

Protein-Pattern Changes and Morphological Effects Due to Methionine Starvation or Treatment with 5-Azacytidine of the Phorbol-Ester-Sensitive Cell Lines HL-60, CCL-119, and U-937

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Methionine starvation causes changes in the protein pattern of HL-60 promyelocytic leukemia cells as observed by two-dimensional electrophoresis. One group of proteins is apparently modified, appearing in new positions. A further series of proteins, including several principal nuclear polypeptides, is substantially diminished. The morphology of a fraction of the cells in the culture changes concomitantly, with condensation and fragmentation of the nucleus and eventual remodeling of the cell to a "grape-cluster" appearance. Similar effects are produced by a DNA methylation inhibitor, 5-azacytidine, but not by various other toxic agents tested. A defect in DNA methylation, either by depletion of *S*-adenosyl-L-methionine (the methyl donor) or by inactivation of the relevant enzyme, may be responsible. The T-lymphoblastoid line CCL-119 and the histiocytic lymphoma line U-937 also show these effects, but most fibroblast, epithelial, and lymphoblastoid lines do not. These changes can be largely prevented in each of the three susceptible lines by prior treatment with the tumor promoter, phorbol myristate acetate (PMA), an agent known to cause differentiation in at least two of the lines. The results thus suggest interesting relationships between methionine metabolism, protein and structural changes in the cell nucleus, and PMA-induced cell differentiation.

Additional Keyphrases: *methionine metabolism • electrophoresis, two-dimensional • production of the "Metmin" set of proteins • cancer*

The amino acid methionine is considered essential in human metabolism (1). Nevertheless, it is common in experiments with cultured cells to limit or omit non-radioactive methionine from normal growth media as a means of increasing incorporation of added radioactive [³⁵S]methionine into newly synthesized proteins. In most cell lines (fibroblasts or lymphoblastoid lines, for example), this treatment appears to have little or no effect on the pattern of protein synthesis over the brief (<1 to 18 h) labeling periods used. In some human-cell lines, however, we have observed a series of specific protein alterations after transfer to methionine-deficient media, which suggest interesting relationships between methionine metabolism and differentiation. The effect involves primarily the appearance of a set of novel polypeptides we have called the "Metmin" group, and diminished accumulation of principal nuclear proteins. These effects are accompanied by morphological changes that ultimately lead to cell death.

Effects caused by methionine starvation are particularly intriguing in view of the range of processes in which the

amino acid participates. In addition to its roles in the initiation of protein synthesis and as a structural constituent of almost all proteins, it serves, in the form of *S*-adenosyl-L-methionine (SAM), as the donor of aminopropyl groups in the reactions of polyamine biosynthesis, and as the source of methyl groups in the methylation of lipids and of DNA. The last reaction involves modification of the genetic material itself, and hence is considered a likely candidate as a means of controlling the expression of some genes.

Regulatable aspects of methionine metabolism have been extensively studied in hopes of clarifying the puzzling differences in methionine utilization by normal and some tumor cells (2-13). Tetrahydropteroylglutamate methyltransferase (EC 2.1.1.13, methionine synthase), for instance, can be induced by folate, methionine, or vitamin B₁₂ (10). In addition, there is evidence that methionine metabolism differs considerably among in vitro cell lines, and between fetal and adult tissues. Tisdale (3, 4) and others (14) have shown that some tumor lines display an absolute requirement for the preformed methyl groups of methionine, whereas most untransformed cells appear to be able to use homocysteine in combination with 5-methyltetrahydrofolate and vitamin B₁₂, the substrates and cofactor required for enzymatic regeneration of methionine. Tisdale concludes that the methionine-requiring Walker-carcinoma line is likely to have a very high consumption of methionine rather than a defect in its metabolism (3), although the methionine sink in such cells remains unidentified. Normal fetal and adult human liver tissue also differ in some aspects of methionine metabolism; methionine synthase activity is twice as great in the livers of premature infants as in livers of adults (15), and the other folate enzymes are altered as well. Methionine metabolism is thus expected to be regulatable by extracellular conditions and to vary, sometimes substantially, among cell types.

The participation of SAM in DNA methylation suggests that methionine starvation could result in hypomethylation of newly synthesized DNA during cell division. Any such effect could influence the expression of a variety of genes. Studies of the inducibility of the mouse metallothionein-I gene by cadmium (16), and of the relative levels of expression of ovalbumin in chicken oviduct vs liver (17) and of human globin genes in various cell types (18) suggest that the decreased DNA methylation is related to increased expression of those genes. Experimental manipulations designed to induce hypomethylation of DNA, in particular treatment with the drug 5-azacytidine (5-AC), have been shown to derepress several genes on previously silent copies of an X-chromosome (19); to cause mouse embryo cells to form myotubes, chondrocytes, and adipocytes (20); to produce macrophage-like cells from a mouse pre-B lymphoma (21); and to increase expression of total Hb in human thalassemia patients (22). In addition it has been shown that some human cancer cells display hypomethylation of a variety of genes (23). This wide range of phenomena indi-

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¹ Nonstandard abbreviations: SAM, *S*-adenosyl-L-methionine; 5-AC, 5-azacytidine; and PMA, phorbol myristate acetate.

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cates that many genes are sensitive to DNA methylation effects and that some of these genes are related to differentiation. The ability of 5-AC, an inhibitor of DNA-methylase enzyme (24), to cause hypomethylation by a pathway presumably quite different from any direct effect on the availability of methionine makes it possible for us to show that the expression of Metmin proteins may be more closely related to effects of hypomethylation than to the metabolism of the amino acid itself.

Our interest in methionine starvation and the possible mechanisms by which its effects are generated stems from experiments on the cell line HL-60, derived from a human promyelocytic leukemia (25). This line can be made to differentiate *in vitro* by treatment with phorbol ester tumor promoters such as phorbol myristate acetate (PMA), yielding a cell that in many respects resembles a macrophage (26). By analyzing the proteins synthesized by HL-60 cells with and without PMA treatment, we discovered a series of differences that we initially attributed to differentiation. However, because methionine-deficient media were used in the first experiments, only later, with the inclusion of complete-medium controls, did it become clear that some of the major differences were attributable to the effects of methionine starvation on the untreated HL-60 cells. These include effects that never appeared in the PMA-treated, differentiated cells. The intriguing possibility that such a basic difference may reveal an important defect in the methionine metabolism of HL-60—and, as we discovered later, other PMA-responsive human cell lines—led us to examine the phenomenon in greater detail.

Materials and Methods

Cell culture. HL-60 cells were obtained from both Dr. E. Huberman (Argonne National Laboratory) and the American Type Culture Collection. For experimental manipulation, cells were maintained in 24-well microculture plates in an atmosphere of 50 mL/L CO₂, 95% relative humidity, at 37 °C. For microscopic examination, cells were centrifuged onto glass microscope slides in a Cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA 15143), and stained with Giemsa stain. Methionine-free RPMI 1640 medium was prepared from a Selectamine Kit (GIBCO, Grand Island, NY 14672) and normal (methionine-containing) medium was prepared by addition of methionine. All media contained 100 mL of fetal bovine serum per liter and antibiotics.

Cell fractionation. Detergent-insoluble cytoskeleton/nuclei preparations were made by collecting cells onto small squares of glass fiber filter (GF/A; Whatman Inc., Clifton, NJ 07014), placed over round polycarbonate filters on a standard vacuum filtration apparatus (Bio-Rad Laboratories, Richmond, CA 94804), taking care to control the vacuum to avoid drying the filter. The cells thus immobilized were extracted with five filter-saturating applications of ice-cold buffer "A" consisting of, per liter, 3 mL of NP-40 detergent (Particle Data Inc., Elmhurst, IL 60126), 0.32 mol of sucrose, 10 mmol of Tris HCl (pH 7.8), 3 mmol of MgCl₂, 1.1 mmol of PMSF, and 0.5 mmol of dithiothreitol. Filters were then blotted to remove excess dampness and placed in yellow Eppendorf disposable pipette tips that were held in small tubes. Thirty microliters of the solubilizing solution described below was applied to solubilize remaining proteins, and the liquid was collected in the tube by centrifugation. This step was repeated once with a further 30 µL of solubilizing solution.

Cytoplasm (nucleus-free) and karyoplast (nucleus-containing) membrane-bounded cell fragments were prepared by enucleation with cytochalasin B. Cells were incubated for 30

min at 37 °C in complete medium plus 20 µg of cytochalasin B (Sigma Chemical Co., St. Louis, MO 63178) per milliliter. The cells, in 100 µL of medium, were then layered atop a step density gradient consisting of 100 µL of Ficoll-Paque (Pharmacia Inc., Piscataway, NJ 08854) above 30 µL of 900 mL/L Percoll (Pharmacia) plus 100 mL of 10-fold concentrated phosphate-buffered isotonic saline per liter. The gradient (total volume 230 µL) was set up in an LP-42 centrifuge tube and spun in the LP-42 rotor (Beckman Instruments, Fullerton, CA 92634) at 20 000 rpm for 20 min, then gently decelerated to rest. Cell fragments from the upper (cytoplasm) or lower (karyoplast) interface were collected, diluted with medium, pelleted in a capillary-bottom Microfuge tube, and solubilized.

Two-dimensional electrophoresis. Samples of cellular proteins were prepared by dissolving cells in a solubilizing solution containing, per liter, 9 mol of urea, 20 mL of NP-40, 10 mL of 2-mercaptoethanol, and 20 mL of ampholytes (pH 9–11; LKB Instruments, Gaithersburg, MD 20877). Two-dimensional electrophoresis was performed as described previously, with the 18 × 18 cm ISO-DALT system (27, 28), and with ampholytes consisting of a mixture of 1 part pH 3.5–5 and nine parts pH 3.5–10 (LKB). Gels were stained, destained, dried, and exposed to Kodak XAR film for autoradiography. Quantitation of spots was performed with the TYCHO I computer system (29).

Results

Methionine Starvation Results in the Appearance of a Set of Novel Proteins in HL-60 Cells

Culture of HL-60 cells for 24 h in RPMI 1640 medium lacking methionine (aside from that contained in the added fetal bovine serum or added as radiolabel) results in the appearance of a specific set of at least seven major polypeptides visible on two-dimensional gels (Figure 1, Table 1). These proteins are not observed in cells starved similarly for leucine or phenylalanine, which are taken up by the same transport system as is used for methionine (system L, ref. 32). Starvation for serine, which is taken up mainly by systems A + ASC (32), also fails to result in appearance of the Metmin proteins, essentially ruling out the induction of a general amino acid transport system as the basis for the effect. Metmin accumulation is not detectable in cells grown and labeled in fresh complete medium (i.e., normal culture conditions) and only begins to appear when the methionine concentration drops below about 5% of the usual RPMI 1640 concentration, which is 15 mg/L. Cells grown in methionine-deficient medium supplemented with L-homocysteine (20 mg/L), tetrahydrofolic acid (0.1 mol/L), and vitamin B₁₂ (7.5 µmol/L) do not produce Metmins, indicating that cellular pathways can generate sufficient methionine by remethylation of homocysteine to prevent starvation. Homocysteine alone does not prevent starvation when it is used to supplement methionine-deficient medium that is more than a few weeks old, at which time the medium seems to be essentially folate-free. Starvation for glucose, treatment with hydroxyurea, sulfhydryl poisons, the protein synthesis inhibitor cycloheximide, or the RNA-polymerase inhibitor α -amanitin do not produce the effect.

The set of proteins appearing as a result of methionine starvation does not include proteins associated with other well-known sets such as the heat-shock (33), mitochondrial (34), interferon-induced (35), or cadmium-induced (N. L. A., unpublished) proteins. One protein (Metmin:1) appears to correspond to a member of the Inmono set observed by Willard (36) as characteristic of leukocytes from patients with mononucleosis, indicating that at least some Inmono

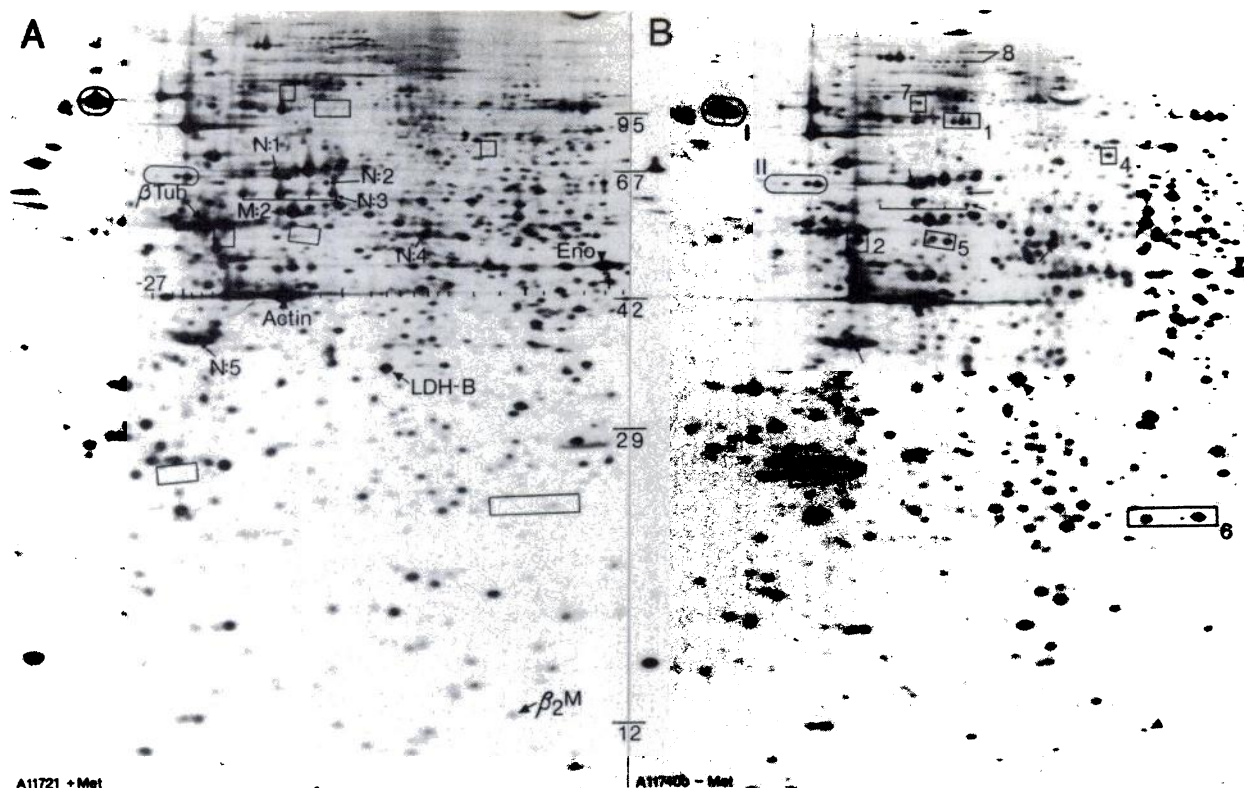


Fig. 1. Two-dimensional electrophoretic patterns of HL-60 human promyelocytic leukemia cells labeled with [35 S]methionine for 18 h in the presence (A) or absence (B) of normal medium concentrations of non-radioactive methionine

Rectangular boxes (labeled 1–7 in panel B) contain the major Metmin proteins, the appearance of which here is ascribable to methionine starvation. A pair of high- M_r protein trains, showing possible conversion of a larger to a somewhat smaller form, are labeled 8 (panel B). Major nuclear (N:1–5) and mitochondrial (M:2) proteins are labeled in panel A; N:1, 2, and 3 are substantially decreased by methionine starvation. N:1 is tentatively identified as lamin B (30). Actin, lactate dehydrogenase (LDH-B), enolase (ENO), and the β -subunit of tubulin (β -Tub) are labeled for reference. Ovals I and II in panel B circumscribe proteins that appear to show increased molecular mass (I) and charge (II) heterogeneity in methionine-starved cells. The vertical scale is approximate SDS/molecular mass (in kDa); the horizontal scale running through actin in panel A represents the positions of creatine kinase (EC 2.7.3.2) charge standards (31)

Table 1. Characteristics of Major "Metmin" Proteins

Metmin	M_r , kDa ^a	Isoelectric position ^b	No. of charge isomers
1	100	–15, –16	3
2	52	–21	1
3	25	–24.7, –26.2	2
4	78	–6.2	1
5	52	–16.2, –17.2	2
6	22	–2.7, –5.1	2
7	125	–17.6	2

^a Subunit molecular mass estimated by reference to muscle-protein standards (45).

^b Isoelectric point (in 9 mol/L urea) as estimated by comparison with high-resolution internal charge standards produced by carbamylation of rabbit muscle creatine kinase (31).

proteins probably were produced during labeling of leukocyte samples in methionine-deficient medium rather than in vivo. Based on the novelty of most of the methionine-starvation protein set, we denote its members as Metmin:1–8 in accordance with the nomenclature for protein sets used previously (29). Pulse-labeling experiments designed to investigate the "induction" of Metmin "synthesis" showed instead that these proteins are likely to be the products of the modification of pre-existing normal cellular proteins. HL-60 cells pre-labeled in complete medium and then shifted to unlabeled, methionine-deficient medium for a period of 39 h show efficient radioactive labeling of the Metmin proteins (Figure 2). Cells similarly labeled, but incubated instead in normal medium, do not. If new synthe-

sis of Metmin proteins had been triggered by transfer to deficient, unlabeled medium then the Metmin proteins would not have been labeled. Conversely, cells grown in unlabeled complete medium and then labeled in methionine-deficient medium show a variable, but often high, ratio of total Metmin (detected by Coomassie Brilliant Blue staining of gels) to radioactively labeled Metmin (detected by autoradiography). This result indicates that large quantities of existing, "cold" precursor protein (present in the cells before starvation) may be converted to Metmin polypeptide before appreciable quantities of newly labeled precursors become available. Although we have, in general, no indication of which normal protein is modified to yield each Metmin, in the case of Metmin:8 it is tempting to associate the protein with a nearby spot-train of slightly higher M_r , which disappears as Metmin:8 appears (Figure 1).

Several other interesting changes take place as a result of methionine starvation in addition to the appearance of Metmins. Several of the predominant nuclear proteins almost disappear from the pattern [Nuc:1–3 (N:1–3) in Figure 1], although at least two other nuclear polypeptides do not appear to be affected. Nuc:1 is tentatively identified as Lamin B (30), a protein likely to be important in controlling nuclear architecture. In addition, there are subtle but reproducible effects involving increased acidwards charge-modification of a highly conserved, lysine-poor, trimethyl-lysine-inducible protein ("Everlast," oval-encircled II in Figure 1B; N. L. A., unpublished), and an upwards shift of part of the density associated with a major cytoplasmic phosphoprotein ("Tbird," oval-encircled I in Figure 1B; N. L. A., unpublished).

5-Azacytidine Treatment Also Results in Appearance of Metmin Proteins

HL-60 cells grown in complete medium (with methionine at normal concentrations) can be made to produce the Metmin proteins by treatment with 5 to 10 μmol of 5-AC per liter (Figure 2). As in the case of methionine starvation, drug treatment efficiently produces Metmin proteins in prelabeled cells, indicating that the process is one involving modification of existing molecules. Treatment with 5-AC results in the appearance of the same set of proteins as does methionine starvation, and in about the same relative amounts. The drug also produces a substantial reduction in total protein synthesis, making rather difficult the radiographic detection of newly labeled proteins. Stained gels, showing total protein rather than newly labeled molecules, nevertheless show high production of Metmin 15 to 40 h after 5-AC treatment. The decrease in accumulation of normal nuclear proteins observed after methionine starvation is also observed after treatment with 5-AC. Screening of a range of potentially cytotoxic drugs indicates that treat-

ment with cytosine arabinoside (10–100 $\mu\text{mol/L}$) can produce moderate amounts of Metmin, while high concentrations of adenosine arabinoside (1 mmol/L) and oxalic acid (20 mmol/L) can produce low amounts.

Localization of Metmin Proteins in HL-60 Cells

Cytoskeletal/nuclear cell fractions prepared by extraction of methionine-starved HL-60 cells with NP-40 contain a large proportion of the Metmin protein in the cell (Figure 3). Table 2 shows the approximate proportion of each Metmin protein present in such preparations as determined by quantitative densitometry of the relevant spots on two-dimensional gels. When the nuclear protein Nuc:1 is used for normalization, 24–89% of the various Metmins are found in the NP-40-insoluble residue, while less than 5% of the LDH-B (soluble-phase), β_2 -microglobulin (membrane-bound), or Mitcon:2 (mitochondrial) protein remains. Metmin proteins are also enriched in karyoplasts (mainly nuclei) and deficient in cytoplasts prepared by cytochalasin B enucleation of Metmin-containing cells, indicating that

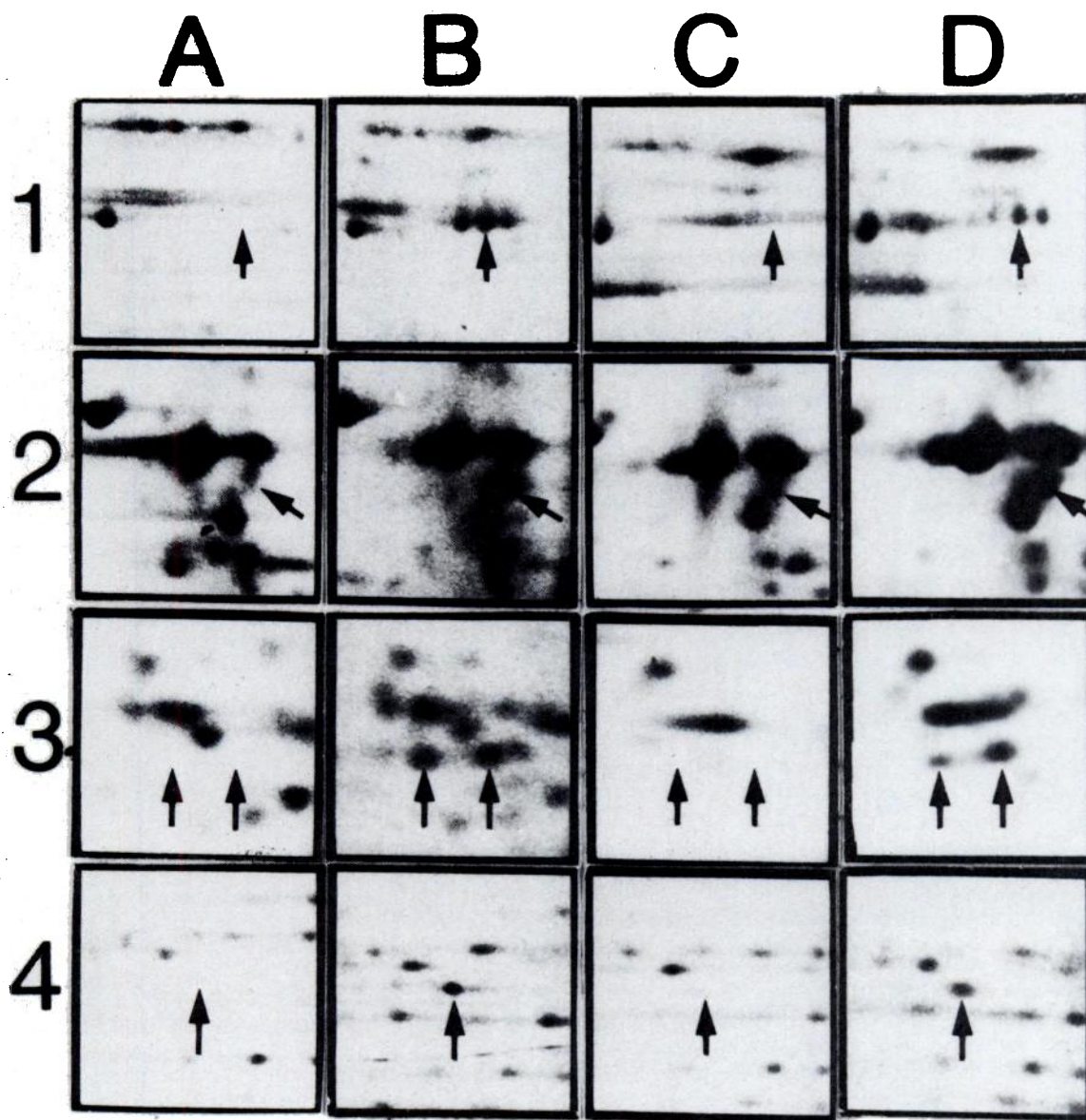


Fig. 2. Sections of two-dimensional gels showing regions surrounding Metmin proteins 1–4

(A) Cells labeled in complete normal medium. (B) Cells labeled as in (A), but with 5-azacytidine (10 $\mu\text{mol/L}$), resulting in appearance of Metmin proteins. (C) Cells prelabeled for 24 h in complete normal medium, then incubated for 39 h in non-radioactive complete medium. (D) Cells prelabeled as in C, but subsequently incubated for 39 h in non-radioactive medium minus methionine. Metmin proteins appearing in D must arise by modifications of normal proteins labeled during prelabeling in complete medium

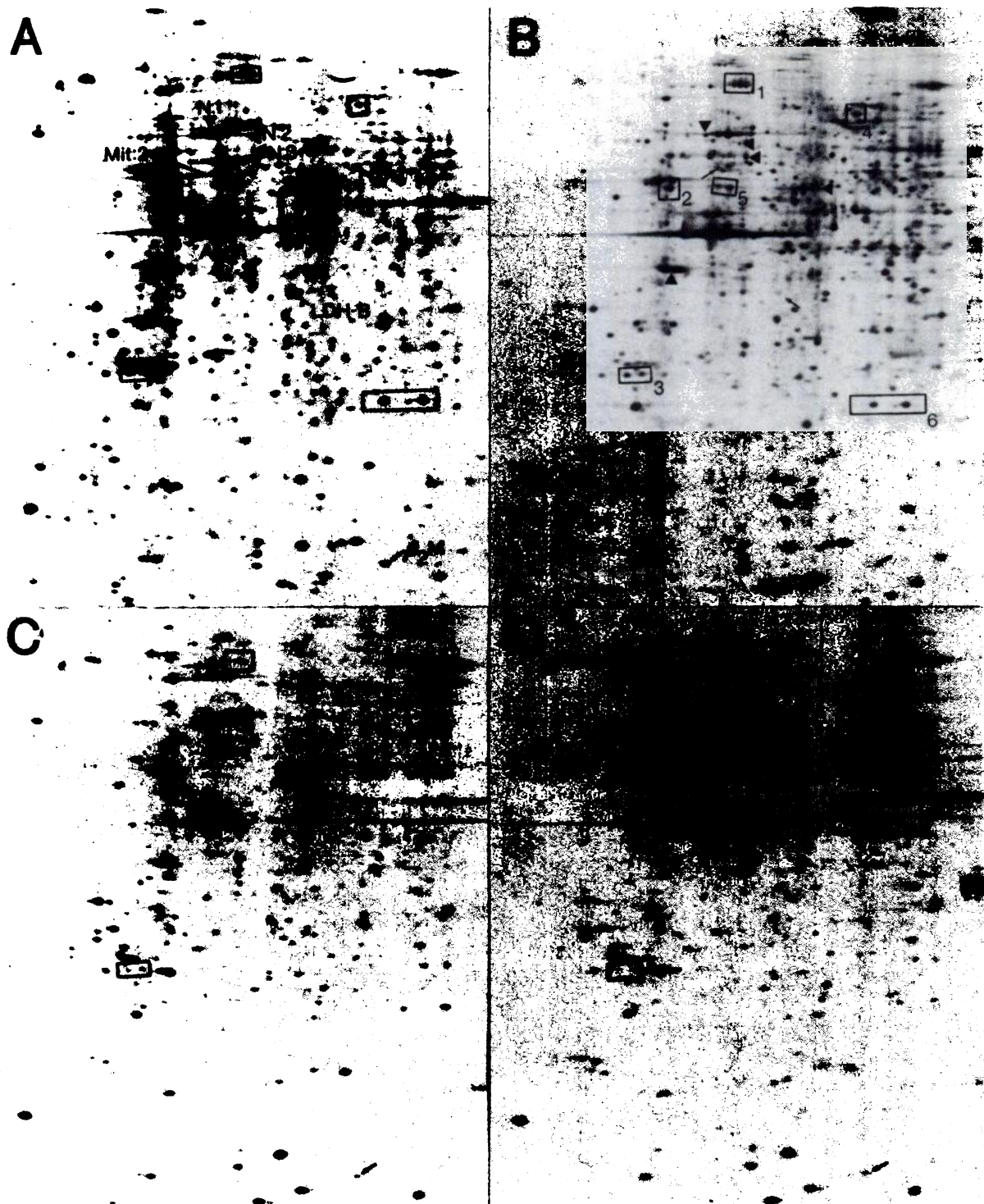


Fig. 3. (A) Two-dimensional pattern of whole HL-60 cells labeled with [³⁵S]methionine in methionine-deficient medium. (B) Pattern for the nuclear/cytoskeletal pellet prepared as described in *Materials and Methods*. Nuclear cytoskeletal and Metmin proteins remain, but mitochondrial proteins (e.g., Mit:2) and soluble enzymes (LDH-B) are extracted. (C) Karyoplast (nucleoplast) fraction and (D) cytoplast (nucleus-free) fraction of Metmin-containing HL-60 cells treated with cytochalasin B: nuclear and Metmin proteins appear only in the karyoplast fraction

they are nuclear rather than attached to the cytoplasmic cytoskeleton (Figure 3). LDH-B and β_2 -microglobulin are evenly divided between the fractions, while Mitcon:2 is slightly enriched in karyoplasts, owing to the tendency of heavy mitochondria to partition into the denser, nucleus-containing fragment of the cell. We carried out the enucleation experiments with cells showing less than maximal Metmin concentrations, in order to avoid the complications introduced into such procedures by the changes in cell morphology described below.

Prevention of Metmin Production in HL-60 Cells by Pretreatment with Phorbol Myristate Acetate

HL-60 cells treated with the active phorbol ester tumor promoter, PMA, attach to tissue-culture plastic and begin to differentiate towards monocytic/macrophage forms (26). Within two days, this differentiation produces almost complete resistance to the Metmin-increasing and nuclear-protein-decreasing effects of methionine starvation (Figure 4) or 5-AC treatment. The prevention of Metmin production

Table 2. Partition of Metmin Proteins between Subcellular Structures

	Fraction remaining after NP-40 extraction ^a	Fraction of total in karyoplast fraction ^b
Metmin:		
1	0.53	0.94
2	0.30	0.89
3	0.28	0.83
4	0.89	0.88
5	0.50	1.00
6	0.27	0.65
7	0.24	1.00
Nuc:		
1	1.00	1.00
2	1.45	1.00
3	1.15	0.94
LDH-B	0.04	0.44
β_2-M	0.05	0.49
Mitcon:2	0.05	0.69

Results obtained by computer-based analysis of the autoradiograms of Fig. 3. Proteins are as indicated in Figs. 1 and 3. ^aFraction of protein found in whole cells that remained after extraction with pH 7.8 NP-40/sucrose extraction buffer A (described in *Methods*). ^bFraction of total (cytoplasts + karyoplast) present in karyoplast. Results were normalized on the abundance of Nuc:1.

in PMA-treated cells as compared with controls (both labeled in methionine-deficient medium) constitutes the largest class of differences observable in the PMA induction system. PMA induces a variety of other changes in HL-60, presumably reflecting monocytoid differentiation, which are independent of Metmin effects—i.e., they occur in either methionine-deficient or complete media (37). Cells concurrently starved for methionine and treated with PMA for 24 h show only a slight diminution of Metmin production as compared with methionine-starved controls, suggesting that the PMA effect involves differentiations changes (taking at least a day) rather than rapid regulational changes (such as the PMA-induced protein phosphorylations, which usually are complete within several minutes of treatment).

Metmin Production in Other Cell Types

Methionine starvation causes the appearance of Metmin proteins (Figure 4) in the cell line CCL-119, a T-lymphoblastoid line that can be made to differentiate into a suppressor-like cell by treatment with PMA (38), and in U-937, a human histiocytic lymphoma cell line (39) whose insulin responsiveness is altered by PMA (40). Metmin proteins are not detectable in methionine-starved fibroblasts (at least 70 lines were tested; 41) or epithelial cells (HTB-3, BT-20), and are generally absent in all normally attached cell lines. They are produced only occasionally and in low amounts by a non-PMA-inducible lymphoblastoid line such as GM607 or GM1500. Fresh normal human peripheral lymphocytes show low concentrations; high concentrations are seen in mononucleosis (Metmin:1 being the same as Inmono:2 of ref. 36) or in some cases of leukemia (44). The latter instances are characterized by the presence of "immature" cells, perhaps similar in some ways to the susceptible cultured-cell lines used. As in the case of HL-60 cells, PMA-treated (differentiating) cells of the lines CCL-119 and U-937 are no longer capable of producing Metmin in response to methionine starvation (Figure 4).

Morphological Changes Accompanying Metmin Production

The production of Metmin proteins is associated with the appearance of a peculiar altered morphology in some cells of the culture (Figure 5). As viewed by light microscopy, the nuclear material becomes dense, compact, and fragmented into a cluster of small bodies. The cell itself becomes similarly fragmented, and looks like a cluster of grapes. At this stage the cell/cluster still excludes Trypan Blue and is probably still at least partly functional. Other cells in the same culture can appear completely intact, and at least some of these are viable as evidenced by their ability to continue growth after restoration of methionine. The cell/clusters finally disperse into small independent globules, many of which retain a dense fragment of chromatin, and which appear to retain membrane integrity for one or more

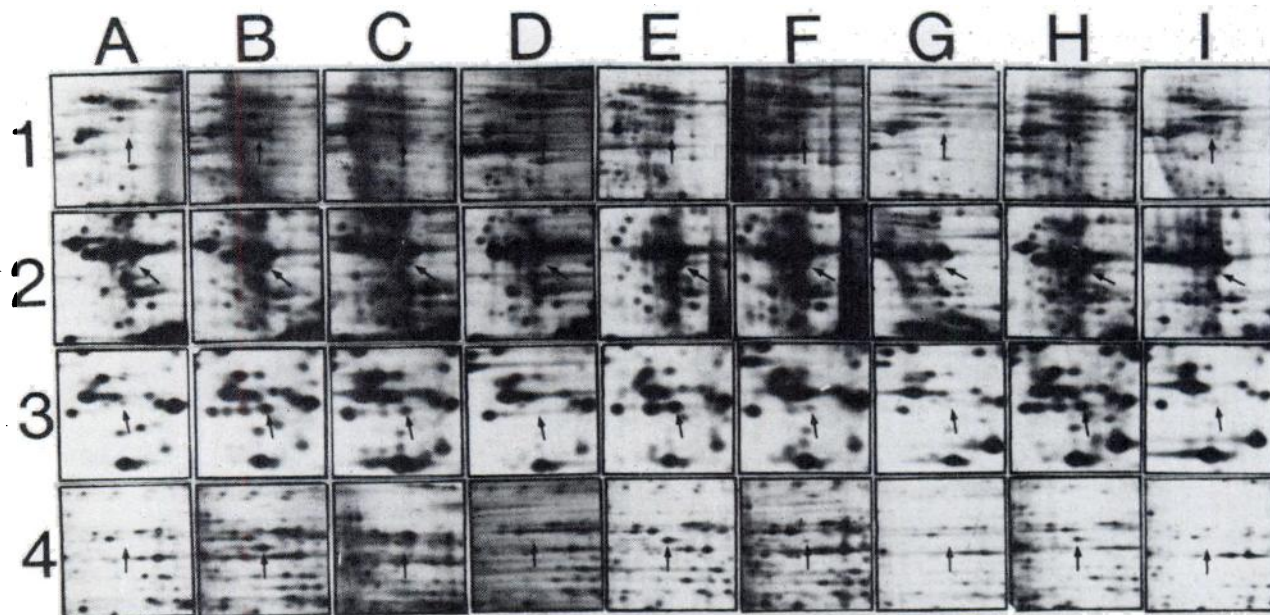


Fig. 4. Regions of two-dimensional patterns surrounding Metmins 1-4 (indicated by arrows)

(A-C) HL-60 cells, (D-F) CCL-119 cells, and (G-I) U937 cells labeled in complete medium (controls: A,D,G), in methionine-deficient medium (B,E,H) showing appearance of Metmins, and labeled in methionine-deficient medium (C,F,I) after pretreatment for 24 h with PMA (10^{-7} mol/L). PMA treatment largely prevents appearance of Metmins

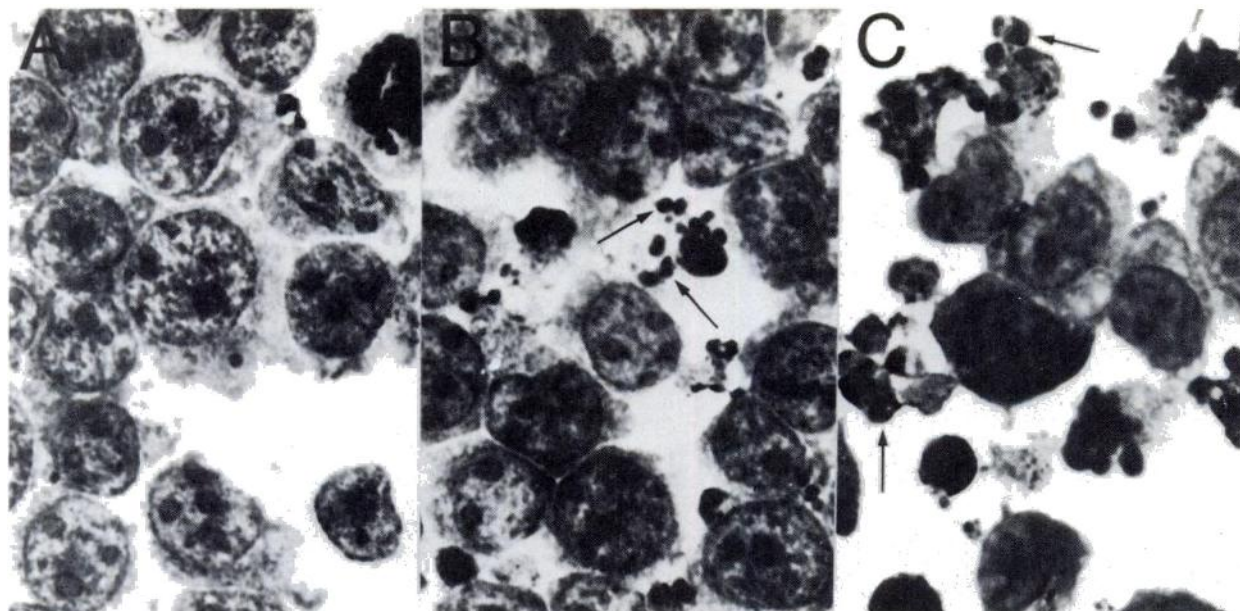


Fig. 5. Giemsa-stained cytocentrifuge preparations of HL-60 cells grown in complete medium (A) and after two (B) or three (C) days of incubation in methionine-deficient medium

Dense bodies (arrows) surrounded by bulbs of cytoplasm accumulate in the culture as normal cells progressively disappear. The morphological changes parallel the appearance of Metmin proteins

days. Those cell types that do not produce Metmin proteins during methionine starvation (fibroblasts, most lymphoblastoid cells examined, etc.) do not appear to show the grape-cluster morphology.

Discussion

A well-defined group of proteins (the Metmin set) is produced in cells of the human promyelocytic leukemia line HL-60 by either starvation for methionine or treatment with 5-azacytidine. A concomitant reduction in certain nuclear proteins is observed. The effect appears to be amino acid specific, since starvation for leucine, phenylalanine, or serine does not produce it. Homocysteine, an amino acid which can be *S*-methylated intracellularly to yield methionine, can substitute for methionine in Met-deficient medium so long as there is sufficient tetrahydrofolate (and vitamin B₁₂) to provide regenerating methyl groups. Treatment with 5-AC results in Metmin production, but presumably by a different route, because the compound is not believed to participate in or inhibit methionine or SAM biosynthesis. 5-AC does, however, irreversibly inactivate an important cellular user of SAM, namely the DNA methylase involved in maintaining symmetric methylation of CpG sequences in nuclear DNA (24). The two main treatments capable of causing Metmin production are thus likely to have in common an inhibitory effect upon DNA methylation, due in one case to depletion of methyl-donating substrate (SAM) and in the other to inactivation of enzyme.

Subcellular localization studies indicate that Metmin proteins are found predominantly in the cell nucleus. Cells divided into cytoplasm and karyoplast portions by cytochalasin B treatment and centrifugation show a pronounced enrichment of Metmins in the karyoplast (nucleus-containing) fraction. Likewise, preparations of detergent-insoluble cytoskeletons, which include the nuclei as well, also contain the bulk of cellular Metmin. The same approaches show that the principal proteins that decrease after methionine starvation or 5-AC treatment are a subset of normal nuclear proteins. In view of the evidence that Metmin proteins are not synthesized as such, but rather produced by modification of pre-existing molecules, it is tempting to speculate that at

least some Metmins are altered versions of normal nuclear proteins. No clear assignments of precursor-product relationships have yet been made, however.

Interesting morphological alterations occur in cultures subject to treatments associated with Metmin production, and these also point to effects in the nucleus. Initially a small number of cells begin to show condensed, and then fragmented, nuclei, followed by subdivision of the cell to give a bunch-of-grapes appearance. This change gradually appears in a larger proportion of the cells present, until all the cells are dispersed into a suspension of roughly spherical microcells, the whole process taking two to three days. Under the same treatment conditions, insensitive cell types such as fibroblasts or typical lymphoblastoid lines show no evidence of this grape-cluster formation.

The nature of the Metmin-producing treatments, the location of the Metmin proteins, and the morphological changes associated with Metmin production all suggest a relationship between methionine metabolism and a series of protein-modification and structural events in the cell nucleus. The mechanism by which these effects are produced, and why they occur in some tumor cell lines and not in others, remains unclear, however. Several studies have found evidence of abnormal rates of methionine uptake and consumption by tumor cells. Tisdale (2, 3), comparing the methionine-requiring Walker rat mammary carcinoma with other tumor cell lines, observed that substitution of homocysteine for methionine caused a cessation of growth in the Walker line attributable to higher methionine requirement, possibly linked to an enhanced methylating activity. Levels of both methionine-forming methionine synthase and SAM-using tRNA methylase were increased by methionine starvation. Tautt (6) observed that transformed rat embryo fibroblasts take up methionine about twice as fast as do the analogous untransformed cells, and concluded from a kinetic analysis that the probable reason was more rapid depletion of intracellular methionine pools in the tumor cells rather than an alteration in membrane transport. The principal thrust of these various investigations was to explain the fact that some, but not all, transformed cells display an absolute growth require-

ment for methionine even though they apparently have active enzymes capable of making Met from added homocysteine. So far no convincing proposals regarding the nature of an intracellular methionine-sink appear to have emerged. Although in none of the methionine metabolism studies to date have HL-60 cells been used, it seems fair to conclude that a variety of tumor lines display strange, and so far poorly understood, defects in methionine metabolism consistent with an abnormally high requirement for methionine or SAM. Preliminary experiments examining the disappearance of radiolabeled methionine from the medium during culture of HL-60 suggest, as might be expected, that these cells exhaust the amino acid abnormally rapidly while growing at normal rates (N. L. A., unpublished).

Perhaps the most intriguing aspect of the Metmin effect is its relationship to differentiation. HL-60 cells treated with active phorbol ester tumor promoters such as PMA differentiate over a period of days into cells that are in many respects similar to macrophages (26). This differentiated state is no longer as susceptible as the parental cells to methionine starvation. Metmin proteins fail to appear, and the "grape-cluster" morphology is absent when PMA-treated cells are deprived of the amino acid. The effects of 5-AC are similarly diminished. In addition to HL-60, we have examined two other cell lines responsive to PMA and found the same results. After treatment with PMA, the T-lymphoblastoid line CCL-119 and the histiocytic lymphoma line U-937 both show changes in their patterns of gene expression indicative of differentiations events (38, 40). Both produce Metmin proteins and show grape-cluster morphological changes when starved for methionine, and both are essentially resistant to methionine starvation effects after treatment with PMA leading to differentiation. So far we have not tested any other PMA-responsive cell types, and so have not yet found any case in which a PMA-responsive cell failed to show the Metmin effect during methionine starvation. Conversely, we have not yet found an example, among some 20 various cell lines plus 80 fibroblast-like lines examined, of a cell that produced substantial Metmins under the relevant conditions but which, when tested, was not PMA responsive. Fresh peripheral leukocytes from mononucleosis and leukemia patients have not yet been tested for PMA responsiveness by use of two-dimensional electrophoresis, but would, according to the proposed relationship, be expected to show some gene expression changes on PMA treatment. On the basis of the evidence obtained so far we conclude that there may be an important correlation between a cell's ability to differentiate further on PMA treatment and some defect in methionine or SAM metabolism that makes it susceptible to the Metmin and other effects described here.

The influence of PMA-induced differentiation of HL-60 on cellular processes involving SAM is well documented. PMA causes an increase of as much as twofold in phospholipid methylation (42) and increased synthesis of spermidine and putrescine (43). The increase in phospholipid methylation involves an apparent induction of the relevant methyltransferase, but an alteration in SAM levels was not excluded. Increased spermidine and putrescine synthesis did not involve increases in S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50) or ornithine decarboxylase (EC 4.1.1.17) activities, leading to the suggestion that effects other than induction of known enzymes were involved (43). In the present context, we may note that these results are consistent with the hypothesis that SAM or methionine is being rapidly utilized for some unrecognized purpose in untreated

HL-60, and that PMA-induced differentiation restricts this loss, leading to increased availability of SAM for various other uses.

A second hypothesis may explain how some of the effects of decreased methionine (and hence SAM) availability are manifested in sensitive cell lines. DNA synthesis does not appear to be stringently linked to methylation of new CpG sites, and thus does not stop abruptly upon withdrawal of methionine (7). Nevertheless, mammalian cells apparently cannot, in general, continue replicating DNA for very long in the absence of methylation; indeed, this effect may explain in part the properties of 5-AC as a drug. A possible explanation of the morphological changes observed in methionine-starved HL-60 may be that these cells continue to synthesize DNA under circumstances in which it cannot be methylated. Accumulation of unmethylated DNA may disrupt nuclear structures, and the observed decrease in normal nuclear proteins may result from the failure of newly synthesized nuclear proteins to find a suitable site in the unmethylated DNA. Partial degradation of free nuclear proteins, as well as others, could result in the appearance of the Metmin polypeptides. PMA-treated cells would not show such an effect because they can no longer divide, and hence do not synthesize unmethylatable DNA. Under this hypothesis, HL-60 and other susceptible cells would suffer from an uncoupling of DNA replication control from methylation capacity. The survival (without growth) of non-PMA-inducible cells for extended periods in methionine-deficient media would reflect the fact that they do not try to divide.

If the suspected relationship between susceptibility to the Metmin effect and PMA-responsiveness is confirmed, then we would expect by analogy that treatment with 5-AC should have a much more toxic effect on methionine-starvation-sensitive, PMA-sensitive cells than on normal ones. Only a few leukemias show high Metmin production under conditions of methionine starvation (44), and these may be far more sensitive to the drug than the others. Since PMA responsiveness can be observed rather easily in most cases (causing attachment of cells to plastic culture dishes), this correlation might provide a useful predictive measure of the efficacy of 5-AC treatment.

These studies demonstrate the applicability of two-dimensional electrophoretic methods to the detailed analysis of pharmacological effects. They also suggest a new class of drug effects, namely specific cleavage of pre-existing cellular proteins in response to drug treatment.

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