THE SYNTHESIS OF NINETY PROTEINS INCLUDING ACTIN THROUGHOUT THE HE LA CELL CYCLE

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ABSTRACT

Abundant cytoplasmic proteins pulse-labeled with [35S]methionine at specific times throughout the HeLa cell cycle were analyzed with two-dimensional gel electrophoresis. More than 300 proteins could be resolved in this way. The frequency of appearance of label in the most abundant 90 proteins, ranging from 4% to <0.1% of the total methionine incorporated, was determined at six time points in the cell cycle. 84 of these proteins were made as a similar proportion of the total at all times during the cell cycle. A nonmuscle actin protein (spot 1) identified by molecular weight and isoelectric point represented 2-4% of the total methionine incorporated at all the time points. Only six proteins were found which varied by greater than fourfold during cell division, four appearing to represent a greater proportion of the total synthesis during the period at or immediately surrounding M (spots 31b, 44, 53, and 70d). Two appear to represent a smaller percentage of total synthesis during the early (spot 78) or the total (spot 74) G2 period.

KEY WORDS 2-dimensional gels · cell cycle · synchrony · protein synthesis · actin

The mammalian cell in culture grows and divides in a well regulated and timed series of events. During S phase the DNA is synthesized, during M the cell divides, and between these are two gaps: G2, post DNA synthesis, and G1, postmitosis (16). Cell surface changes occur during the cycle as do the activities of a variety of enzymes (16). Protein synthesis drops 75% at mitosis as a result of a block in initiation (5). Most RNA synthesis is shut off at mitosis except for some small molecular weight species (23, 25). The mRNA persists through mitosis, however (21, 8). The synthesis of bulk chromosomal bound proteins goes on throughout the cell cycle (19) although some small proteins may be made only in S (6). These proteins showed different profiles on sodium dodecyl sulfate (SDS) gels depending on the cell cycle and changes in the rate of synthesis before DNA synthesis (20, 2). Kolodny and Gross (10) analyzed total cytoplasmic proteins synthesized during the HeLa cell cycle on one-dimensional SDS gels and reported variations in certain proteins.

More detailed analysis of proteins is made possible by the two-dimensional technique (isoelectric focusing followed by SDS electrophoresis) developed by O'Farrell (15). We analyzed proteins synthesized by cells in early and late S, G2, M, and thymidine-arrested G1 by the two-dimensional technique. We have found that, of the 90 relatively abundant cytoplasmic proteins studied by frequency analysis, only eight proteins vary by greater than fourfold during the cell cycle. Nonmuscle actin (7, 17, 24) was found to represent a relatively constant percentage of total incorporation (2-4% of total) at all times studied.

MATERIALS AND METHODS

Cell Culture

HeLa (S3) cells were grown in suspension at 37°C in Eagle's medium (4) supplemented with 7% horse serum (Flow Laboratories, Inc., Rockville, Md.). Cell cultures were tested by Microbiological Associates, Walkersville, Md., and found to be free of mycoplasma. Cells were
Electrophoresis of Proteins

acrylamide, and 5% ampholines (LKB Instruments, England Nuclear, Boston, Mass., 6.7 Ci/mmol) incorporated and autoradiography (see Results). Cells used for autoradiography were labeled with 10 μCi/ml [35S]methionine for 30 min, and nuclear and cytoplasmic fractions were obtained. Colcemid and nuclei plus and minus colcemid.

frequency Analysis

Gels were stained with Coomassie brilliant blue to visualize protein standards and then fluorographed, dried, and exposed to X-ray film by the method of Laskey and Mills (12). Film was developed after 1.5, 3, 6, 12, 24, 48, and 96 h of exposure to the gel. The spots appearing at 96 h on the 6 h post release cytoplasm sample were labeled 1 (actin) through 90. Other gels were compared with this standard gel, and as many of the 90 spots as possible were identified by number on the basis of molecular weight, isoelectric points, and relative positions. The time of appearance of all spots on the X-ray film was then determined, and each numbered spot was assigned a relative intensity number.

We assume that the amount of radioactivity in a peptide spot is inversely proportional to the time required for a visible spot to develop (15). Relative Intensity (R.I.) for a spot appearing on film at a given time can be calculated by the following formula:

\[
R.I. = \frac{E_{n} \times E_{1}}{n_{1} \times E_{n} + n_{2} \times E_{n} + \cdots + n_{n} \times E_{n}}
\]

where \( E_{n} \) = longest exposure time used; \( E_{1} \) = exposure time at earliest time with all \( E \)'s increasing by a factor of 2 from the previous \( E \); and \( n_{1}, n_{2}, n_{3}, \ldots, n_{n} \) = the number of new spots seen at times 1, 2, 3, etc. The denominator in the equation is therefore the weighted sum of all the peptides counted.

The relative intensity numbers were compared for each spot at all times throughout the cell cycle. The appearance of spots not present in the standard gel was noted and the relative frequency of these spots was determined in an analogous manner. The relative frequency of a readily identifiable spot varied about twofold from gel to gel at the same time in the cell cycle. All gels were electrophoresed and analyzed on at least two or three separate occasions to normalize for the twofold variation.

Electrophoresis of Histones

The technique was that of O'Farrell (15). The first dimension was isoelectric focusing with 9 M urea, 4% acrylamide, and 5% ampholines (LKB Instruments, Inc., Rockville, Md.; pH 5–8: pH 3.5–10 at 4:1 ratio) in cylindrical gels. The second dimension was a 10% acrylamide discontinuous SDS gel (pH 8.8) with a 5% stacker gel (pH 6.8). Protein standards of known isoelectric points and molecular weights were routinely run as standards. Sample volume applied to the first dimension was adjusted so that 106 counts were applied. The concentration of the protein samples was determined by the method of Lowry et al. (14). Specific activities of the protein samples were 28,000–53,000 cpm/μg for cytoplasmic proteins and 6,000–25,000 cpm/μg for nuclear proteins in S, G\(_1\), and G\(_2\), and 16,000–22,000 cpm/μg and 10,000–14,000 cpm/μg for M cytoplasm and nuclei plus and minus colcemid.

Cell Synchronization and Labeling

Cells were trapped at the G\(_1\)/S boundary by a double thymidine block (20) with 2 mM thymidine. A portion of the cells were released from the second thymidine block by resuspending them in fresh medium at 37°C. Under these conditions, S lasts for 7–8 h and S and G\(_2\) about 9.5–10.5 h. At 3, 6, 9, 11, and 13 h after release from thymidine, a portion of the cells were resuspended at 2 x 10⁶ cells/ml in medium with one-tenth the normal amount of methionine, 7% dialyzed horse serum, and 100 μCi/ml [35S]methionine (Amersham Corp., Arlington Heights, Ill., 700–1,400 Ci/mmol). The cells were incubated with the radioactive label for 1 h at 37°C, washed, and lysed with a small volume of reticulocyte standard buffer (RSB) (10 mM NaCl, 10 mM Tris pH 7.4, 1.5 mM MgCl\(_2\) plus 1.0% NP-40 (Particle Data, Inc., Elmhurst, Ill.). Cytoplasm and nuclei were individually treated with pancreatic ribonuclease at 20 μg/ml at 4°C for 10 min after adjusting the solution to 0.3 M NaCl. Concentrations were adjusted to those of O'Farrell's sample buffer (15).

Mitotic cells were prepared by resuspending cells in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y. [Gibco]) and allowing them to attach to 250-μl plastic culture bottles (Nunc) for 1 h. Cells that did not adhere were removed. 3 h later, the bottles were again gently shaken and nonadhering cells removed. 30 min later, the bottles were shaken and the rounded metaphase cells were detached with a small volume of medium containing colcemid at 0.6 μg/ml and nuclei plus and minus colcemid.

Synchrony was determined by [3H]thymidine (New England Nuclear, Boston, Mass., 6.7 Ci/mmol) incorporation (20) and autoradiography (see Results). Cells used for autoradiography were labeled with 10 μCi/ml [3H]thymidine in suspension for 10 min, washed, resuspended in RSB, and fixed on slides with ethanol:chloroform:acetic acid (6:3:1).

Electrophoresis of Proteins

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fraction in a modified two-dimensional system. The first dimension consisted of an acid: urea gel plus DF 16 with 12% acrylamide (1) in cylindrical gels followed by a discontinuous 22% acrylamide gel (11) in the second dimension. Calf thymus histones (Sigma Chemical Co., St. Louis, Mo.) were used as markers. Gels were stained, fluorographed, and exposed for various times as described above.

**Chymotryptic Peptide Analysis of Actins**

Purified rabbit skeletal muscle actin (18) was the generous gift of Dr. James Schwartz. HeLa actin labeled with [³⁵S]methionine was purified from cell cytoplasm on DNase agarose (13) and produced a single spot on two-dimensional gels.

Spot one protein and DNase-agarose purified actin were located on the SDS (second) dimension gel by exposing the gel to X-ray film, cut out with a razor blade, and electrophoretically eluted. Eluted proteins were precipitated by the addition of 20% trichloroacetic acid on ice after addition of 100 μg of rabbit muscle actin as carrier. The pellet was collected after centrifugation at 8,000 × g, washed three times with acetone, resuspended in 0.375 ml of a solution containing 0.125 Tris-HCl (pH 6.8), 0.001 M ethylenediamine tetraacetic acid, 0.5% SDS, and incubated at 100°C for 2 min. The reaction mix was transferred to 37°C. 20 μg of chymotrypsin were added, and the mix was incubated for 30 min. The peptides generated were analyzed on a 15% acrylamide SDS gel (18), stained with Coomassie blue, fluorographed and exposed to X-ray film (12).

**RESULTS**

**Cell Synchrony**

Cells obtained by shake-off or colcemid arrest and shake-off were examined in the light microscope. 85–90% of the shake-off cells were judged mitotic by appearance. Over 90% of the colcemid-arrested cells were mitotic. Cells not treated with drugs proceeded through mitosis and changed appearance to interphase cells within 30 min of harvesting. We also observed the 75% drop in amino acid incorporation in the mitotic cells as compared to an unsynchronized population seen by others (8, 21).

Cells blocked twice by thymidine were monitored for synchrony by two techniques: autoradiography and histone synthesis. Cells labeled with [³[H]Tdr were fixed to slides and processed for autoradiography. The number of cells with grains on the nuclei was determined from the population of synchronized cells pulse-labeled at various times. The data shown in Table I indicate that, at 2.5 and 5 h post thymidine release, over 90% of the cells are synthesizing DNA at an appreciable rate and are hence in S phase. At 8–10 h post release, 97% of the cells cease DNA replication and enter G2. At 12 h post release, the number of cells with visible chromosomes and mitotic plates increases dramatically and represents 18% of the total cells. By these criteria, the cells are better than 90% synchronized.

We also monitored the amount of incorporation of methionine into proteins behaving like histones on gels during the cell cycle in our synchronized population. The data are shown in Table II. The amount of incorporation into proteins comigrating with calf thymus H2A, H2B, H3, and H4 histones at 3 and 6 h post release from thymidine block was >10 times that incorporated into cells at 0, 9, 11, and 13 h after release. It was also >20 times that incorporated into mitotic shake-off and colcemid-arrested cells. Histone synthesis occurs primarily during the S phase (3), and by these criteria our population is well synchronized.

**TABLE I**

| Hours post TdR release | With grains | No grains | With labeled nuclei | Cells with visible chromosomes |
|------------------------|-------------|-----------|---------------------|------------------------------|
| 2.5                    | 160         | 5         | 97                  | 23.7                         | 0                            |
| 5                      | 158         | 17        | 90.3                | 39.4                         | 0                            |
| 8                      | 18          | 82        | 18                  | 15                           | 3                            |
| 10                     | 3           | 97        | 3                   | 13                           | 5                            |
| 12                     | 6           | 109       | 5.2                 | 11                           | 18                           |
| 15                     | 6           | 94        | 6                   | 12                           | 2                            |

**TABLE II**

| Time of labeling | Relative intensities of H2A, H2B, H3, and H4 |
|------------------|---------------------------------------------|
| 0 h of [³[H]Tdr release | 0.1 |
| 3 h of [³[H]Tdr release | 1.0 |
| 6 h of [³[H]Tdr release | 0.9 |
| 9 h of [³[H]Tdr release | 0.1 |
| 11 h of [³[H]Tdr release | 0.1 |
| 13 h of [³[H]Tdr release | 0.1 |
| Mitotic cells | .05 |
| + Colcemid | .05 |

* 1 h of [³[H]Tdr labeling |

† 30 min of [³[H]Tdr labeling

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Rapid Communications 835
Protein Analysis

The synchronized cells were pulse-labeled with methionine at 0, 3, 6, 9, 11, and 13 h after release from thymidine and during mitosis with shaken-off or colcemid-arrested cells. More than 300 cytoplasmic proteins could be resolved by electrophoresis on an isoelectric focusing gel at final pH 4.8–7.0 followed by SDS gel electrophoresis (see Fig. 1, A and B). Analysis of the relative amount of radioactivity in the various proteins demonstrates that they range from 4 (for protein 1) to 0.25% (for protein 17) of the total methionine incorporated at 6 h post release (See Table III). Comparison of the patterns obtained at various times shows remarkable similarities. Very few spots seem to differ from gel to gel and from time to time in the cell cycle. We then undertook a spot-by-spot analysis of the frequency distribution of 90 of the most characteristic and easily identifiable spots. Some data are shown in Tables III and IV. We conclude that throughout the cell cycle a large number of the 90 proteins that we have analyzed vary by less than a factor of two in their rate of synthesis as a percentage of total. Not all the data are shown for spots with twofold or less variation (Table III), merely some representative examples. The synthesis of just a few proteins varies by more than twofold (see Table IV). All such examples are shown in the table. Protein numbered 53 appears at much higher frequencies in cells labeled 11 h after thymidine release (corresponding to late G2). Proteins 31b and 70d appear at higher frequencies in cells labeled at 13 h (very early in G1). Protein 44 seems to be made both at M and early G1, while proteins 74 and 78

![Figure 1 Autoradiogram of [35S]methionine-labeled cytoplasmic proteins. Cells were labeled with methionine, and cytoplasmic proteins were electrophoresed in the first dimension in an isoelectric focusing gel. The second dimension was an SDS discontinuous gel. (A) cells labeled in mid S (6 h after TdR release); (B) cells labeled in M/G1 (early) (13 h post TdR release).](image)

Table III

Relative Intensities of Invariant Proteins During the Cell Cycle

| Spot no. | Hours post TdR: Cycle stage: | Relative intensity* of total (%) |
|----------|-----------------------------|---------------------------------|
|          | 0  | 3  | 6  | S | 9  | G1 | 11 | G1 + M | 13 | M + G1 | Mt |
| 1        | 3  | 4  | 4  | 4 | 2  | 2  | 2  | 2     | 2  |        |    |
| 2        | 4  | 4  | 3  | 4 | 2,5 | 2 | 2  | 2     | 2  |        |    |
| 9        | 0,4 | 1  | 1  | 0,5 | 0,6 | 0,5 | 0,8 |       |    |        |    |
| 12       | 0,2 | 0,2 | 0,3 | 0,4 | 0,3 | 0,2 | 0,4 |       |    |        |    |
| 17       | 0,18 | 0,2 | 0,25 | 0,17 | 0,1 | 0,2 | 0,2 |       |    |        |    |
| 21       | 1,5 | 2  | 2  | 2  | 2  | 2  | 2  | 1     |    |        |    |
| 51       | 0,2 | 0,2 | 0,3 | 0,2 | 0,2 | 0,2 | 0,3 |       |    |        |    |
| 54       | 0,3 | 0,27 | 0,27 | 0,4 | 0,5 | 0,5 | 0,4 |       |    |        |    |
| 82       | 2,5 | 2  | 2  | 2  | 2  | 3  | 2,5 |       |    |        |    |

Only a few representative examples of the 84 invariant proteins are shown.

* For calculation of R.I., see Materials and Methods section.
† Summation of data from ± colcemid mitotic shake-off, 30-min pulse-labeled.
show marked decreases at G₂ and G₁ plus G₀, respectively. In general, however, the large majority (84/90) of cytoplasmic proteins analyzed are synthesized at very similar rates throughout the cell cycle.

We wished to determine whether the similarities of protein spots observed were a result of methionine starvation because of the labeling schema. We therefore labeled cells at several stages (mid S, M, and G₁ blocked) with medium containing normal amounts of methionine and [³⁵S]Met. Analysis of these protein patterns on two-dimensional gels gave results identical to our previous results, indicating that methionine deprivation was not the cause of the very similar patterns.

Identification of Actin

The nonmuscle actins are abundant proteins in nonmuscle cells. We were able to identify spot number one on our gels as actin by its similar mobility with purified marker HeLa actin (9). We confirmed that spot number one was actin by comparing the chymotryptic peptides generated from spot one with those generated from actin isolated from (a) rabbit skeletal muscle (18), and (b) putative HeLa actin isolated on DNase agarose (13) which gave a single spot on a two-dimensional gel. The chymotryptic peptides from the [³⁵S]methionine-labeled protein eluted from spot one comigrated with the fragments obtained from rabbit muscle actin and DNase-agarose purified actin.

Spot number one represents 2–4% of the total methionine incorporated at all stages of the cell cycle (Table III). We have not separated the actins into the β, γ, δ, and ε forms in our gels.

DISCUSSION

We have shown that many cytoplasmic proteins including actin are synthesized at similar frequencies throughout the cell cycle. The cells were all synchronized as determined by visual inspection, thymidine incorporation, autoradiography, and histone synthesis. The rate of synthesis of six proteins has been shown to vary with the cell cycle.

These observations of the synthesis of proteins are dependent to some degree on our methods of cell synchronization. The double thymidine block interferes with normal DNA synthesis, and our criteria for synchrony: thymidine incorporation, histone synthesis, and appearance of mitotic cells are related to DNA synthesis. Other features of the cell cycle may not be tightly coupled to the DNA synthesis clock per se, and our cells may not be synchronous with respect to these other features.

Our results do, however, lead us to conclude that synthesis of many of the cellular “housekeeping” functions proceeds regardless of the state of the DNA synthesis program. The similarities of the proteins synthesized even at M when the total incorporation drops 75% is remarkable. Any fluctuations which occur in the spectrum of proteins synthesized during the decreased initiation rate (5) found at M must be of order of twofold or less. We can therefore conclude that the mRNA’s for the 84 abundant proteins are very efficient messages in the initiation of protein synthesis or that they are present in large amounts of all cell cycle stages. Actin protein which is a ubiquitous cellular component is synthesized at all times during the cell cycle, presumably to maintain vital cellular structure and function.

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