Heritability and Control of Differentiated Function in Cultured Cells

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ABSTRACT An established tissue culture cell line which retains a differentiated function *in vitro* is described. The cell line is of connective tissue origin, and its characteristic property is the synthesis and secretion of acid mucopolysaccharides, mainly hyaluronic acid. This differentiated cell function, the activity of which depends on continuous gene action, was found to be possessed by each of eleven clonal substrains, and is therefore a genetically heritable cell character. Rate of acid mucopolysaccharide biosynthesis falls sharply under the influence of the environmental conditions existing in crowded cultures, and this rate also declines if protein synthesis is directly inhibited with puromycin. Environmental modification of a differentiated product of gene action is thus illustrated in this study.

INTRODUCTION

This report concerns the properties of a new tissue culture cell line which retains *in vitro* a differentiated cell function. The cell line has been termed ARE-2-60 and is of connective tissue origin. Its distinguishing characteristic is the synthesis and secretion of large quantities of acid mucopolysaccharides specifically characteristic of the fibroblasts of the originating tissue. ARE-2-60 is thus similar to the cultured long term cell lines recently described by Morris (1) and Daniel *et al.* (2). In the studies presented here, it was found that the differentiated function of ARE-2-60 cells is heritably transmitted from cell to cell, and that it is subject to environmental control.

Many differentiated cell properties are found to be genomically controlled. It is known for instance that genes carried by the erythroblast determine the composition of its specialized product, hemoglobin, just as the genes carried by the retinal pigment cell determine its specialized activity. The proof classically cited for genomic determination is the Mendelian pattern of inheritance with which these and other specialized characters are transmitted, thus indicating chromosomal location for their determinants. The case of hemoglobin synthesis, however, poses an important further problem. Hemoglobin synthesis has been shown to occur in mammalian reticulocytes which

have already extruded their nuclei (3), and the heritability of the hemoglobin synthesis character therefore demonstrates only that an *ultimate* genetic control exists over this differentiated cell activity. To what extent is genomic activity required for the *maintenance* as well as the initial appearance of a state of cellular specialization? In work published elsewhere (4) it has been possible to show that the histospecific biosynthetic activity of ARE-2-60 cells depends for its maintenance on continuous gene action. Evidence for this was obtained from experiments in which actinomycin D, a potent inhibitor of DNA-dependent RNA synthesis, was found to cause an immediate fall in the rate of synthesis of acid polysaccharides by ARE cells, probably due to decrease in the concentration of the enzymes synthesizing these polysaccharides (4).

The maintenance of differentiated cell function may be critically subject to immediate environmental control as well. Some of the best evidence for this has come from attempts to develop tissue culture cell lines which preserve differentiated character *in vitro*, for it has been found repeatedly, with a wide variety of different tissues, that histospecific cell function alters or disappears almost at once following explantation of specialized cells to the monolayer culture environment. The earliest methodical observations on the immediate effects of exposing differentiated cells to tissue culture environment were those of Champy, published half a century ago (5, 6). Champy showed, for instance, that certain consistent changes in the characteristic cytology of kidney tubule cells could be detected within 4 hours of explantation (5). Recent studies of histospecific enzyme distributions in freshly explanted cells have documented the findings of the early workers in this field. Thus Lieberman and Ove measured the catalase and rhodanese activities of fresh kidney cells after only 48 hours *in vitro* (7). Specific activities of these enzymes had already declined to 2 to 3 per cent of their normal levels in the originating tissue, and the relative enzyme distribution in these primary cultured cells now resembled that characteristic of four overtly undifferentiated long term cell lines of diverse origin which were simultaneously studied. Burlington (8) has reported similarly that D-amino acid oxidase and glucose-6-phosphatase, both characteristically high in kidney cells, had totally disappeared from these cells within 4 days in primary culture. In an example involving a different tissue, Ebner *et al.* (9) studied the fate of milk-synthesizing enzymes in differentiated mammary gland cells placed in monolayer culture. Lactose synthesis was found to cease within 29 hours after culturing, and UDP–galactose-4-epimerase activity was completely absent from the assayed mammary cells a few days later. The rapidity with which specialization loss occurs in these cases, the multiplicity of the specific biochemical sites affected, and the apparent uniformity of the effect over the

whole cell population clearly indicate the direct responsibility of the environmental alterations to which the cells have been subjected.

It is probable that most of the cultured cells displaying apparent loss of the overt histospecific functions characterizing their ancestral tissue nonetheless retain competent genes for those functions. Thus differentiation loss in short term cultured cells has often proved to be experimentally reversible, as it has in some long term cell lines as well. One of the classical early demonstrations of this phenomenon was that of Doljansky (10) who showed that melanin synthesis by cultured retinal pigment cells would cease after several *in vitro* transfers, but that these undifferentiated cells could be induced at will to resume massive pigment synthesis merely by altering the medium. The stimulus evoking differentiated response in a long undifferentiated cell line may be hormonal, as in the HeLa cell line of cervical epithelial origin; HeLa cells respond histospecifically to sex hormones and to adrenal cortical steroids at physiological levels (11–13). Similarly, connective tissue lines such as the L-cell line react in a connective tissue–specific way to hydrocortisone (14). Among the most interesting cases is that of collagen production by undifferentiated long term fibroblast cell lines: two different culture lines of this type have been observed suddenly to begin the synthesis of authentic extracellular collagen fibers when certain general environmental alterations were imposed, in the one case the substitution of a serum-free medium (15) and in the other the substitution of suspension for monolayer culture conditions (16). The evidence thus shows clearly that cultured cells may retain the genes which determine the histospecific cell functions of their originating tissue; despite the overtly "dedifferentiated" character of these cells there has been no permanent loss of genomic competency.

On the other hand, examples of gene loss undoubtedly exist among the established cell lines, for in the karyological shuffle accompanying the tissue culture transformation from which these lines have arisen (17), it is likely that the altered chromosome balances and the individual chromosomal aberrations typical of the heteroploid long term cultured cell would result in the effective absence of some particular genomic competency. At least one clear case has been recorded in which the differentiated function of a short term cell line disappeared at transformation. Castor *et al.* (18) have described a hyaluronic acid–secreting synovial cell line which underwent a characteristic tissue culture transformation to give rise to a heteroploid, long term cell line, and in the course of the transformation this cell line totally lost its ability to synthesize hyaluronic acid although environmental conditions had remained constant.

A long term cell line which retains differentiated function *in vitro*, then, is to be regarded *de facto* as a cell line which has preserved competent genetic

units to direct its differentiated activity (despite the karyological upheavals attending the construction of its heteroploid genome) and also as a cell line possessing environmental control mechanisms for the government of its differentiated cell function which are either neutralized or stimulated, rather than repressed, by the tissue culture environment. Both these properties are apparently possessed by ARE-2-60.

EXPERIMENTAL

Origin of the ARE-2-60 Cell Line[1]

During the winter of 1960 a series of primary cultures was initiated from the connective tissue of the stripped eye of 1 to 4 day old weanling rats. Connective tissue in general is known to contain large quantities of the acid mucopolysaccharides (AMPS) hyaluronic acid and chondroitin sulfate (19), secreted by the fibroblasts composing the tissue (20, 21). Vitreous fluids are also known to be rich in hyaluronic acids. Rat eye connective tissue was chosen for several reasons: it is highly cellular, in contrast, for example, to fully developed skeletal cartilage; it is easy to identify and to obtain in clean form; and it was found to yield uniformly better growth than did bone or skin explants under similar conditions. The tissue was minced with cataract knives on alcohol-sterilized teflon disks inserted in 60 mm Petri dishes and the fragments, 0.1 to 1.0 mm in size, were exposed to a 1.0 per cent isotonic trypsin solution for 30 minutes at 37°. At the end of this time the treated tissue fragments were centrifuged free of the trypsin solutions, suspended in growth medium, and pipetted into fresh Petri dishes. Within a day or two radial migration of fibroblastic cell types could be observed surrounding the explants, followed by evident proliferation, and when growth permitted, the new monolayer cultures were trypsinized and secondary cultures set up in 250 ml kimax culture bottles.

While this work was in progress, the detailed account by Morris (1) appeared in which the establishment of a polysaccharide-producing fibroblastic cell line from calvarial bone was described, and the growth medium subsequently used in these studies was partly based on Morris' medium "A-3". The medium used here was composed of 15 per cent fetal calf serum, 30 per cent bovine amniotic fluid, 25 per cent Puck's "saline F," and 30 per cent Puck's micromolecular constituent solution "N-16" (22) with antibiotics and cysteine added.

The secondary bottle cultures grew slowly. Sometimes a month or more would pass without subculture being required. When the cultures were about 2 months old, media exposed to the monolayers for several days were harvested

[1] This nomenclature is conventional. "ARE-2-60" denotes a culture line initiated February, 1960, and constituted of Altered Rat Eye cells.

and analyzed for acid mucopolysaccharides (AMPS) with the methods described below, and it was possible to show that the cultured cells were actively secreting AMPS.

Transformation[2]

In order for the advantages ideally obtainable from a tissue culture system to be realized, a *bona-fide* "long term" or "established" cell line is a necessity for the following reasons: (*a*) It is difficult to exclude rigorously the presence in short term cultures of persistent (*i.e.* never divided) original tissue cells. Though in the minority, these cells might conceivably be responsible for most of the tissue-characteristic functions observed in the culture. (*b*) It is common for short term cultures to be far more heterogeneous in cell type and in division rate than are established cell lines. (*c*) Short term cultures represent fundamentally unstable cell-environment systems since most of them tend either to become necrotic or to undergo transformation after several months or less. (*d*) The cells of an established cultured line have immense practical advantages, for they proliferate continuously at rates nearly as high as any known for mammalian cells, and they can be frozen, cloned, plated, or subcultured with far greater ease than their short term progenitors.

Of four independently originated short term rat eye strains (all shown to secrete AMPS) only one survived longer than 3 months, the others undergoing spontaneous necrosis. The fourth line transformed, giving rise to a culture of established cell line type. Characteristic morphological patterns of tissue culture transformation were first observed in one of the four replicate culture bottles then representing this short term strain in an examination 109 days and five transfers after its initiation. Subsequently, at 140 days, a second culture was observed to undergo transformation, but was later lost. The other two cultures became necrotic and died. When first noted the new (transformed) cell type was present in two foci of extremely heavy proliferation in an otherwise quiescent, moderately populated monolayer. The new cells were quite obviously different from their progenitors: they were less than half their size, they grew more densely on the bottle surface, and they displayed a dramatically superior adaptation to the tissue culture environment, as measured by their successful colonization and overgrowth of the culture within the following 2 weeks. This was the culture which initiated the ARE-2-60 cell line.

Besides the observation of a transformation event three other definitive

[2] The term "transformation" as here used refers specifically to the abrupt process of cultured cell alteration by which most of the established cell lines have been reported to arise. The tissue culture phenomenon thus conventionally denoted is not to be confused with the phenomenon of DNA transformation, to which it bears no known relation despite the unfortunate similarity in terminology.

characteristics of an established mammalian cell line are: (*a*) high plating efficiency; (*b*) heteroploid karyotype; (*c*) maximum mean generation time in the range of 16 to 25 hours. All these criteria were satisfied by the transformed cells.

PLATING EFFICIENCY Low known numbers of monodisperse cells were seeded into Petri dishes. The number of colonies larger than 8 cells was counted after 10 to 12 days of growth and compared to the number of cells

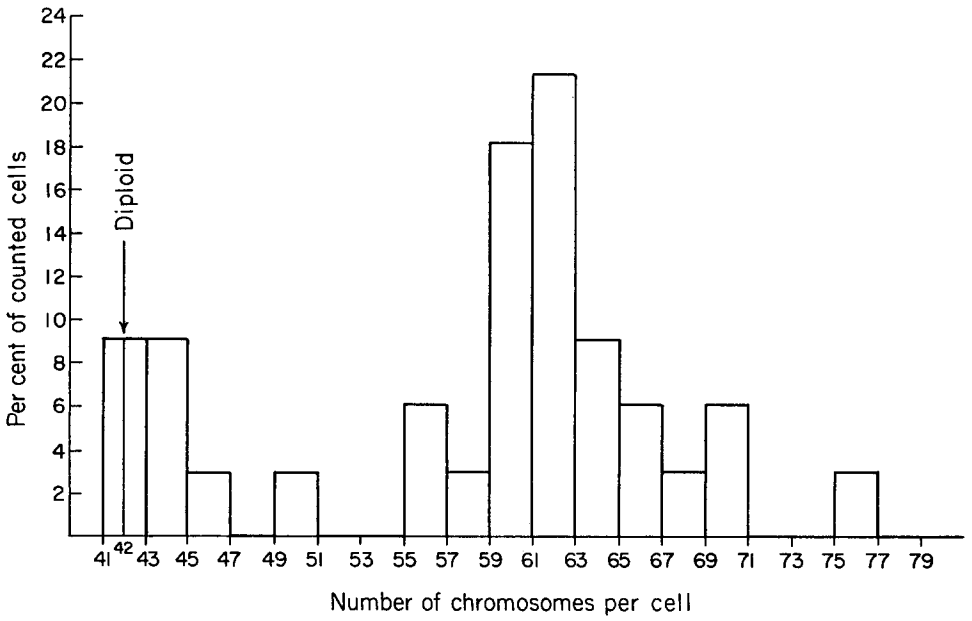

FIGURE 1. Distribution of chromosome number in ARE-2-60 cells.

planted. The ARE-2-60 cell line was found to possess a plating efficiency $\geqq 52$ per cent. By comparison, plating efficiency of the pretransformed cell type was only 1.1 per cent.

KARYOTYPE The distribution of chromosome numbers per cell in the ARE cell line was studied with a minor modification of the procedures of Hsu and Kellog (23) in which the 1.0 per cent trypsin concentrations normally required for monodispersion of these cells were used rather than the lower concentrations recommended by those authors. The results obtained are summarized in Fig. 1, which describes a typical heteroploid tissue culture population with a major peak in the subtetraploid region, a small peak (18 per cent) in the immediately hyperdiploid area, and wide scatter in the distribution as a whole.

MEAN GENERATION TIME Maximum mean generation time was found with the usual procedures to be 22.6 hours, well within the range characteristic of long term mammalian cell lines.

Polysaccharide Secretion by the ARE Cell Line

When the overlying culture medium is decanted from a monolayer of ARE-2-60 cells after several days of growth, it is immediately obvious that its viscosity has increased during its exposure to the cells. This was in fact the initial observation of Vaubel in 1933 (24), the first to note AMPS secretion by monolayer cultures of connective tissue origin; high viscosity is characteristic of the high molecular weight acid polysaccharide, hyaluronic acid.

ASSAY OF CULTURE MEDIA AMPS were routinely estimated in harvested media by a slight modification of the procedures designed by Bollet (25, 26) and modified by Morris (1) to meet the same purpose. Following a preliminary centrifugation to remove any cells present, the media were treated at room temperature overnight with equal volumes of N NaOH. The samples were then chilled, neutralized with 5 N HCl, and the protein was precipitated by the addition of cold PCA to 6 per cent final concentration. After standing at 0–2°C for 60 minutes the samples were centrifuged at 10,000 × *G* for 90 minutes. The supernatants were then dialyzed individually against cold running distilled water overnight. The next day the dialysates were treated with a 1.0 per cent protamine sulfate solution (0.40 ml of protamine sulfate solution added per original 10 ml medium volume) in order to precipitate the acid polysaccharides. Precipitates were now collected by centrifugation for 60 minutes at 10,000 × *G* in the cold. Finally the precipitates were dissolved in 2.5 ml of 2.0 M, pH 5 acetate buffer per original 10 ml of medium, and duplicate 1.0 ml samples were analyzed for hexuronic acids by the method of Dische (27) as improved by Bitter and Ewins (28). These procedures gave quantitative information on the concentration of hexuronic acids present initially in a cold acid-soluble, but macromolecular form precipitable with protamine sulfate. Recoveries of relevant amounts of hyaluronic acid and/or chondroitin sulfate added to initial blank medium samples were always in the range of 78 to 86 per cent. Corrections for use of glucuronolactone standards in the Dische carbazole procedure and for the efficiency of recovery of added AMPS yielded absolute values for hexuronic acids present in the culture media.

THE NATURE OF THE POLYSACCHARIDES SECRETED It would be expected that the major AMPS component synthesized by the ARE cell line would be hyaluronic acid, since this is the experience of those who have studied the spectrum of polysaccharides secreted by other cultured cell systems (1, 29–31). Direct verification of hyaluronic acid synthesis and secretion was obtained

in an experiment in which medium containing 2.5 μc *N*-acetylglucosamine-1-C^{14}/ml was added to established, growing, cultures. The medium was harvested after 2 days and after alkaline extraction, deproteinization, and dialysis as above, the supernatant was lyophilized and redissolved in a solution containing 2.5 mg carrier hyaluronic acid/ml. Paper electrophoresis at 33 v/cm on acid alcohol–washed, Whatman 3 mm paper, in 0.01 N pH5 acetate buffer, then followed. In 9 hours it was possible to achieve separation of the toluidine blue metachromatic marker spot from insoluble material at the

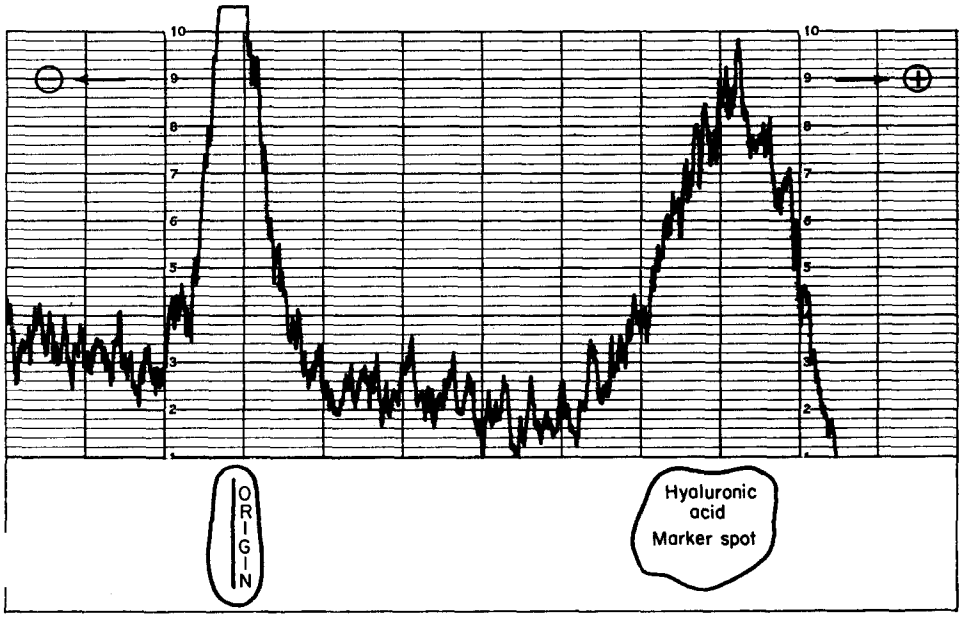

FIGURE 2. Identification of hyaluronic acid in an AMPS-containing extract from medium exposed to ARE-2-60 cells for 2 days. The medium initially contained the AMPS precursor *N*-acetylglucosamine-1-C^{14}, and the trace represents the distribution of radioactivity along the paper electrophoresis strip here superimposed on it. See text for details.

origin (Fig. 2). The paper was then scanned in a gas-flow windowless strip scanner and as can be seen in Fig. 2 the only activity peak moving toward the anode corresponds in position to the hyaluronic acid marker.

In another type of experiment details of which are given below, *N*-acetylglucosamine-1-C^{14} was supplied to the cells as a precursor utilized for the synthesis of cell-bound AMPS. UDP-*N*-acetylglucosamine has been shown to be incorporated directly into hyaluronic acid in studies of Dorfman *et al.* (32). It was found here that ARE cells incorporated this precursor in a form rendered insoluble in water, weak acids, and phosphate buffers after fixation with the AMPS precipitant cetylpyridinium chloride. Subsequent treatment

with 1.0 per cent testicular hyaluronidase (in pH 6, M/10 phosphate buffer for 30 minutes at 37°C) released the incorporated activity into a soluble form. The specificity of this enzyme preparation is such that it does not differentiate effectively between chondroitin sulfates A and C and hyaluronic acid; the experiment shows only that one or all of these closely related connective tissue products were being synthesized. Although data concerning the total spectrum of AMPS species synthesized by ARE-2-60 cells were thus not obtained, it is clear that the specific connective tissue polysaccharide, hyaluronic acid, is being synthesized by these cells and it is probable that this is their major or their sole product.

TABLE I

TOTAL AMPS PRODUCTION BY CULTURES OF VARYING CELL NUMBER*

Cell No. (10^6 cells)	No. of samples	Average μg AMPS/ sample	Average $\mu\mu$g AMPS secreted/cell
1–2	3	55.6	37.0
2–3	13	68.8	27.5
3–4	19	63.5	18.1
4–5	11	65.3	14.5
5–6	9	77.3	14.1
6–7	8	77.8	12.0
7–8	5	59.2	7.9

* Media exposed to cultures for 5 days were analyzed for total AMPS as described in text. All values shown are corrected for the native AMPS content of the media before exposure to ARE cells.

AMOUNT OF AMPS SYNTHESIZED The rate of synthesis for export of AMPS by these cells was found to vary under certain conditions over an approximately 12-fold range. At the maximum synthesis rates measured for the uncloned stock ARE cell line, 18.3×10^{-12} gm of AMPS (calculated as hyaluronic acid) is synthesized per average cell per day. This cell contains about 242×10^{-12} gm of protein as calculated from measurements by the method of Lowry *et al.* (33) applied to the cold TCA precipitates of counted, washed, monodisperse cells. Such a cell may thus secrete daily an amount of AMPS equal to 8 or 9 per cent of its total weight of protein (no measurements were made of the relative amounts of AMPS remaining in the cytoplasm).

External Control of AMPS Synthesis Rate

In order to obtain an idea of the amount of variation in AMPS synthesis rates medium samples which had overlain ARE cells for 5 days were collected over a 6 month period and analyzed. Cell numbers at the end of the 5 day growth period were obtained with hemocytometer counts. The cultures

had been initiated in the course of stock transfers with known, randomly varying inocula ranging from 0.4×10^6 to 2.0×10^6 cells per bottle. Data obtained from total AMPS assay in these media are presented in Table I. Total AMPS synthesis is evidently about the same per culture per unit time over an eightfold range of cell numbers, which is to say that it declines sharply on a per cell basis for cells in higher cell populations (Table I). These results imply that AMPS synthesis rate per cell is adversely sensitive to the environmental conditions developing in crowded cultures, conditions which also inhibit rate of cell proliferation. Experiments were next carried out to determine more directly the relation among crowding environment, growth rate, and AMPS synthesis rate per cell.

TABLE II

RELATION BETWEEN RATE OF AMPS SECRETION AND RATE OF GROWTH*

Time interval	μG AMPS synthesized in time interval per culture	Initial cell No. (N_o) (10^6 cells)	Final cell No. (N_t) (10^6 cells)	MGT	AMPS synthesis rate (10^{-12} gm/cell hr.)
days				*hrs.*	
0–1	25.24	0.92	1.97	22.6	7.62
1–2	3.43	1.97	3.04	29.5	0.58
2–3	1.98	3.04	3.77	38.5	0.24

* Each value represents the mean of determinations on eight replicate cultures. Rate of synthesis of AMPS per cell per hour was calculated from the expression

$$R = \frac{(\mu g \text{ AMPS in time interval}) \ln N_t/N_o}{t(N_t - N_o)}$$

Replicate cultures of 1×10^6 cells/bottle were set up and growth permitted to proceed for 1, 2, or 3 days. The initial rate of exponential growth is not maintained by ARE cells after they have reached a population of approximately 2×10^6 cells/bottle, when visible crowding sets in on some areas of the bottle surface, though the cell populations continue to expand and frequently attain eventual levels of 6 to 10×10^6 cells/bottle. Cell counts were obtained at the end of the 1, 2, or 3 day growth periods mentioned, and the media were collected and analyzed for total AMPS hexuronic acids. Approximate mean generation times (MGT) and rates of per cell synthesis of AMPS could then be calculated for the time intervals 0 to 24, 24 to 48, and 48 to 72 hours, assuming exponential pattern of growth (though at slower rates) in the 24 to 48, and 48 to 72 hour periods after seeding. The results are presented in Table II. The data demonstrate a control mechanism governing the rate of AMPS synthesis which is extremely sensitive to relatively slight environmental alterations. Thus for a slight increase in the ap-

proximate mean generation time from 22.6 to 29.5 hours there has been a collapse of per cell AMPS synthetic activity to less than 10 per cent of the initial value. This experiment, as well as that of Table I, suggested further that active AMPS synthesis depends on the maintenance of a maximum rate of protein synthesis and that possibility was now investigated directly with the aid of the specific protein synthesis inhibitor, puromycin.

PROTEIN SYNTHESIS AND THE AMPS SYNTHESIS RATE For this experiment the lowest possible levels of puromycin were utilized, so that any effect found might be attributable to the specific action of this agent rather than to secondary cell damage. Replicate cultures were set up at 1×10^6 cells per bottle, and after the first 24 hours had passed (no cell division occurs during this time) the medium was changed to one which contained puromycin. After a second 24 hour period the cells were harvested, counted in quadrupli-

TABLE III

EFFECT OF PUROMYCIN ON RATE OF AMPS SECRETION*

μG puromycin/ml	Per cent initial cell No. after 24 hrs.' growth	Cellular AMPS synthesis rate (10^{-4} gm AMPS/gm protein hr.)	Per cent control rate of AMPS synthesis
Control	218	20.5	100
0.5	151	1.45	7.1
2.0	131	3.64	17.8
4.0	131	3.47	16.9
4.5	50	0.00	0.0

* Each value represents the mean of determinations on four replicate cultures.

cate, and their media analyzed for total AMPS. In the 24 to 48 hour interval control cells increased in number 218 per cent on the average, as shown in Table III, and puromycin-treated cells either increased to a lesser extent or began to die, *i.e.* fall off the monolayer surface (cell death was monitored by counts of cell number in samples of the overlying medium). Increases in cell number given in Table III for the lower doses of puromycin may represent only the completion of cell divisions already in late stages of preparation at the time of puromycin addition. Whether or not this is the case, it is evident in Table III that even at those doses where puromycin inhibition of protein synthesis was so mild that some net cell increase did occur, AMPS synthesis rates suffered an 80 to 90 per cent decline. The highest puromycin dose tested was 4.5 μg/ml, the 50 per cent lethality level. It was found that uptake of 0.25 μc C^{14}-L-tyrosine/ml (final specific activity 5.2 μc/mg medium tyrosine) into the total protein of cells surviving 24 hours' exposure to this concentration of puromycin was still 55 per cent of the control value. Specific

activity of total cell protein was obtained by counting in a low background, gas-flow counter, diluted, dried aliquots of the same labeled protein solutions as were used for estimations of total cell protein by the Lowry procedure (32). Total protein of surviving puromycin-treated cells had an activity of 45 CPM/μg protein while that of control cells was 82 CPM/μg protein. It can be concluded from the experiment of Table III that AMPS synthesis by ARE-2-60 cells is more sensitive to low levels of puromycin than is either growth or total protein synthesis.

Since the rate of AMPS synthesis apparently depends on the amount of new protein being made, it is possible that the rapid formation of AMPS-synthesizing enzymes is required to maintain a high rate of AMPS production. This explanation implies the rapid turnover of these enzymes. Turnover of total ARE cell protein was measured, under the conditions of rapid growth found to stimulate optimal AMPS synthesis. The method used was taken from Eagle *et al.* (34), and depends on a comparison between the specific activity of the new net protein synthesized by growing cells from a labeled precursor, and the specific activity of that precursor in the culture medium. In these experiments tyrosine was selected as the protein precursor. The tyrosine content of the cold acid-soluble fraction of the undefined growth medium was estimated with an adaptation of the Folin-Ciocalteau reaction, yielding a value of 42 μg free tyrosine/ml medium. To this medium, high specific activity C^{14}-tyrosine (New England Nuclear) was added to a final specific activity of 3115 CPM/μg tyrosine. A logarithmically growing population of cells of known size was exposed to the isotope-containing medium for 24 hours and then harvested. The increase in cell number, obtained from hemocytometer counts, provided an exact index of net protein increase based on the measured value of 242 μg protein/10^6 cells. From the specific activity, the amount of newly synthesized cell protein was calculated to total 898 μg, though the amount of net protein increase actually measured was 689 μg. Considering the exponential rate of growth, the excess protein synthesized represents a rate of turnover of 0.9 per cent total protein/hour. This result is consistent with the idea that under optimal conditions, the AMPS-synthesizing machinery turns over rapidly, and turnover rates for some individual proteins undoubtedly exceed the total protein turnover rates measured here.

Clonal Inheritance of the Property of AMPS Synthesis

The "broken coverslip" technique was used to initiate eleven separate clonal strains of ARE-2-60 cells. Small fragments of standard Corning No. 1 coverslips were placed in 60 mm Petri dishes and covered with a cell suspension containing about 100 totally monodisperse cells. About a week to 10 days after plating the Petri dishes were inverted and the adhered coverslip frag-

ments viewed in a darkfield dissecting microscope under bright direct light at × 90. Fragments bearing single colonies were then transferred to individual Petri dishes. Eventually the clone would overgrow onto the surface of its new container. When the clonal populations had attained sufficient density they were trypsinized and seeded into culture bottles. The whole procedure was then repeated, in order to preclude the possibility that the final clone might actually stem from an accidental clump of two or more cells. With the dispersion and examination procedure used such clumps were never seen in the preparative inocula, but only by cloning twice is it possible to be confident of the single cell origin of the derived clones. The clones eventually obtained were tested for the secretion of AMPS into the medium. Each clone was found to possess this property, despite differences in gross growth pattern and in individual cell size and appearance. An experiment

TABLE IV

CLONAL SYNTHESIS OF CELL-BOUND AMPS*

Clone	Total AMPS CPM/ 10^6 cells	Per cent of total counts as AMPS	μG protein/ 10^6 cells	AMPS CPM/ mg protein
A	106	57	373	284
B	101	54	228	443
C	107	49	552	194
D	126	63	250	504

* The values given for total AMPS CPM were obtained as hyaluronidase-removed CPM averaged from four coverslips counted per clone. Counts which remained after the hyaluronidase treatment are believed to have been incorporated in protein since these counts, unlike AMPS counts (4), are abolished without any time lag in cells exposed to high doses of puromycin.

quantitatively evaluating synthesis of cell-bound AMPS by four of these clones was next performed, as follows: 7×10^5 cells were planted on 22 mm square Corning No. 1 coverslips inserted individually in small sealable Petri dishes. After 12 to 16 hours, during which 99 per cent or more of the cells attach to the glass substratum (no cell divisions are observed within this period) the coverslips were transferred to medium containing 5 μc *N*-acetyl-glucosamine-1-C^{14}/ml. Incubation with this label was allowed to proceed for 60 minutes. The coverslips were then removed, rinsed in three large volumes of warm saline G (22) for 1 minute, 1 minute, and 5 minutes respectively, and then fixed for 40 minutes in 0.75 per cent cetylpyridinium chloride in 90 per cent absolute ethanol–10 per cent formalin. The fixative was arrived at empirically, starting with one suggested by Williams and Jackson, who were the first to use the AMPS precipitant cetylpyridinium chloride for histological purposes (35). After fixation the coverslips were rinsed in water, dried at 60°, and then counted directly in the low-background counter (36).

Since only 200 to 400 μg of protein are present in 7×10^5 ARE cells, self-absorption correction per coverslip is negligible. Above 8×10^5 cells/coverslip, counted radioactivity was not linear with cell number, probably due to piling up of the cells when planted. After counting, the coverslips were recovered and exposed to 1.0 per cent testicular hyaluronidase in M/10, pH 6, Na/K phosphate buffer for 30 minutes at 37°. They were then rinsed in water, dried, and recounted. Since it can be shown that almost no counts are lost from coverslips repeatedly incubated at this stage in the buffer alone, the counts lost from the cell sheet in the hyaluronidase solution can be regarded as newly synthesized AMPS. In Table IV the results of a comparative experiment with four clones are shown: although there are some differences in cell-bound AMPS production on a specific activity basis among these clones, production per cell is about the same. It is to be noted that there is no quantitative correlation between the amount of synthesis of cell-bound AMPS and the amount of AMPS secretion by cells of these clones. Thus, while cells of clone D can be shown to secrete over twice as much AMPS per unit time as do cells of the ARE stock cell line or cells of other clones including clone B, cells of the latter clone apparently synthesize cell-bound AMPS at a rate nearly equal to that measured for clone D in the experiment of Table IV.

DISCUSSION

The demonstration that clonal strains of ARE-2-60 synthesize AMPS proves that this differentiated character is heritably transmitted from cell to cell. Since AMPS synthesis is actinomycin-sensitive (4), this specialized cell function can be considered to be DNA-directed. The ability to synthesize AMPS therefore represents a genomic specialization, in this case one inherited from the remotely distant period when the histogenesis of the ancestral connective tissue occurred. The fact that all eleven of the clones tested possess the overt AMPS synthesis character suggests that it is present in virtually all ARE cells.

The ARE cell, then, possesses an inherited, actively functioning DNA locus responsible for the maintenance of AMPS synthesis. This synthesis is also subject to sensitive control by environmental factors. It is well known that rates of protein synthesis decline in crowded monolayer cultures, and it was found here that crowding stops AMPS synthesis in ARE cells. Since direct interference with protein synthesis also was found to inhibit AMPS synthesis (Table III), it is possible to interpret the effects of crowding on the rate of AMPS synthesis as the result of a requirement for a high rate of synthesis (and turnover) of the protein units composing the AMPS production machinery. However, environmental factors may in actuality affect AMPS

synthesis independently of protein synthesis. In either case it is clear that the rate of AMPS synthesis, the functional product of a particular genomic activity, can be modified externally.

The mechanisms by which such response to external conditions operates remain a matter of speculation. Whether the primary step in the control of a responsive cellular process such as AMPS synthesis is genomic or is essentially local and non-genomic, is unknown. If DNA-dependent RNA synthesis in ARE cells is stopped, an initial decline in the rate of AMPS synthesis is detectable within 20 minutes, and by 8 hours AMPS synthesis rate has fallen to half its initial value (4); evidently AMPS synthesis is dependent on continuous gene action in these cells. It is therefore possible to envision a control system which governs the rate of AMPS synthesis by means of varying the intensity of primary gene action; *i.e.*, the rate with which DNA-dependent RNA molecules are produced. One candidate for the role of an *in situ* regulator of the intensity of gene action is chromosomal histone, which has been shown experimentally to repress DNA-dependent RNA synthesis (37, 38). Among the attractive elements of a scheme proposing histones for this function is an observation suggesting that histone distribution responds to pertinent alterations in extracellular conditions: Allfrey *et al.* have shown that *in situ* turnover rates for the histones of pancreatic acinar cells are immediately affected by feeding, which is a stimulus specifically inducing enhanced enzyme secretion by these cells (39). Elucidation of the mechanisms by which gene action in differentiated cells is affected by external events constitutes a most important objective in the attempt to understand the functional mechanics of the differentiated state.

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