GENE EXPRESSION IN DIFFERENTIATED CELLS

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This communication is concerned with the dynamics of gene control over specific biochemical functions in a differentiated cell. Direct experimental access to the intracellular link between gene action and the target cell characters has become feasible with the accumulation of evidence demonstrating that the synthesis of specific RNA's on the genomic DNA template is the actual primary mechanism of gene function in the cell and with the development of the highly effective inhibitor of that synthetic reaction, actinomycin D. In the experiments to be presented, this agent was used to block nuclear RNA synthesis in a cultured mammalian cell type which has retained *in vitro* a differentiated cellular activity. The effect of actinomycin treatment on this cell function and on a specific mitochondrial enzyme activity was then followed. Actinomycin treatment was found to inhibit the specific cell function immediately, while, in contrast, the mitochondrial enzyme activity appeared to remain impervious to the effects of this agent for long periods.

The cell line whose development made possible these studies is of connective tissue origin. Termed ARE-2-60 and described in detail elsewhere,[1] its differentiated property consists of the continuous synthesis and secretion into the culture medium of acid mucopolysaccharides characteristic of connective tissue. The major advantages of experimental material of this nature are twofold: it permits study of cellular differentiation as a genetically inherited or genomically specified cell property, and it provides for the expression of such properties an environment which is devoid of complex (i.e., variable) tissue-tissue interactions. Since the cells grow in a monolayer on a glass substratum, even cell-cell interactions are strikingly reduced as compared to the case in normal tissue, organ culture, or artificial cell aggregates.[2]

Differentiated cellular properties are determined ultimately by information carried in the cell genome. The well-known Mendelian transmission of variant hemoglobin forms serves as a classic illustration of this fact, for hemoglobin is a protein whose mass production constitutes the end-product of a highly specialized cell, the reticulocyte. The particular question to which this investigation is directed is the degree of immediate control exercised by the active genes in another differentiated system. Does a specific cytoplasmic function such as the secretion of some histospecific cell product proceed independently once the synthesizing machinery is set up, or is continuous gene action a requisite for the maintenance of differentiated activity?

*Origin and Characterization of the ARE-2-60 Cell Line.**—The cultured cell line used in these experiments originated three years ago as one of a series of primary monolayer cultures arising from explanted fragments of 1–4 day old weanling rat eye connective tissue. All details of the procedures by which the cultures were initiated and handled are given elsewhere.[1]

The ARE cell line behaves as a typical permanent cell line with respect to growth rate (mean generation time is 22.6 hr), plating efficiency ($\geqslant 52\%$), and karyotype (heteroploid, modal chromosome number near $3n$). All ARE cells are descended from one culture in which a characteristic tissue culture transformation event was observed to occur about four months after explantation of the primary fragments. The useful property of the ARE line, AMPS secretion, was detected

before transformation and has been maintained without quantitative variation since. Eleven clonal strains have been obtained,[1] all of which possess this histospecific property, thus clearly demonstrating the inheritance of a specialization initially set up during the histogenesis of the ancestral tissue of origin.

Connective tissue fibroblasts are known to secrete large quantities of hyaluronic acid and chondroitin sulfates.[3, 4] The major AMPS product synthesized and secreted by the ARE cell line appears to be hyaluronic acid, though other AMPS are not excluded.[1] In this respect, ARE-2-60 resembles fibroblast cell lines described by C. C. Morris[5] and also by Daniel *et al.*[6] In the experiments which are now to be presented, it was cell-bound rather than secreted AMPS synthesis which was measured. Details of the procedures employed are given in reference 1; in brief, ARE cells were planted on glass coverslips, exposed to N-acetylglucosamine-1-C^{14}, and fixed, and the radioactivity in the cells was counted directly in a low-background gas-flow counter.[7] The coverslips were then exposed to a solution of testicular hyaluronidase and recounted. Counts lost from the fixed cells during the hyaluronidase treatment can be considered to represent newly synthesized AMPS.[1]

In monitoring ARE cell function under various conditions, it was found that the per cell rate of AMPS synthesis was exceedingly sensitive to conditions affecting the general rate of protein synthesis. Thus, in experiments utilizing low doses of the protein synthesis inhibitor puromycin, it could be shown that even at levels of puromycin inhibition so mild that some net cell increase occurred during the 24-hr period of the experiment, AMPS synthesis and secretion were inhibited 80–90 per cent. This result set the stage for the experiments which now follow, for it suggested the possibility that in this continuously growing cell type active nuclear mediation might be required for the maintenance of the rate of protein synthesis on which the AMPS production system depends.

Effect of Actinomycin D on Synthesis of RNA, Protein, and Cell-Bound AMPS.— It is known that actinomycin functions by suppressing RNA-DNA polymerase activity,[8] apparently by complexing specifically and firmly with the DNA primer,[9, 10] and it has been shown in other tissue culture systems that actinomycin inhibits almost completely the synthesis of all cell RNA.[11, 12] The small amount of RNA synthesized in cells 95 per cent inhibited by actinomycin treatment does not resemble cell DNA in base composition[8] and in fact appears to consist mainly of s-RNA.[11] The irreversible nature of actinomycin action makes it possible to consider cells which have been exposed to the drug and then transferred to actinomycin-free medium as "chemically enucleated" cells.

In Figure 1 are presented the pooled results of several experiments in which RNA synthesis and AMPS synthesis were measured simultaneously in replicate coverslip preparations incubated for one hr with the respective C^{14} precursors at given intervals following a 20-min actinomycin treatment. The result of the experiments summarized in Figure 1 is clearcut: when 97 per cent of all cell RNA synthesis is blocked by actinomycin D, the rate of AMPS synthesis begins to decline immediately, without appreciable lag, and it continues to decline as long as it is followed. It is evident that in order for this differentiated trait to be expressed, *continuous nuclear activity* is required in the form of DNA-dependent RNA synthesis. This system is therefore characterized by immediate nuclear control. Parallel experiments were conducted to measure the uptake of C^{14}-L-leucine into protein at various intervals after actinomycin treatment. It was found that rate of total protein synthesis (CPM/coverslip/one-hr incubation) declined at essentially the same rate as does AMPS synthesis (Fig. 2). Franklin and Baltimore have published essentially

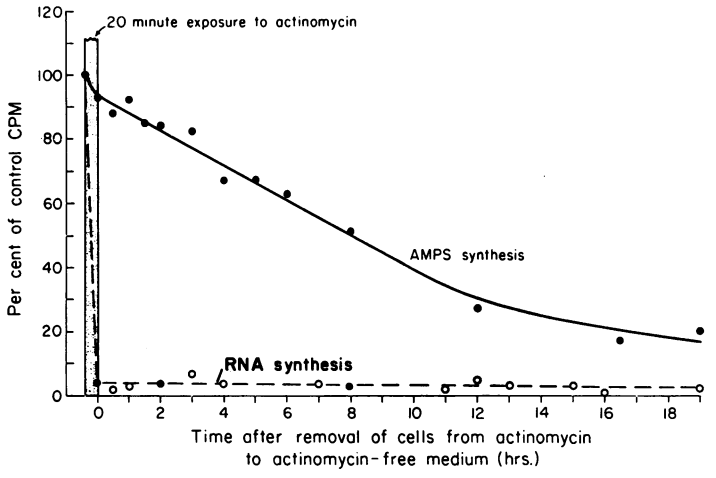

FIG. 1.—Effect of actinomycin D on synthesis of RNA and cell-bound AMPS. After exposure to actinomycin D for 20 min, coverslip cultures were transferred to actinomycin-free medium where they remained for the periods of time given along the abscissa. They were then incubated with C^{14}-N-acetylglucosamine or C^{14}-orotic acid for one hr, rinsed, fixed, and counted as described in text. Heavy black dots represent an average of three or four independent determinations, each in duplicate; open circles represent single determinations.

similar data showing the effects of actinomycin D on total protein synthesis in L-cells.[12]

In these experiments, isotopic labeling was carried out in actinomycin-free medium containing 5 μC/ml C^{14}-orotic acid (for RNA), 1 μC/ml C^{14}-L-leucine (for protein), or 5–10 μC/ml N-acetylglucosamine-1-C^{14} (for AMPS). Ninety-nine per cent of the counts incorporated from orotic acid were removable by incubation in 0.1 per cent crystalline RNAase. Protein samples were fixed in cold TCA in the same way as RNA samples. Twelve to sixteen hr after planting (7×10^5 cells/coverslip), the cells adhering to the coverslip surface were exposed for 20 min to actinomycin D, at a final concentration of 5 μg/ml, then rinsed free of the agent and transferred to clean medium. No cell division occurs in the period between planting and exposure to actinomycin.

Protein Turnover and Cell-Bound AMPS Synthesis Rate.—The effect of actinomycin on AMPS synthesis could be due to its interference with protein synthesis, in particular to interference with the synthesis of the enzymes which construct the AMPS polymers (or their components). If this were the case, then a parallel experiment to those of Figure 1, but where protein synthesis itself (rather than RNA synthesis) is blocked, should cause a sharper fall in AMPS synthesis rates than was found in the actinomycin-treated cells. Such was in fact the result (Fig. 2) when the effect of 50 μg puromycin/ml on AMPS synthesis was tested. At this dose, over 96 per cent of the normal protein synthesis was obliterated. Since puromycin inhibition is not irreversible, the 50 μg/ml concentration was maintained in the isotope-containing media. AMPS synthesis in the one-hr incubation periods with N-acetylglucosamine-1-C^{14} at the given time intervals after the start of puromycin treatment drops about five times faster than it does if RNA synthesis is blocked instead. The short rise in AMPS synthesis at 15 min and 30 min above the control values (Fig. 2) is not understood.

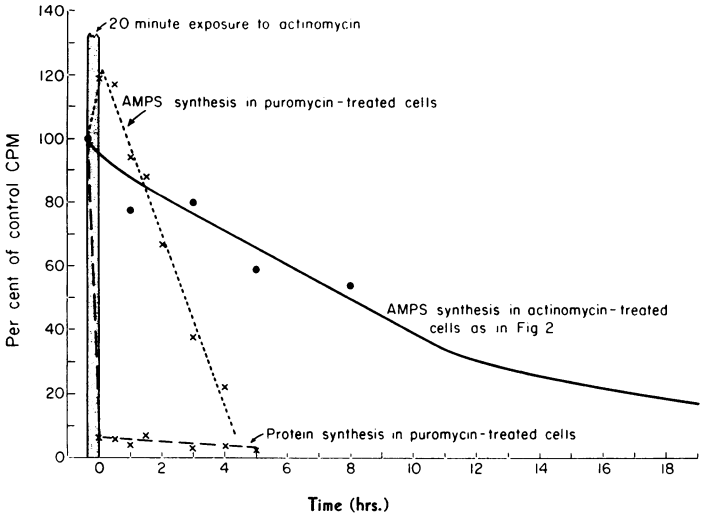

FIG. 2.—Comparison between the effect of actinomycin and the effect of puromycin on cell-bound AMPS synthesis. Heavy black dots represent the relative uptake of C^{14}-leucine into cell protein at various times following 20-min exposure to actinomycin. AMPS synthesis and protein synthesis in puromycin-treated cells were measured as described in the text from uptake of N-acetylglucosamine-1-C^{14} and C^{14}-leucine after various periods of exposure to 50 μg/ml puromycin.

It would thus appear that the turnover of one or several key enzymes constituting the AMPS synthesizing apparatus is so high that it must be continuously resynthesized to maintain the normal AMPS synthesis rate. This is in line with the finding that total protein turnover even in these rapidly growing cells is very high. At the rate of protein turnover characteristic of ARE cells,[1] half the protein present at any given time would have been replaced within three days, despite the rapid accretion of new protein occurring simultaneously as a function of the characteristically high rate of cell proliferation. Using another tissue culture cell line growing at comparable rates, Eagle *et al.* reported the very similar value of 1.0 per cent protein turnover/hr.[13] Since these turnover values refer to total cell protein, specific protein constituents are probably characterized by much higher turnover rates. Fast turnover of the AMPS-synthesizing machinery associated with a constant flow of apparently short-lived, newly synthesized RNA molecules is the simplest explanation of the results presented in Figures 1 and 2.

It is in any case evident that those RNA molecules on which AMPS synthesis depends must turn over at a high rate, since decreased AMPS production is detected within 20 min of actinomycin treatment (the shortest time the resolution of the method permits). As total protein synthesis appears to fall at about the same rate as AMPS synthesis in ARE cells and shows no lag before fall-off of synthesis rate in similarly treated L-cells,[12] the result obtained with the specific AMPS marker has a certain generality. Little is known about the rates of turnover of RNA molecules synthesized by RNA-DNA polymerase in nucleated cells. One system from which information bearing on this question has been derived is that of the isolated calf thymus lymphocyte nucleus. In this nucleus, the bulk of the RNA synthesized in a given period of time is messenger-type RNA, resembling cell DNA in base composition.[14] The evidence suggests that this RNA retains its activity to

stimulate nuclear protein synthesis for only about 20 min from the time of its own construction.[15] Thus, thymus lymphocyte messenger RNA would appear to possess a high turnover rate, since it is rapidly synthesized and rapidly inactivated. However, it is not profitable to speculate on which RNA component is the critically affected one in actinomycin-treated ARE cells. As Allfrey and Mirsky have pointed out,[17] both messenger and ribosomal RNA appear to be synthesized from a DNA template, and in rapidly growing cells both are synthesized at a high rate so that empirical distinctions between them tend to blur.

The Effect of Actinomycin on Succinic Dehydrogenase Activity.—In order to gain a better perspective on the immediacy of nuclear control in ARE cells, it was decided to test the effect of actinomycino n an enzymic activity of a different type. The widely distributed mitochondrial enzyme succinic dehydrogenase was chosen, since if there is some component of the cellular machinery which is not so immediately dependent on nuclear control, it might well be sought in a cytoplasmic organelle such as the mitochondrion. The idea that mitochondria are to be regarded as cell organelles is an old one.[16] Recent support for the view that mitochondria are relatively autonomous cytoplasmic units (i.e., cell organelles) derives from studies of Fletcher and Sanadi[18] on mitochondrial turnover. These authors labeled rat liver mitochondria with methionine S^{35} and acetate C^{14} and then followed the loss of label from cytochrome C and several other mitochondrial components. They found that radioactivity in all the mitochondrial fractions declined at the same rate, with a half-life of 10 days, almost twice the half-life known for total liver cell proteins.[19, 20] As the authors note, the picture obtained is that of breakdown (and synthesis) of the whole mitochondrial entity at once, and this result is in accord with the organelle theory. The report by Chevremont *et al.*[21] that mitochondria in chick fibroblasts will display under certain conditions a feulgen-positive material which becomes labeled with thymidine-H^3 (DNA) might also be regarded as oblique support for the organelle thesis.

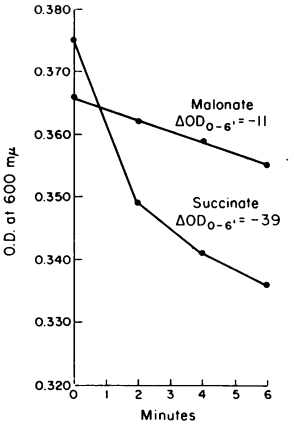

FIG. 3.—Succinic dehydrogenase activity of cell homogenates. The reaction cuvettes each contained 24.5 μM succinate, 6 μM KCN, 30 μg EDTA, 11.2 μg dichlorophenol indophenol, and 500 μg crystalline bovine serum albumin, besides the cell extract to be tested for succinic dehydrogenase activity. The exact composition of the stock solutions was as in Rogers.[26] The procedure was as follows: 0.50 ml KCN EDTA-BSA solution and 1.55 ml 0.01 *M* Na succinate solution or 0.01 *M* Na succinate containing 0.14 *M* malonate were mixed and allowed to equilibrate for 3 min in the Beckman spectrophotometer. The dye was then added (0.075 ml), the contents were mixed rapidly, and a zero-time reading of *OD* at 600 mμ was taken. Readings were taken at 2, 4, and 6 min thereafter, preceded in each case by stirring within 15 sec.

Mitochondria, however, are also subject to central genomic control. Ephrussi and co-workers[22] have demonstrated single-factor Mendelian transmission of mitochondrial respiratory enzyme systems in yeast. C. Raut has described other yeast mutants in which specifically mitochondrial cytochrome enzymes are shown to be controlled by a single Mendelian factor.[23, 24] In another organism, Neurospora, cytochrome-free genetic mutants have also been identified.[25] Measurement of succinic dehydrogenase activity in actinomycin-treated ARE cells would indicate

whether genomic control over this mitochondrial enzyme is characterized by the same immediacy as shown for AMPS and total protein synthesis in these cells.

Twelve to sixteen hr after planting, replicate ARE cultures were thus exposed to 8 μg/ml actinomycin for 20 min, the monolayer was rinsed and covered with fresh medium, and at given intervals thereafter the cells were harvested. Cell homogenates were then prepared as described below. Parallel coverslip cultures with the same ratio of actinomycin to cells were used to monitor RNA synthesis throughout the experiment with the same procedures as utilized in the experiment of Figure 1. The results of two experiments measuring succinic dehydrogenase in actinomycin-treated cells are shown in Table 1, expressed as mμM succinate reduced per mg of protein.

TABLE 1

SUCCINIC DEHYDROGENASE ACTIVITY AND RNA SYNTHESIS AFTER ACTINOMYCIN TREATMENT

Time after actinomycin	Experiment I		Experiment II	
	Per cent of control RNA synth.	mμM succinate reduced/mg P	Per cent of control RNA synth.	mμM succinate reduced/mg P
Control (0 hours)	100	75.6	100	75.2
2	1.8	147.6	4.8	120.8
8	2.6	90.0	3.3	
12			3.9	114.8
17	1.8	142.0		
23			3.6	105.2

Each value is based on duplicate determinations carried out on two independent replicate cultures.

It is evident from the data of Table 1 that the specific activity of succinic dehydrogenase shows no decline in cells suffering near-total blockage of nuclear RNA synthesis for many hours. In fact, succinic dehydrogenase activity has increased somewhat in the actinomycin preparations, perhaps as a by-product of the general derangement of synthetic metabolism wrought by the actinomycin treatment (total protein/10^6 cells remained constant throughout the experiment).

Succinic dehydrogenase activity was measured with a method adopted from the procedure of K. Rogers[26] originally intended for the estimation of succinate. This method depends on succinic dehydrogenase-mediated electron transport between succinate and the blue dye 2,6-dichlorophenol in the presence of cyanide to block further cytochrome-mediated transport pathways. Succinic dehydrogenase is specifically inhibited by malonic acid, and the activity of this enzyme was expressed as the *difference* in the decline in OD at 600 mμ of the contents of two Beckman cuvettes, identical except for the presence of about 10 times as much malonate as succinate in one of the cuvettes. Details of the assay procedure are given in the legend to Figure 3. The cell extracts used for the enzyme activity measurements were prepared as follows: Several million cells were washed in the cold in 0.06 *M* Na/K phosphate buffer, and, after standing at 0–2° for 30 min under these hypotonic conditions, they were 100 per cent broken by three min of motor-driven homogenization in a 2 ml Tenbroek homogenizer. Homogenates were then dialyzed at 0–2° against four 1:50 volumes of 0.06 *M* pH 7 phosphate buffer containing 0.01 *M* Na succinate. Duplicate 0.40 ml samples were taken for protein analysis by the Lowry procedure,[27] and the remaining homogenate was kept in the cold until 15 min before measurement of enzyme activity. Figure 3 shows curves typical for such preparations. According to a linear standard curve for these reaction conditions,[28] an OD change of 0.010 represents the disappearance of 4 mμM succinate, and thus, in the case portrayed in Figure 3, the succinic dehydrogenase in the preparation catalyzed the reduction of 11.2 mμM of succinate during the six min of observation.

Discussion.—The fact that AMPS production by ARE cells falls without lag when DNA-dependent RNA synthesis is blocked serves as a demonstration that AMPS synthesis is a genomically controlled cell character. The use of actinomycin D thus constitutes a novel method for showing the dependence of specific cellulular

functions on gene action, one which is complementary to the classical methods.

The main focus of attention in this paper is the degree of immediacy which characterizes genomic control over specific cell functions. AMPS synthesis, an inherited differentiated character of the ARE-2-60 cell line, has been shown to cease when gene action is discontinued, as does the total protein synthesis of these cells. The apparent decay of AMPS synthetic activity in actinomycin-treated ARE cells displayed a half-time of eight hr, and, since AMPS synthesis was shown to be directly dependent on protein synthesis, that value constitutes a quantitative index of the immediacy of gene control over this cellular function. In the same cell, however, it was found that as long as 23 hr after obliteration of RNA synthesis the specific activity of the mitochondrial enzyme succinic dehydrogenase remains undiminished. Thus, gene action determining this enzyme system is characterized by a low degree of immediacy. Nonetheless, it is to be stressed that the evidence available at this time demonstrates clearly the existence of an ultimate genetic control over mitochondrial systems though it also suggests a large degree of mitochondrial autonomy. In considering the vital dynamics of intracellular function, the *immediacy* with which a given cell character is controlled by its genomic determinants is one of the main pieces of information necessary for understanding the manner in which that character is integrated into cellular metabolism. It is therefore of considerable interest to find that highly diverse degrees of immediacy in gene control coexist within the same cell.

Turning to what is known about immediacy of gene control in other systems, the main example among the cells of vertebrates is the reticulocyte. Hemoglobin synthesis in the mammalian reticulocyte remains active for days after loss of the cell nucleus, thus proving the existence of ribosomal protein synthesis systems[29] which do not require immediate gene action in order to maintain function, although it is well known that hemoglobin synthesis is under genetic control. Of added interest here is a recent observation that actinomycin D does not affect reticulocyte hemoglobin synthesis.[11] Whether the reticulocyte model is analogous to the succinic dehydrogenase system studied here depends on whether the latter enzyme was being synthesized in the course of the experiment, which is unknown. On the basis of present information, the situation is different in bacteria. Time lag between gene action and the appearance of the target character has been studied in various ways in bacteria and has been found to be of exceedingly short duration. For example, β-galactosidase production in *E. coli* zygotes was detected within two min of the introduction of the gene for β-galactosidase in a mating experiment of Riley *et al.*[30] In another case, Lacks and Hotchkiss studied amylomaltase formation in pneumococcus mutants constitutively lacking this enzyme, following introduction of the gene for its synthesis by wild type transforming DNA, and it was found that amylomaltase formation began after a lag of only six min.[31] Other examples involving virus-induced enzyme synthesis could be cited, e.g., the appearance of deoxycytidylate hydroxymethylase in T-2 infected *E. coli*.[32] All of these microbial systems illustrate an extreme of immediacy in gene control over the respective cell functions, compared to the case in the differentiated vertebrate cells studied. The contrast serves to draw attention to one of the fundamental properties of nucleated differentiated cells, the existence of mechanisms moderating and controlling the inherited genomic potentialities carried by these cells. We know that genomic

control is exercised through the selective repression and activation of certain genetic loci in certain cells. It is apparent that an even more subtle mode of genomic control exists, represented by variations in the immediacy with which gene action is reflected in the target cell activities. Since cell differentiation is the result of these processes of genomic specialization and modulation, investigation into their nature and mechanism is among the fundamental problems in developmental biology; for it is now known that gene activity may be necessary for the maintenance of a character as well as for its first appearance.

Summary.—Actinomycin D was used to study gene control over specific cellular activities in an established cultured cell line preserving a differentiated biosynthetic function *in vitro*. It was found that the specific biosynthesis characteristic of these cells was subject to immediate genomic control, while, by contrast, specific activity of a mitochondrial enzyme was not affected by actinomycin treatment.

* This nomenclature is conventional. "ARE-2-60" denotes a culture line initiated February 1960 and constituted of altered rat eye cells.

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