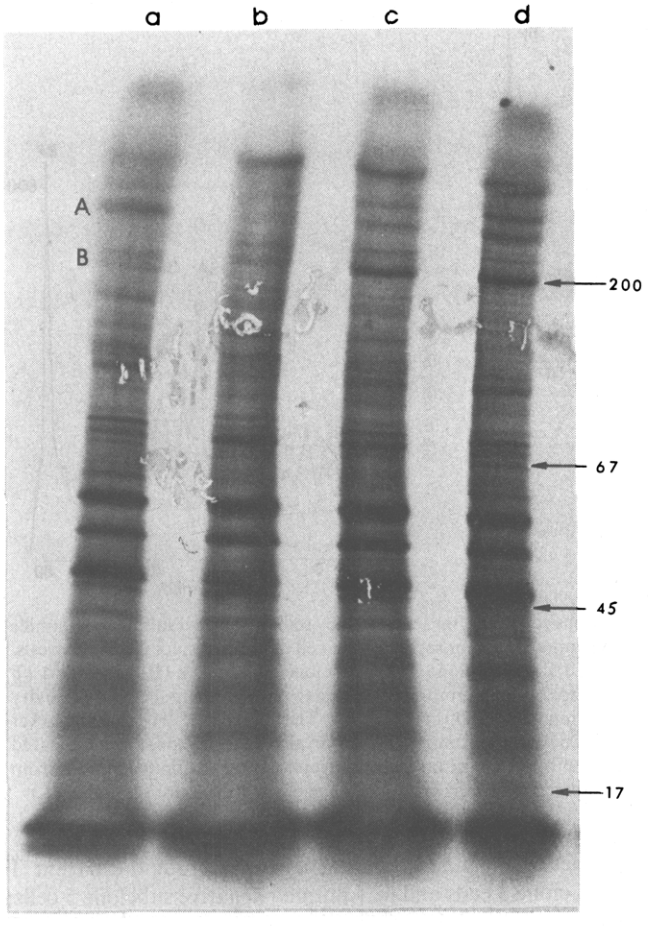

FIGURE 4: Autoradiography of gel slab separation of radioactivity in labeled membrane preparations. See Experimental Section for details. (a) N AL/N, (b) SV AL/N, (c) subclone 1, (d) subclone 5. Numbers next to arrow on right indicate molecular weights ($\times 10^{-3}$), from positions of ^{14}C-labeled marker proteins.

vealed a significant and reproducible difference only in peak b, which may be correlated with cell type. The percent of radioactive counts from two SV40-transformed cell lines, SV AL/N and T AL/N subclone 1 cells, was low (2.3 and 2.8%, respectively) in this peak, significantly lower (~50%) than that from T antigen negative N AL/N (7.4%), T AL/N clone 3 cells (5.2%) and subclone 5 cells (5.6%). Another difference in a high-molecular-weight radioactive component also may be apparent in peak c, where the amount from SV AL/N cells (3.4%) was about 40% below that from any other cells. There was no other significant difference.

Autoradiography of Gel Slabs. Labeled membrane components separated by sodium dodecyl sulfate–8% acrylamide gel slab electrophoresis were also located by autoradiography. A high-molecular-weight component was present and prominent in preparations from N AL/N cells, but was reduced or absent in membrane preparations from T antigen positive SV AL/N cells (band A in Figure 4). This component (band A) was present in the T antigen negative subclone 5 in larger amounts than in the T antigen positive subclone 1. Both of these observations completely agree with those obtained by the counting of radioactivity, given above (peak b in Figure 3, and Table II) and also are (in part) apparent in the staining patterns (Figure 2). Autoradiography also showed differences in this figure in a somewhat lower-molecular-weight region (band B in Figure 4), which corresponds to the region of peak c in Figure 3. The prominent band B was present in the autoradiogram of preparations of subclones 1 and 5, but it was much less obvious in preparations of N AL/N and SV AL/N cells. This band appeared as a doublet in preparations from N AL/N cells. It was reduced (peak c, Figure 3) in both SV AL/N and N AL/N cells, and it may have been counted as a single peak in computing values for Table II.

Mycoplasma Contamination and Control Experiments. Pulse-labeling studies were carried out on the uptake of radioactive amino acids into the various peaks. Near the completion of such studies, however, contamination by mycoplasma was detected in various cell lines. Mycoplasma contamination was detected in sucrose density gradients by the appearance of bands at 1.20 g/mL, from [^{3}H]uridine label from extracellular components from tissue-culture fluids from these cell lines. This result was subsequently confirmed and the contaminant was identified (*M. hyorhinis*; Del Giudice, personal communication). Efforts to completely eradicate mycoplasma in these cell lines by various antibiotic treatments (Barile, 1973) were not successful, although a schedule of Kanamycin followed by chlorotetracycline treatments (see Experimental Section) significantly reduced mycoplasma contamination. Since there was no assurance that membrane amino acid metabolism may not be influenced by mycoplasma (or by antibiotics used to control mycoplasma infection), the pulse-labeling studies were discontinued. However, the proteins and the differences, as presented above, were undoubtedly of cellular origin. Repeated membrane preparations from cell lines partially "cured" of mycoplasma gave distributions and patterns of protein components identical to that in Figures 2 and

TABLE III: Summary of Properties of Cell Lines.

Cell	Tumorigenicity (TD_{50})[a]	Saturation density[b]	Growth in viscous suspension[c]	Virus genome rescued	SV40 antigens		
					T	Surface[d]	Transplantation[e]
N AL/N	10^7	Low	Low	−	−	−	
SV AL/N	10^8	High	High	+	+	+	+
T AL/N	10^2	High	Intermediate		−	−	
T AL/N clone 3	10^2	High	Low		−	−	
Subclones 1 and 2	10^4	Intermediate	High	+	+	+	+
Subclones 5 and 6	10^2	High	Low		−	−	−

[a] Approximate median tumorigenic cell dose which induces lethal tumor in syngeneic AL/N mouse (cf. McFarland et al., 1975). [b] Cells grown attached to Falcon petri dish (cf. McFarland et al., 1975). [c] Ability of cells to form colony in viscous Methocel (cf. McFarland et al., 1975). [d] Ability of cells to absorb out cytolytic activity of an SV40-specific antiserum, determined by microassay (Pancake and Mora, 1974, 1976). [e] Determined by immunization and rejection experiments (cf. Chang et al., 1977).

3. Also, an independently cultivated mouse embryo cell line, NIH Swiss SV 3T3 clone 5, was used to carry out similar membrane preparations, and protein components were separated by sodium dodecyl sulfate-8% acrylamide gel slab electrophoresis. This cell line was never infected by mycoplasma (G. Todaro, personal communication). The major membrane protein components from these cells were present in relative positions and amounts (Figure 5) which were similar to those of the major protein bands observed in the various AL/N cell lines described above. Note that the amount of band 3 is much reduced compared to bands 1 and 2 from this SV40-transformed cell line.

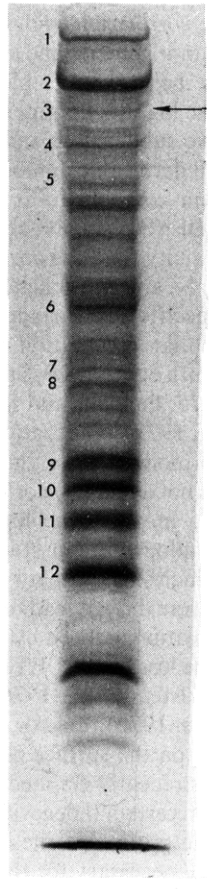

FIGURE 5: Separation of protein components from a SV40-transformed independently obtained cell line (NIH Swiss SV 3T3 clone 5), free of mycoplasma contamination. For conditions of electrophoresis and detection with Coomassie blue stain, see Experimental Section and Figure 2. Input membrane protein: 50 μg. Band 3 (peak b) position is shown by arrow.

Discussion

The most significant observation was that, in membrane preparations obtained from the various kinds of exponentially growing AL/N mouse embryo fibroblast cells, the protein components, as separated by sodium dodecyl sulfate-8% acrylamide gel slab electrophoresis, showed very similar patterns. This was so, regardless of whether the cells were normal or tumorigenic, whether transformed spontaneously or by SV40, whether from a mass cell population or from cloned cells, or whether from SV40 antigen positive or negative sister subclones derived from the cloned parental cell. A small difference was noted in the staining pattern in the high-molecular-weight region. A single major glycoprotein component of approximately 250 000 apparent molecular weight, probably the same as the LETS glycoprotein (cf. Hynes, 1976), was much reduced (or absent) in the SV AL/N mass cell line, but was present in the N AL/N mass cell line.

After metabolic labeling of exponentially growing fibroblasts with ^{14}C-labeled amino acids for 7 h, a similar change was noted, which was also detectable when contrasting the SV40 T antigen positive and negative sister subclones. Thus, metabolic labeling of exponentially growing cells for 7 h revealed a consistent decrease in biosynthesis of a specific protein (or a differential turnover) in all SV40 antigen positive cells under study, as compared to their SV40 T antigen negative counterparts.

Autoradiography of separated labeled protein components from gel slabs (Figure 4) was consistent with the radioactive counting patterns (Figure 3). In SV40 T antigen positive cells under study, as compared to SV40 T antigen negative cells, autoradiography also showed a reduction in a labeled protein component in the same high-molecular-weight region.

The *deficiency* in a single membrane protein component was observed when comparing exponentially growing cells. It appears to be a common correlate of the presence and functioning of the SV40 genome, as detected by its various antigens (see Table III). Various SV40-specific antigens, such as T (Henderson and Livingston, 1974; Livingston et al., 1974; Del Villano and Defendi, 1973), cell surface (Smith et al., 1970; Smith and Mora, 1972; Pancake and Mora, 1974, 1976; Luborsky et al., 1976), and transplantation antigens (Drapkin et al., 1974; Chang et al., 1977), have been detected, solubilized, and partially characterized by appropriate biologic assays. These antigens have a molecular weight below 100 000; obviously,

they represent a small, and by the above methods, undetectable fraction of the proteins in the cell membrane.

No correlation was discernible in exponentially growing cells between membrane protein constituents and saturation density in the cell lines investigated (Table III). Apparently, in these cell lines the ability of cells to grow to high density in monolayers in attachment is not a simple correlate of any protein change in the membranes, detectable by the techniques employed.

The ability of all SV40-transformed cells, including the clonal cell lines, to grow without anchorage in viscous Methocel suspension, as contrasted to the corresponding non-SV40-transformed cells, including the T antigen negative sister subclones, appears to correlate with the observed membrane difference in these cells. Further work is necessary before it can be concluded that the reduced amount of the high-molecular-weight (glyco-) protein constituent(s) is a general correlate of anchorage dependent cell growth.

There is no simple correlation evident in the cell lines investigated between tumorigenicity and any membrane protein changes detectable by the methods employed. Both the normal N AL/N cells, and the highly tumorigenic T AL/N Cl 3 and its T antigen negative subclones 5 and 6, have a similar, a significant, and a well detectable amount of the 250 000 molecular weight protein component. It was shown that the SV40-transformed cells (SV AL/N, and subclones 1 and 2) have lower tumorigenicity than the corresponding T antigen negative cells (T AL/N, and subclones 5 and 6, respectively), because the SV40-specific cell surface and transplantation antigen(s) cause cellular recognition and rejection in the syngeneic mouse (Smith et al., 1970; Smith and Mora, 1972; McFarland et al., 1975; Pancake and Mora, 1976; Chang et al., 1977; Mora et al., 1977). Thus, any observed difference, such as in the high-molecular-weight (ca. 250 000) cell-membrane protein, is not a (direct) correlate of acquired cellular tumorigenicity, in the geneologically related well-matched cell lines employed in our study. This is consistent with a recent study which showed that spontaneous neoplastic transformation in culture did not lead to the loss of the LETS protein, and transformation induced by chemical carcinogens or by viruses led to the loss of the LETS protein in most, but not in all, cases (Pearlstein et al., 1976; also cf. Hynes and Macpherson, in Hynes, 1976). Loss or different distribution of the LETS proteins on the surface of cells as observed by antibody staining was recently claimed to correlate with the oncogenic phenotype in certain (adenovirus-2 transformed rat) cells, but not with the portion of the adenovirus-2 genome which is thought to be necessary for the maintenance of the transformed phenotype in culture (Chen et al., 1976). Obviously, further work on geneologically related and well-matched cell lines is necessary before any general conclusion may be made.

Acknowledgments

We thank Ms. Lynda Couvillion for the T AL/N clone 3 and subclonal cells and for the biologic characterization of these cells. Dr. Chungming Chang carried out the surface antigen studies.

References

Anderson, J. L., Martin, R. G., Chang, C., Mora, P. T., and Livingston, D. M. (1977), *Virology* (in press).

Atkinson, P. H. (1975), *J. Biol. Chem. 250,* 2123-2134.

Atkinson, P. H., and Summers, D. F. (1971), *J. Biol. Chem. 246,* 5162-5175.

Barile, M. F. (1973), in Contamination in Tissue Culture, Fogh, J., Ed., New York, N.Y., Academic Press, p 131.

Chang, C., Pancake, S. J., Luborsky, S. W., and Mora, P. T. (1977), *Int. J. Cancer 19,* 258-266.

Chen, L. B., Gallimore, P. H., and McDougall, J. K. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3570-3574.

Del Villano, B. C., and Defendi, V. (1973), *Virology 51,* 34-46.

Drapkin, M. S., Appella, E., and Law, L. W. (1974), *J. Natl. Cancer Inst. 52,* 259-264.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606-2624.

Fogh, T., and Fogh, H. (1969), *Ann. N.Y. Acad. Sci. 172,* 15.

Grower, M. F., and Bransome, E. D. Jr., (1970), in The Current Status of Liquid Scintillation Counting, Bransome, E. D., Jr., Ed., New York and London, Grune and Stratton, pp 263-269.

Henderson, C., and Livingston, D. M. (1974), *Cell 3,* 65-70.

Holley, R. W. (1975), *Nature (London) 258,* 487-490.

Hynes, R. O. (1974), *Cell 1,* 147-156.

Hynes, R. O. (1976), *Biochim. Biophys. Acta 458,* 73-107.

Hynes, R. O., and Bye, J. U. (1974), *Cell 3,* 113-120.

Kiehn, D. E., and Holland, T. T. (1970), *Biochemistry 9,* 1716-1728.

Laemmli, U. K. (1970), *Nature (London) 227,* 680-685.

Livingston, D. M., Henderson, I. C., and Hudson, J. (1974), *Cold Spring Harbor Symp. Quant. Biol. 39,* 283-289.

Luborsky, S. W., Chang, C., Pancake, S. J., and Mora, P. T. (1976), *Biochem. Biophys. Res. Commun. 71,* 990-996.

Maizel, J. V., Jr. (1971), in Methods of Virology, Vol 5, Maramarosh, K., and Koprovsky, H., Ed., New York, N.Y., Academic Press, pp 171-246.

McFarland, V. W., Mora, P. T., Schultz, A., and Pancake, S. (1975), *J. Cell. Physiol. 85,* 101-111.

Mora, P. T. (1973), in Membrane Mediated Information; Biochemical Functions, Kent, P. W., Ed., Oxford and Lancaster, Medical Technical Publications, pp 65-84.

Mora, P. T. (1976), in Cell Surfaces and Malignancy, Mora, P. T., Ed., Fogarty International Monograph 24, Washington, D.C., U.S. Government Printing Office, pp 249-256.

Mora, P. T., Brady, R. O., Bradley, R. M., and McFarland, V. W. (1969), *Proc. Natl. Acad. Sci. U.S.A. 63,* 1290-1296.

Mora, P. T., Pancake, S. J., Luborsky, S. W., Chang, C., and Coll, J. (1977), *Adv. Chem. Ser.* (in press).

Pancake, S. J., and Mora, P. T. (1974), *Virology 59,* 323-327.

Pancake, S. J., and Mora, P. T. (1976), *Cancer Res. 36,* 88-94.

Pardee, A. B. (1975), *Biochim. Biophys. Acta 417,* 153-172.

Pearlstein, E., Hynes, R. O., Franks, L. M., and Hemmings, V. J. (1976), *Cancer Res. 36,* 1475-1480.

Pollack, R., Risser, R., Conlon, S., and Rifkin, D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4792-4796.

Rice, R. H., and Means, G. E. (1971), *J. Biol. Chem. 246,* 831-832.

Sanford, K. K., Handleman, S. L., Hartley, J. W., Jackson, J. L., and Ganntt, R. R. (1972), *J. Natl. Cancer Inst. 49,* 1177-1189.

Smith, K. D., Armstrong, T. L., and McCarthy, B. J. (1967), *Biochim. Biophys. Acta 142,* 323.

Smith, R. W., and Mora, P. T. (1972), *Virology 50*, 233-246.
Smith, R. W., Morganroth, J., and Mora, P. T. (1970), *Nature (London) 227*, 141-145.
Takemoto, K. K., Ting, R. C. Y., Ozer, H. L., and Fabish, P. (1968a), *J. Natl. Cancer Inst. 41*, 1401-1409.
Takemoto, K. K., Todaro, G. J., and Habel, K. (1968b), *Virology 35*, 1-8.

Response of Adrenal Tumor Cells to Adrenocorticotropin: Site of Inhibition by Cytochalasin B†

James J. Mrotek‡ and Peter F. Hall*

ABSTRACT: The ability of cytochalasin B to inhibit the steroidogenic response of mouse adrenal tumor cells (Y-1) to adrenocorticotropin (ACTH) was examined with two aims: to consider the specificity of the inhibitor and to determine at what point(s) in the steroidogenic pathway it acts. Cytochalasin B did not inhibit protein synthesis or transport of [^{3}H]-cholesterol into the cells nor did it alter total cell concentration of ATP. Together with previous evidence, this suggests that the effects of cytochalasin observed are relatively specific in these cells. Cytochalasin inhibits the increase in conversion of [^{3}H]cholesterol to 20α-[^{3}H]dihydroprogesterone (20α-hydroxypregn-4-en-3-one: a major product of the steroid pathway in Y-1 cells) produced by ACTH but does not inhibit conversion of cholesterol to pregnenolone by mitochondrial and purified enzyme preparations from Y-1 cells and bovine adrenal, respectively. Cytochalasin does not inhibit the conversion of pregnenolone to 20α-dihydroprogesterone but was shown to inhibit increased transport of [^{3}H]cholesterol to mitochondria resulting from the action of ACTH. These findings indicate that cytochalasin acts after cholesterol has entered the cells and before it is subjected to side-chain cleavage in mitochondria. In view of the known action of cytochalasin on microfilaments, it is proposed that these organelles are necessary for the transport of cholesterol to the mitochondrial cleavage enzyme and that at least one effect of ACTH (and cyclic AMP) is exerted upon this transport process. The specificity of the effects of cytochalasin is considered in relation to this conclusion.

A previous report from this laboratory demonstrated that cytochalasin B inhibits the steroidogenic response of mouse adrenal (Y-1) tumor cells to ACTH[1] (Mrotek and Hall, 1975); cytochalasin does not inhibit the basal or unstimulated production of steroids. Because cytochalasin is known to inhibit the function of microfilaments, it was proposed that these organelles may play a part in the response of Y-1 cells to ACTH. Moreover, it appeared that the inhibitor acts before the conversion of cholesterol to pregnenolone which is the step of steroid biosynthesis stimulated by trophic hormones (Hall and Young, 1968; Hall and Koritz, 1965; Karaboyas and Koritz, 1965). It was decided to study the effects of cytochalasin B on Y-1 cells from two points of view, namely, to investigate the specificity of action of cytochalasin and to determine the site(s) on the steroidogenic pathway at which inhibition of the action of ACTH is exerted by this inhibitor.

Experimental Section

Culture of Cells. Mouse adrenal tumor cells (Y-1) (American Type Culture) were grown as monolayers in plastic tissue culture dishes (60 × 15 mm) as described previously (Mrotek and Hall, 1975). Cells were allowed to reach confluence before use in these studies. The method of incubating cells with various agents such as ACTH and cytochalasin has also been reported (Mrotek and Hall, 1975). [7α-^{3}H]Cholesterol (0.5 μCi; 0.05 pmol/flask) was added to the incubation medium in 50 μL of dimethyl sulfoxide-ethanol (1:1, v/v).

Assay of Medium and Cells. Following incubation, the steroid content of the medium was measured either as total steroids using a fluorimetric assay (Callard et al., 1965) or as 20α-dihydroprogesterone using a radioimmunoassay (Maroulis and Abraham, 1975). When mitochondria and mitochondrial enzymes were examined, pregnenolone was determined by a radioimmunoassay which has been described previously (Hall et al., 1975).

Levels of ATP in cells plus medium were determined by a fluorimetric method (Means and Hall, 1968). In some studies, cells were incubated with L-[U-^{14}C]phenylalanine (0.5 μCi/10^7 cells) and incorporation of ^{14}C into protein was measured (Means and Hall, 1967).

When cells were incubated with [7α-^{3}H]cholesterol, 20α-dihydro[^{3}H]progesterone was isolated by extracting the medium three times with diethyl ether after addition of carrier 20α-dihydroprogesterone. The combined extracts were taken to dryness and applied to thin-layer chromatograms (Kowal and Fiedler, 1968). 20α-Dihydro[^{3}H]progesterone was measured by liquid scintillation spectrometry and recovery of carrier steroid was determined by A_{240}; values shown in the accompanying data are corrected for recovery which varied within the range 62-83%. The identity of 20α-dihydro[^{3}H]-progesterone was confirmed by recrystallization with authentic 20α-dihydroprogesterone. When cells were incubated with

† From the Department of Physiology, California College of Medicine, University of California, Irvine, Irvine, California 92717. *Received October 22, 1976.* Supported by National Institutes of Health Grants CA03159-02 and AM15621.

‡ Present address: Department of Biological Science, North Texas State University, Denton, Texas 76203.

[1] Abbreviations used: dibutyryl-cAMP and db-cAMP, $N^6,O^{2'}$-dibutyryladenosine 3′,5′-monophosphate; pregnenolone, 3β-hydroxypregn-5-en-20-one; 20α-dihydroprogesterone, 20α-hydroxypregn-4-en-3-one; ACTH, adrenocorticotropin.