Pre-adipocyte determination either by insulin or by 5-azacytidine

(CHEF cells/adipocytes/differentiation/DNA methylation)

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ABSTRACT   CHEF/18 is a diploid Chinese hamster cell line of embryonic origin, which is fibroblastic in structure, but behaves like a mesenchymal stem cell line in its ability to differentiate into adipocytes, myoblasts, and chondrocytes. With these cells, adipocyte formation has been divided experimentally into two stages: (i) determination of pre-adipocytes, which have lost the ability to form other cell types while retaining their fibroblast structure; and (ii) commitment or terminal differentiation, in which lipids accumulate, adipocyte structure develops, and cells lose the ability to divide. This paper reports that the first stage can be induced by exposure to 5-azacytidine or 2'-deoxy-5-azacytidine, drugs that also induce CHEF cells to form other mesenchymal cell types, or by growth with added insulin. Pre-adipocytes are distinguished from CHEF stem cells by (i) their inability to form other mesenchymal cell types; and (ii) their rapid accumulation of lipid in response to added insulin. The possibility is discussed that both insulin and the cytidine analogs promote differentiation by the same mechanism, namely changes in the pattern of DNA methylation.

Do changes at the DNA level mediate cellular differentiation? Recent studies describing the multipotentiality of embryonic fibroblastic cell lines have provided novel material with which to examine this question. The ability of ageing 3T3 cells occasionally to undergo conversion to adipocytes was described by Green and colleagues (1–4). Subsequently, Jones and colleagues (5–8) found that the cytidine analogs azacytidine and deoxyazacytidine could mediate this conversion of 3T3 cells to adipocytes as well as the differentiation of a different embryonic mouse fibroblastic cell line, 10T 1/2 (9), to form not only adipocytes but also myoblasts and chondrocytes. Jones and Taylor proposed that the cytidine analogs act by interfering with methylation after incorporation into newly replicated DNA (8).

The possibility that changes in DNA methylation might be involved in differentiation was proposed by Scarano (10). Subsequently, the discovery of site-specific methylation and restriction enzymes (11, 12) as well as improved methods for detection of methylated bases (12) has made it experimentally feasible to examine changes in methylation associated with defined steps in differentiation. Hypomethylation has been reported in differentiation of Friend erythroleukemia cells (13, 14) and in chemically induced and spontaneous hepatomas (15, 16). In the microbial eukaryotic alga *Chlamydomonas* methylation during gametogenesis and zygote development has been shown to protect chloroplast DNA of maternal origin against the restriction and degradation by endonucleases of paternal homologous DNA that occurs in zygotes and is the molecular basis of maternal inheritance of chloroplast genes (17–20).

These changes are large scale and can be measured analytically. Changes in methylation have also been reported at the level of single recognition sites in DNA; the predominant pattern is decreased methylation of particular sites in genes being transcribed and increased methylation associated with nontranscription (21, 22). The respective functions and regulation of paired methylation–restriction and of methylation *per se* in eukaryotic gene expression remain almost totally unknown.

This paper describes the differentiation in cell culture of a secondary mesenchymal stem cell line with a fibroblastic structure to form myoblasts, chondrocytes, and adipocytes. This stem cell line, consisting of a twice-cloned population of diploid Chinese hamster embryonic fibroblasts called CHEF/18, has previously been studied extensively in this laboratory (23–26). CHEF/18 cells are nontumorigenic and unique in their diploidy and chromosomal stability, which confer a high degree of phenotypic and genetic homogeneity. The conversion of these cells into terminally differentiated mesenchymal derivatives as described below can be induced by exposure to 5-azacytidine (azaCyd) for a single cell cycle. Further, we have discovered that in the absence of azacytidine growth with added insulin alone can convert these stem cells almost 100% to pre-adipocytes. We show that the adipocyte conversion occurs in two separable stages, each induced by insulin. The second stage, from pre-adipocytes to terminally differentiated adipocytes, has previously been shown with 3T3 cells to respond to insulin (3) as well as to other agents (27), but the first stage, from stem cells to pre-adipocytes, has not been previously described in detail, and the role of insulin in this process has not previously been reported. This stage is an important step in embryonic determination (28).

MATERIALS AND METHODS

CHEF cells were grown and maintained as described (23) on plastic in α minimal essential medium (Kansas City Biological, Kansas City, MO) plus 5% or 10% fetal calf serum (Microbiological Associates, Bethesda, MD) plus antibiotics (penicillin at 100 units/ml and streptomycin at 100 μg/ml) in a humidified incubator with 6.5% CO_2 at 37°C. Cells were passed in T-25 flasks (Corning) and plated in 60-mm (Falcon) and 100-mm (Lux Scientific) dishes. Chondrocytes and myoblasts were identified by morphology. To identify adipocytes, cells attached to plastic were fixed with formalin, stained with oil red O (1–4), and counterstained with hematoxylin. Chromosomes were analyzed by trypsin/Giemsa banding as described (25). azaCyd was obtained from Sigma; 2'-deoxy-5-azacytidine (dazaCyd) was a gift from Howard Green; bovine insulin was from Sigma.

RESULTS

Multiple Cell Types Arise After Treatment of CHEF/18 Cells with azaCyd or with dazaCyd. When a young growing culture of CHEF/18 cells is treated with azaCyd or dazaCyd for as short a time as 24 hr, then grown to confluence and maintained for 3–4 weeks, various differentiated cell types arise on the confluent monolayers. Cell types identified as myoblasts,

Abbreviations: azaCyd, 5-azacytidine; dazaCyd, 2'-deoxy-5-azacytidine.

chondrocytes, and adipocytes were found in these monolayers; examples are shown in Fig. 1. Other cell types, as yet unidentified, were seen as well. Although pre-adipocytes beginning to accumulate fat droplets could be recognized in cell populations treated with azaCyd and grown on standard medium, addition of insulin led to rapid lipid accumulation and facilitated their identification.

Each cell type arose predominantly at a particular drug concentration: pre-adipocytes at 3 μM azaCyd or 0.3 μM dazaCyd, myoblasts at 3, 6, and 10 μM azaCyd or 1 μM dazaCyd, and chondrocytes at 10 and 30 μM azaCyd. Because CHEF/18 cells are clonal in origin, these preliminary results already suggest that CHEF cells are in fact a clone of mesenchymal stem cells, and that the same cells can differentiate into any one of the differentiated cell types, given the proper external conditions. Here we describe conditions that favor the adipocyte pathway.

Conditions for Adipocyte Conversion by azaCyd. CHEF/18 cells of comparable population age (from a frozen stock at passage 17) were grown with either 10% or 5% fetal calf serum (similar results at both concentrations) and fixed and stained after 3–4 weeks at confluence. Dose responses after treatment for 24 hr with 1, 3, 6, 10, and 30 μM azaCyd were compared at plating densities of 10^3, 10^4, and 10^5 cells per 60-mm dish, with and without added insulin. azaCyd at 1 μM had no effect, 3 μM and 6 μM were optimal and indistinguishable, and virtually no adipocytes were seen at 10 or 30 μM azaCyd. No differences were detected in cell populations treated with azaCyd for 1, 2, or 3 days; 1-day treatments were used in subsequent
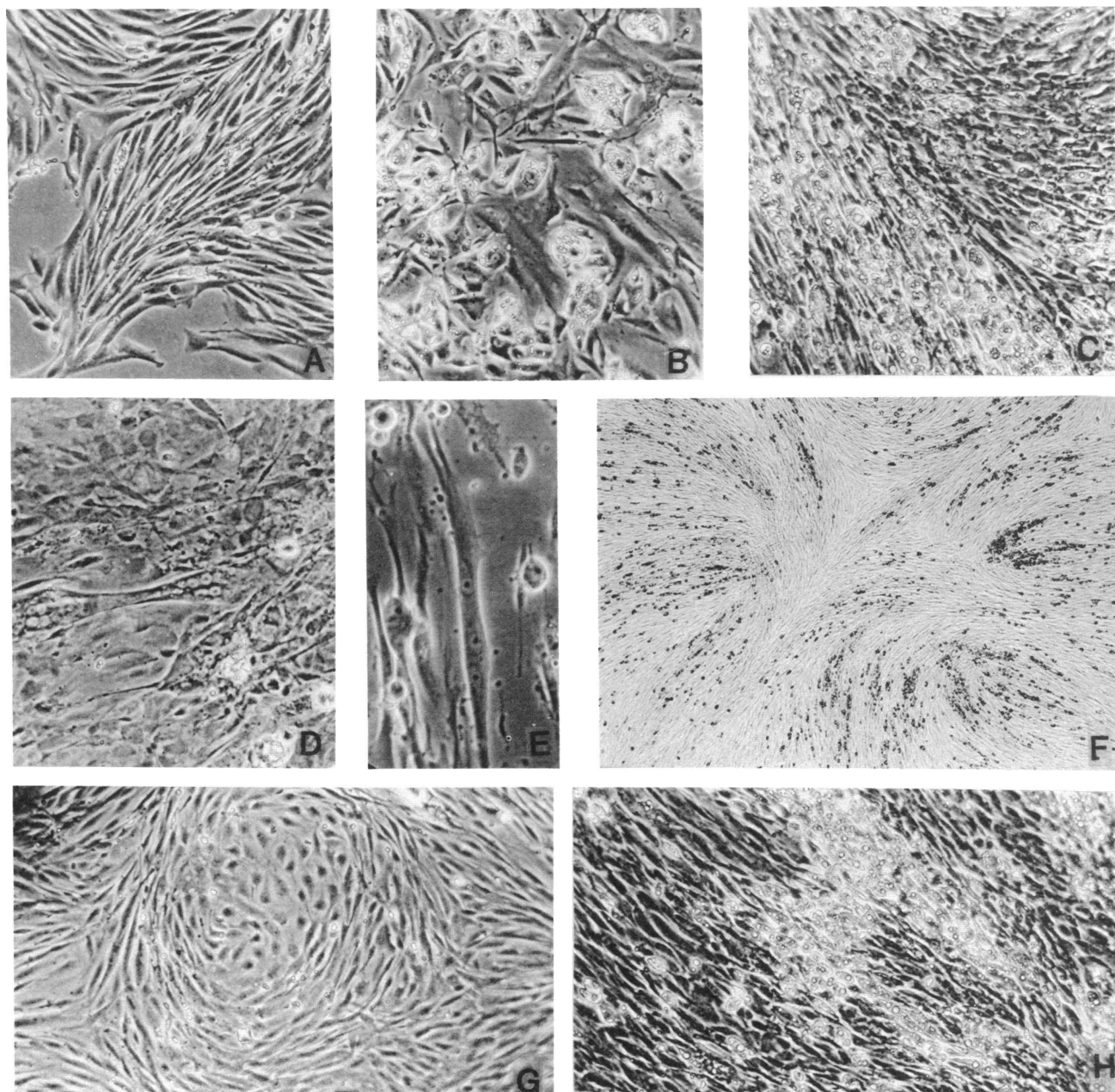

FIG. 1. azaCyd- and insulin-mediated differentiation of CHEF/18. (A) Untreated CHEF/18. (B, C, and H) Adipocytes after 3 μM azaCyd and 18 days of growth with insulin at 10 μg/ml. (D and E) Myoblasts–myotubes after 3 μM azaCyd and 21 days of growth. (F) Adipocyte pattern in colony of preadipocyte line II/3. (G) Chondrocytes after 10 μM azaCyd. (A, C, D, G, and H, ×100; B and E, ×160; F, ×25.)
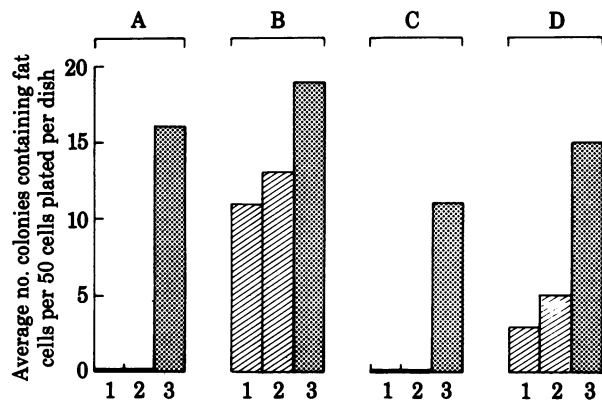
FIG. 2. Induction of adipocytes by azaCyd or by insulin. The numbers under the bars indicate growth medium: 1, α medium; 2, α medium plus glucose at a high concentration (4.5 mg/ml); 3, α medium plus insulin at 10 μg/ml. Treatment A, no azaCyd, cells fixed after 3 weeks; treatment B, no azaCyd, insulin added for 7 days after 3 weeks; treatment C, 3 μM azaCyd, cells fixed after 3 weeks; treatment D, 3 μM azaCyd, insulin added for 7 days after 3 weeks.

studies. In treatments with azaCyd, concentrations of 0.1, 0.3, and 1.0 μM were compared, and 0.3 μM was found to be optimal, confirming previous reports (8) that the deoxy compound is about 10-fold more effective than azaCyd. Because of difficulties in obtaining dazaCyd most subsequent experiments were performed with azaCyd.

It was noted that control plates that were not treated with azaCyd, but did receive added insulin in parallel with the treated cultures, also contained adipocytes at the end of the experiments. After these observations were made, experiments were designed to examine the possibility that insulin itself might play a role in determining adipocyte differentiation.

Pre-adipocyte Determination by Insulin. The ability of CHEF/18 cells to give rise to colonies containing adipocytes was examined by plating 50 or 10^3 cells per dish in: (i) α regular medium (glucose at 1 mg/ml); (ii) α medium plus a high concentration of glucose (4.5 mg/ml); (iii) α medium plus insulin (10 μg/ml). One set of plates was treated with 3 μM azaCyd for 24 hr, the other set was untreated. After 21 days, half the dishes were fixed and stained with oil red O and hematoxylin; the rest were shifted to α medium plus insulin plus the high concentration of glucose for 7 days and then fixed and stained. The results with cells plated at 50 cells per dish shown in Fig. 2 come from one experiment, but similar results have been obtained in several independent experiments.

No colonies were seen that consisted entirely of lipid-accumulating cells, but rather lipid accumulation was confined principally to sectors (see Fig. 1). The extent of adipocyte formation, as revealed by staining with oil red O, was not uniform from one colony to another but was extensive enough so that scoring was unambiguous. In dishes seeded with 10^3 cells, cultures were confluent when scored. However, clusters or sectors of fat cells could readily be counted after staining with oil red O.

The results show: (i) cells treated with either azaCyd or insulin form adipocytes; (ii) addition of a high concentration of glucose without insulin has no effect; (iii) addition of insulin with or without azaCyd pretreatment leads to adipocyte differentiation; and (iv) in month-old cultures, virtually every colony contained some fat-producing cells, in dishes seeded with 50 cells. Approximately twice as many fat-producing centers were found in confluent monolayers of dishes seeded with 10^3 cells per dish (data not shown). Thus under the best conditions, seeding 50 cells per dish, adipocyte conversion occurs in virtually every colony. At higher plating densities, a plateau is seen, as reported by Green and Kehinde (4).

The most important observation is that a similar number of adipocyte-containing colonies were found whether or not the cells had been pretreated with azacytidine, provided they had been grown with insulin. This result provides evidence that insulin alone, in the absence of azaCyd, is effective in the adipocyte differentiation of CHEF/18 cells.

The experiment summarized in Table 1 was designed to examine parameters influencing the conversion of stem cells to preadipocytes; age of the culture, number of doublings, time at confluence, and time of insulin addition with azaCyd-treated and untreated CHEF/18 cells. Culture age and doublings were regulated by the seeding density: cultures seeded at 10^3 cells grew 15 days before confluence, with a minimum of 10–11 doublings; those seeded at 10^4 cells grew for 10 days before confluence, with a minimum of 7–8 doublings; those seeded at 10^5 cells grew for 6 days before confluence with a minimum of 3–4 doublings. Despite some lethality after azaCyd treatment, drug-treated and untreated cultures at each plating density reached confluence, i.e., about 10^6 cells per dish, at about the same time. Following confluence, each set of plates was sampled twice per week and insulin was added for 3 or 7 days; plates were then fixed and stained with oil red O and hematoxylin.

We found that the first fat-containing cells arose in the untreated cultures 20–23 days after plating, regardless of plating density or time at confluence, provided the cells had received insulin for 3 or preferably for 7 days. Fat-forming cells appeared at a higher frequency in the azaCyd-treated cultures. Several

Table 1. Time course of adipocyte conversion of azaCyd-treated and untreated CHEF/18 cells

No. of cells seeded	Treatment*	Time to confluence, days	Population doublings to confluence[†]	Time to first appearance of adipocytes,[‡] days	No. of colonies with adipocytes at first appearance[§]	Final no. of colonies containing adipocytes[§]
10^3	+azaCyd	15	10–11	18 + 3	25	40
	−azaCyd	15	10	18 + 3	4	17
10^4	+azaCyd	10	7–8	13 + 3	51	62
	−azaCyd	10	7	13 + 7	4	20
10^4	+azaCyd	6	4–5	16 + 7	47	48
	−azaCyd	6	4	16 + 7	5	42

* Other cell types besides adipocytes were seen in cultures treated with azaCyd.
[†] At confluence, 1 × 10^6 cells per dish on +azaCyd dishes; 1.3 × 10^6 cells per dish on −azaCyd dishes.
[‡] First number represents days in medium without added insulin. Second number represents days with added insulin required to produce colonies with rounded, oil red O-staining cells.
[§] Because plates were confluent, colonies were not sharply demarcated; adipocytes were mainly clustered at colony centers, thus permitting quantitation.

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Table 2. Comparative time course of adipocyte conversion of CHEF/18 and pre-adipocyte cell lines*

	7-day cultures[†]		14-day cultures[†]		21-day cultures[†]	
Cell line	+ 3 days insulin	+ 7 days insulin	+ 3 days insulin	+ 7 days insulin	+ 3 days insulin	+ 7 days insulin
CHEF/18	0, 0	0, 0	2, 0	10, 10	3, 4	4, 5
IV/1	0, 0	24, 28	21, 22	55, 57	20, 25	47, 57
IV/2	0, 0	16, 19	24, 22	41	15, 30	49, 50
II/2	0, 0	24, 27	0, 2	50, 54	5, 7	37, 43

* Numbers represent colony centers with oil red O-staining adipocytes per 10^3 cells per 60-mm dish.
† Days of growth in regular medium without added insulin.

additional days were required for maximal appearance of fat-containing cells in all the cultures, especially in the cultures not treated with azaCyd.

These results show that the conversion of CHEF/18 cells to pre-adipocytes takes a minimum of about 3 weeks. The age of the culture is a principal factor in the response to insulin. The results confirm the evidence in Fig. 2 that insulin alone in the absence of azaCyd can convert CHEF/18 cells to adipocytes. It should be noted, however, that azaCyd-treated cells give rise to colonies or clusters with larger masses of adipocytes than do cultures receiving only insulin.

Comparison of CHEF/18 with Pre-adipocyte Cultures. Pre-adipocyte cultures were established by picking fat-cell containing regions from confluent cultures of CHEF/18 cells grown with insulin either treated with azaCyd or untreated. Cell lines IV/1 and IV/2 were derived from azaCyd-treated cells, whereas II/2 came from an azaCyd-untreated culture grown initially with insulin but not terminally differentiated. All three cell lines will grow indefinitely in the absence of insulin but undergo terminal differentiation in its presence. The rate of fat accumulation in response to added insulin was examined in experiments similar to those described above.

Cells of CHEF/18 and the three pre-adipocyte lines growing exponentially on regular medium without added insulin were used as inocula, and plated at 10^3 cells per dish. Insulin was added after 7, 14, or 21 days, and plates were sampled for fixation and staining at 3 and 7 days after insulin was added. As shown in Table 2, the pre-adipocyte lines were readily distinguishable from CHEF/18 in this assay. Seven-day cultures to which insulin was added for a second 7 days showed substantial sectors of round fat-containing cells in about half the final number of such colonies, whereas the first similar colonies were found in CHEF/18 cultures at 21 days (14 + 7). Not shown in Table 2 is the further qualitative difference between CHEF/18 and the pre-adipocyte cultures, which could be seen at a glance. CHEF/18 colonies contained far fewer fat-containing cells at 21 + 7 days than did the PA colonies at 7 + 7 or 14 + 7 days. Thus, the kinetics of appearance of rounded, fat-globule-containing cells in response to insulin is dramatically and reproducibly different in CHEF/18 and in pre-adipocyte cells.

The pre-adipocyte cell line that was not treated with azaCyd differed (II/2) from the azaCyd-treated lines (IV/1 and IV/2) in the slowness of its response to insulin after 14 or 21 days growth in α medium. Whether this slow response is characteristic of insulin-induced pre-adipocytes is not yet known.

Chromosomes. Chromosomes of the three pre-adipocyte lines in Table 2 were examined by Giemsa banding and compared with the CHEF/18 cells previously described (25). The IV/1 and IV/2 lines were indistinguishable from CHEF/18, whereas the II/2 cells were similar except for an extra chromosome 3p present in some cells.

DISCUSSION

This paper reports that CHEF/18, the Chinese hamster embryo fibroblastic cell line (23–26), behaves very much like a secondary mesenchymal stem cell line, capable of differentiating into adipocytes, myoblasts, or chondrocytes. In the adipocyte conversion, two stages have been distinguished in this work: stage I, called determination, in which the multipotentiality of the stem cells becomes limited to the adipocyte pathway; and stage II, called commitment, in which the pre-adipocytes undergo terminal differentiation mediated by insulin. Biochemistry of the stage II process is being investigated in several laboratories (27, 29, 30). The stage I process has not been previously examined in detail.

We have found that the stage I process can be activated by exposure of the cells to azaCyd or dazaCyd for a single cell cycle or by growing the stem cells for 3–4 weeks with added insulin. The products of the stage I process, whether induced by azaCyd or by insulin, are pre-adipocytes, which are not yet terminally differentiated and can be grown indefinitely in culture in the absence of added insulin.

Are CHEF/18 cells in fact pre-adipocytes? The answer is clearly no, for several reasons.

(*i*) CHEF/18 cells are a clonal diploid population and therefore genetically uniform. Under optimal conditions, virtually every colony-forming cell can give rise to a colony containing adipocytes. Thus, adipocyte differentiation is not a selection process at the cellular level. Nonetheless, under different conditions CHEF/18 cells can give rise to other mesenchymal cell types.

(*ii*) CHEF/18 cells can give rise to several differentiated cell types, whereas pre-adipocytes have only two options: either to grow as such in the absence of added insulin or to terminally differentiate in its presence. In experiments designed to test this point, pre-adipocyte cultures were treated with azaCyd. No other differentiated cell types were detected.

(*iii*) Pre-adipocytes grown without added insulin resemble CHEF/18 stem cells morphologically. They can be distinguished operationally by the kinetics of their response to insulin. (see Table 2)

The stage II process, as studied with 3T3-L1 pre-adipocytes (2), can be induced not only by insulin but also by a variety of other agents, of which an effective combination is the glucocorticoid dexamethasone plus 3-isobutyl-1-methylxanthine, an inhibitor of cAMP phosphodiesterase (27). Spiegelman and Green (29) have described changes in the rates of synthesis of several proteins occurring during adipose conversion of 3T3-derived pre-adipocytes, and they have shown that the changes are regulated by the amounts of translatable mRNAs and furthermore that mRNA availability is itself noncoordinately regulated for various of the conversion-related proteins. Rosen and

colleagues (31, 32) have described an insulin-induced phosphorylation of ribosomal protein S6 that occurs during conversion of 3T3-L1 cells. Both findings indicate the complex nature of the stage II process.

Is the stage I process also complex, and can it be induced by agents other than azaCyd or insulin? As presently understood, insulin has two distinct sets of actions, the metabolic activities exemplified by glucose oxidation, and the growth-promoting activities, seen as promotion of DNA replication and cell division (33). In general, the metabolic activities respond to insulin concentrations about 1/1000th of those required for growth promotion. Growth promotion may be mediated by one of a family of insulin-like polypeptides, the somatomedins (34), through a different set of receptors than those that bind insulin with high affinity. It is clearly important to distinguish whether the stage I effect on differentiation described in this paper is mediated more effectively by insulin or by an insulin-like factor.

Adipose conversion was originally described by Green and coworkers (1–4) as an infrequent spontaneous event occurring in ageing Swiss 3T3 monoloyer cultures, in the absence of added insulin. As yet we have seen no comparable spontaneous conversion with CHEF/18 cells. Further differences between the two cell lines in adipose conversion include the following: (i) CHEF/18 cells are Chinese hamster diploids and of clonal origin, whereas 3T3 is a heteroploid uncloned mouse line with about 65 chromosomes ($2n = 40$). (ii) Every CHEF/18 cell that forms a colony is capable of adipose conversion with added insulin, whereas the process is rare in 3T3. (iii) During adipose conversion 3T3 cells form clusters of fat-containing cells in colony centers, whereas CHEF/18 cells frequently form sectors as shown in Fig. 1.

Our pre-adipocyte clones respond to added insulin with different patterns of adipose conversion. Clones such as IV/1 respond rapidly, as shown in Table 2, and, when plated at 50 or 100 cells per plate, each plating unit undergoes conversion (data not shown). Clone IV/2 responds somewhat more slowly than IV/1, and clone II/2 is even slower, with fewer colonies forming adipose cells within a given time—i.e., 4 weeks. These clonal differences in time and frequency of adipocyte conversion (or commitment) are reproducible on subculture. A myogenic clonal cell derived from a mouse teratocarcinoma (35) has been shown to give rise to subclones that differentiate into adipocytes and fibroblasts as well as to myoblasts, and separate subclones show heritable differences in which cell type is predominant (36). These clonal differences are analogous to those we have described in this paper.

The arrangement of terminally differentiating adipocytes in pre-adipocyte colonies (Fig. 1) does not appear to be clonal but rather resembles nonclonal pattern formation, as if the stage II process was regulated by environment or cell–cell interactions. The differences in time and frequency of adipocyte commitment, however, from one pre-adipocyte line to another are clearly clonal and heritable on subculture.

What is the nature of the genetic control? Here we return to the role of azaCyd and what it may be telling us about the process. Jones and Taylor proposed that azaCyd mediates differentiation by inhibiting DNA methylation and found a decrease in methylation of the nuclear DNA from myotubes compared with the cells from which they arose (8). Similarly, decreased methylation has been observed in Friend erythroleukemia cells after differentiation induced in cell culture (13,

14). Regulated changes in methylation could provide a metabolic switch, as suggested also by the evidence that links transcription with the loss of methylation at specific sites in DNA (21, 22). Because insulin mediates the stage I conversion of CHEF/18 stem cells to pre-adipocytes as effectively as does azacytidine or deoxyazacytidine, it is a fair inference that insulin itself may act directly or indirectly by affecting DNA methylation.

1. Green, H. & Kehinde, O. (1974) *Cell* 1, 113–116.
2. Green, H. & Meuth, M. (1974) *Cell* 3, 127–133.
3. Green, H. & Kehinde, O. (1975) *Cell* 5, 19–22.
4. Green, H. & Kehinde, O. (1976) *Cell* 7, 105–113.
5. Constantinides, P. G., Jones, P. A. & Gevers, W. (1977) *Nature (London)* 267, 364–366.
6. Constantinides, P. G., Taylor, S. M. & Jones, P. A. (1978) *Dev. Biol.* 66, 57–71.
7. Taylor, S. M. & Jones, P. A. (1979) *Cell* 17, 771–779.
8. Jones, P. A. & Taylor, S. M. (1980) *Cell* 20, 85–93.
9. Reznikoff, C. A., Brankow, D. W. & Heidelberger, C. (1973) *Cancer Res.* 33, 3231–3238.
10. Scarano, E. (1967) in *Comprehensive Biochemistry*, eds. Florkin, M. & Stotz, E. H. (Elsevier, New York), Vol. 28, p. 55.
11. Arber, W. (1979) *Science* 205, 361–365.
12. Smith, H. O. (1979) *Science* 205, 455–462.
13. Christman, J. K., Price, P., Pedrenan, L. & Acs, G. (1977) *Eur. J. Biochem.* 81, 53–61.
14. Christman, J. K., Weick, N., Schoenbrun, B., Schneiderman, N. & Acs, G. (1980) *J. Cell Biol.* 86, 366–370.
15. Lepeyre, J.-N. & Becker, F. F. (1979) *Biochem. Biophys. Res. Commun.* 87, 698–705.
16. Walker, M. S. & Becker, F. F. (1981) *Cancer Biochem. Biophys.* 5, 169–173.
17. Burton, W., Grabowy, C. T. & Sager, R. (1979) *Proc. Natl. Acad. Sci. USA* 76, 1390–1394.
18. Royer, H.-D. & Sager, R. (1979) *Proc. Natl. Acad. Sci. USA* 76, 5794–5798.
19. Sager, R., Grabowy, C. T. & Sano, H. (1981) *Cell* 24, 41–47.
20. Sano, H., Grabowy, C. T. & Sager, R. (1981) *Proc. Natl. Acad. Sci. USA* 78, 3118–3122.
21. Razin, A. & Riggs, A. (1980) *Science* 210, 604–610.
22. Ehrlich, M. & Wang, R. Y.-H. (1981) *Science* 212, 1350–1357.
23. Sager, R. & Kovac, P. (1978) *Somatic Cell Genet.* 4, 375–392.
24. Sager, R. & Kovac, P. (1979) *Somatic Cell Genet.* 5, 491–502.
25. Kitchin, R. & Sager, R. (1980) *Somatic Cell Genet.* 6, 75–87.
26. Smith, B. L. & Sager, R. (1982) *Cancer Res.*, in press.
27. Rosen, O. M., Smith, C. J., Hirsch, A., Lai, E. & Rubin, C. S. (1979) *Recent Prog. Horm. Res.* 35, 477–499.
28. Weiss, P. (1939) *Principles of Development* (Holt, New York).
29. Spiegelman, B. M. & Green, H. (1980) *J. Biol. Chem.* 255, 8811–8818.
30. Roth, J., Lesniak, M. A., Megyesi, K. & Kahn, C. R. (1979) in *Hormones and Cell Culture*, Cold Spring Harbor Conferences on Cell Proliferation (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 6, pp. 167–186.
31. Smith, C. J., Rubin, C. S. & Rosen, O. M. (1980) *Proc. Natl. Acad. Sci. USA* 77, 2641–2645.
32. Rosen, O. M., Rubin, C. S., Cobb, M. H. & Smith, C. J. (1981) *J. Biol. Chem.* 256, 3630–3633.
33. King, G. L. & Kahn, C. R. (1981) *Nature (London)* 292, 644–646.
34. Blundell, T. L. & Humbel, R. E. (1980) *Nature (London)* 287, 781–787.
35. Jakob, H., Buckingham, M., Cohen, A., Dupont, L., Fiszman, M. & Jacob, F. (1978) *Exp. Cell Res.* 114, 403–408.
36. Darmon, M., Serrero, G., Rizzino, A. & Sato, G. (1981) *Exp. Cell Res.* 132, 313–327.