

FLUCTUATIONS IN THE PRODUCTION OF SPECIFIC CELLULAR PEPTIDES DURING THE GROWTH OF ANIMAL CELLS

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ABSTRACT

Patterns of newly synthesized proteins of Vero cells in different growth states were obtained using two-dimensional gel electrophoresis. The 240 most prevalent peptide spots were then compared. Cells in exponential growth and in the stationary phase were found to have patterns of peptide spots characteristic of their state of growth. The transition between these patterns is progressive, and the cells acquire a pattern characteristic of quiescent cells by the late exponential phase. These observations suggest that a series of modulations in gene expression occurs during the transition of growth states in animal cells that leads to the specific appearance or disappearance of certain cellular peptides.

KEY WORDS two-dimensional acrylamide · growth-peptide production · autoradiography · cultured animal cells · density-dependent regulation

Cultured fibroblasts may exist in two metabolically distinct states: growing and resting (13, 18). The transition from one state into the other is reversible. Factors known to mediate this transition include cell density, essential nutrients, and growth factors (reviewed by Holley [10]). This reversible property of cultured cells provides a convenient system for the elucidation of gene activities specifically related to proliferation.

Past studies in this area have centered on the analysis of variations of selected proteins or enzyme activities in cells in different states of growth (2, 3, 6, 8, 9, 14, 16), and have demonstrated that some proteins or enzymes varied in amount or activity under different growth conditions. These findings also provided indications that the expression of genetic information varies with growth conditions. However, in these studies, only selected protein species were investigated. Because the parallel comparison of changes of many gene products is not available, it is not clear whether these growth-related variations in protein production occur simultaneously during the transition of cell growth or in a sequential fashion.

In this study, we monitored the production of

numerous protein species as a measure of gene expression. The newly synthesized cellular proteins were separated by two-dimensional gel electrophoresis. With these gels we were able to measure the variations in production of individual peptides as cells progress through the exponential phase into the stationary phase. Our results indicate that the production of many of these proteins varies during growth and that these variations do not occur simultaneously but at many times through the growth period.

MATERIALS AND METHODS

Cells and Cell Growth

Vero cells used in this study are a line derived from an African green monkey kidney (19). They are epithelial-like and require serum for growth (7). Cells in early exponential phase were used to initiate the growth cycle reported here. Cells were plated at a density of 1×10^4 cells/cm² in 35-mm tissue culture dishes. Growth medium of Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, New York) and 10% (vol/vol) calf serum (Flow Laboratories, Rockville, Md.) was changed daily. Cell number was determined by hemocytometer counting.

Radioisotope Labeling

To label cellular proteins, [³⁵S]methionine (600 Ci/

mmol, New England Nuclear, Boston, Mass.) at 100 μ Ci/ml in methionine-free Dulbecco's modified Eagle's medium plus 10% dialyzed calf serum was used. Growth medium was removed from the monolayer, and 0.5 ml of prewarmed labeling medium was added. Cells were incubated in a 37°C incubator for 1 h with occasional agitation. At the end of the labeling period, monolayers were washed once with ice-cold phosphate-buffered saline (150 mM NaCl, 4 mM KCl, 9.5 mM phosphate, pH 7.2), lysed immediately with 300 μ l of cell lysis buffer (solution A as described by O'Farrell [15]), and stored at -70°C. 1 μ l of the cell lysate was used to measure TCA-precipitable radioactivity.

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (15) except that (a) ampholines (LKB) pH 5-8 and 3.5-10 were used instead of pH 5-7 and 3-10; (b) sodium dodecyl sulfate (SDS)-10% polyacrylamide gels were used for second-dimensional separation. The protein concentration of each sample was estimated as described (12), and comparable amounts of protein were loaded on each gel. For each sample, amounts varying 2.5-fold were subjected to electrophoresis at each run. The pattern of cellular proteins of each sample was determined by at least two independent electrophoresis trials. Those spots consistently present in all four to six determinations were considered as characteristic polypeptides for that particular sample and compared from sample to sample.

Fluorography, as described by Bonner and Laskey (1), was adopted to shorten the length of exposure time approximately tenfold. Usually, the appropriate exposure time was used to bring out approximately the same number of spots in a given area. The pH value of the isoelectric focusing gels was measured using a parallel gel. 0.5-cm gel segments were soaked in 1.5 ml of degassed water for 1 h. The pH value of this eluate was then determined with a pH meter. The reproducibility of identifying polypeptides in the pH range of 6.5-5.3 is absolute. However, peptides migrating in the acidic or basic ends of the first-dimensional gel were sometimes difficult to identify.

The measure of the relative intensity of individual peptide spots on the autoradiograph was used as the measure of the rate of production of that peptide relative to the total assayed. The relative intensity was determined as the length of exposure required before the peptide spot became visible. For example, if spot A became visible after 4 h of exposure while spot B required 8 h of exposure to become visible, we concluded that spot A was twice as intense as spot B, and therefore the rate of production of peptide A was twofold that of peptide B. Exposure times varied from 30 min to 256 h, and the length of exposure was doubled exponentially. (Thus 10 different exposures were used in these determinations.) The assumption in this measurement is that the amount of radioactivity in a peptide

spot is inversely proportional to the time required for a visible spot to develop (15). The relative amount of individual peptides produced during the pulse-labeling period was then expressed as the percentage of the weighted sum of all of the peptides counted. A peptide was weighted by the time of appearance. For example, a peptide that appeared at 1 h was weighted 256-fold relative to one that appeared at 256 h. All separated peptides were counted (several hundred per gel) so that the sum of the relative intensities of the 60 peptides reported in Table I would not be expected to equal 100%.

RESULTS

Patterns of Protein Synthesis in Cells at Different Growth States

Vero cells were plated at a density of 1×10^4 cells/cm². The maximal rate of cell division was achieved immediately after plating. On days 9 and 10, the rate of cell division declined, and the saturation density was reached on day 11 (Fig. 1). To analyze the peptides being produced in cells in different states of growth, day 4 cells (exponential growth) and day 10 cells (stationary phase) were incubated for 60 min in the presence of [³⁵S]methionine, and the cellular proteins were subjected to two-dimensional gel electrophoresis. The protein synthetic patterns are shown in Fig. 2. Out of the large number of peptides in the autoradiographs, 240 of the most prevalent polypeptides were numbered and cataloged. By comparing the intensity of these individual peptide spots with those of other spots in the vicinity, the fluctuation in the production of individual peptides was assayed. The types of variations observed include preferential reduction of production in stationary phase (for example, peptides 23, 24, 27, 30, 38, 39, 68, 88, 112, and 114), and preferential enhancement of production in stationary phase (for example, peptides 76, 80, and 234).

The relative intensity of individual peptides was then determined as described in Materials and Methods for days 2-4 which were arbitrarily designated exponential phase and for days 10-12 which were arbitrarily designated stationary phase. Table I lists the relative intensities for 60 such peptides including those demonstrating the most obvious changes. Again a population of peptides was found to be produced to a greater extent (by at least threefold) in exponential phase cells (23, 24, 27, 30, 38, 39, 68, 88, 101, 104, 112, 113, and 114), while the production of

TABLE I
Relative Production of Individual Peptides

Peptides	Exponential (days 2, 3, and 4)	Stationary (days 10, 11, and 12)	Peptides	Exponential (days 2, 3, and 4)	Stationary (days 10, 11, and 12)
	% of total			% of total	
23	0.2 ± 0.06	<0.01	96	0.09 ± 0.3	1 ± 0.5
24	0.08 ± 0.04	<0.01	97	0.9 ± 0.3	1 ± 0.5
27	1 ± 0.6	0.5 ± 0.03	101	0.9 ± 0.3	0.1 ± 0.07
28	0.1 ± 0.05	0.2 ± 0.06	102	0.7 ± 0.1	1 ± 0.5
29	0.2 ± 0.09	0.2 ± 0.07	104	0.5 ± 0.09	0.1
30	1 ± 0.5	0.1	105	0.6 ± 0.11	0.4 ± 0.2
31	0.3 ± 0.1	0.1 ± 0.07	106	2 ± 0.4	3 ± 1
35	0.2 ± 0.09	0.2 ± 0.1	107	4 ± 0.5	4 ± 1.2
38	1 ± 0.5	0.2	112	0.3 ± 0.2	<0.01
39	0.4 ± 0.07	<0.01	113	4 ± 0.7	0.2 ± 0.1
49	0.8 ± 0.3	0.4 ± 0.2	114	0.3 ± 0.05	<0.01
50	0.6 ± 0.1	0.2 ± 0.10	121	3 ± 0.6	2 ± 0.03
51	0.6 ± 0.1	1 ± 0.6	122	3 ± 0.6	1 ± 0.4
56	0.09 ± 0.06	0.5 ± 0.3	123	3 ± 0.6	2 ± 0.5
57	0.2 ± 0.07	0.4 ± 0.4	124	3 ± 0.6	2 ± 0.4
58	0.8 ± 0.3	1 ± 0.4	148	0.07 ± 0.02	0.06
59	0.4 ± 0.1	1 ± 0.5	149	0.6 ± 0.3	0.1 ± 0.05
66	0.1 ± 0.02	0.1 ± 0.02	162	0.2 ± 0.07	0.09 ± 0.04
68	0.7 ± 0.01	0.01	163	0.2 ± 0.04	1 ± 0.4
69	0.1 ± 0.01	0.1 ± 0.04	165	0.05 ± 0.3	0.06
70	0.2 ± 0.07	0.3 ± 0.05	167	0.3 ± 0.07	1 ± 0.5
71	0.1 ± 0.02	0.2 ± 0.04	169	2 ± 0.02	1 ± 0.2
76	0.09 ± 0.04	1 ± 0.4	174	0.3 ± 0.09	0.5 ± 0.1
80	0.1 ± 0.08	1 ± 0.4	178	0.5 ± 0.02	0.53 ± 0.1
81	0.1 ± 0.08	1 ± 0.5	179	0.5 ± 0.20	2 ± 0.9
83	1 ± 0.5	1 ± 0.4	217	1 ± 0.03	0.8 ± 0.09
85	0.3 ± 0.2	1 ± 0.4	218	2 ± 0.03	0.8 ± 0.1
86	2 ± 1	1 ± 0.4	231	0.05 ± 0.09	1 ± 0.4
88	0.06 ± 0.03	<0.01	232	<0.009	0.08 ± 0.02
94	0.1 ± 0.05	0.04	233	<0.009	0.04 ± 0.01
95	0.3 ± 0.2	0.5 ± 0.2	234	0.009	0.1 ± 0.06

The relative production of individual peptides is determined as described in Materials and Methods. The numbers are the numerical average of three determinations. Also included is the standard deviation for each averaged value. Where a standard deviation is not included, this is because two of the three gels analyzed had no observable peptide spot.

another population of peptides (76, 80, 163, 231, and 234) was found to be preferentially enhanced (again by at least threefold) in stationary phase cells. Thus the differences observed by visual inspection of individual peptide spots are confirmed by the determination of their relative intensity.

Nature of the Transition of Protein

Synthetic Patterns

To monitor the time-course of the variation in the synthesis of individual peptides, the protein synthetic pattern of days 6 and 8 cells was ob-

tained and compared to those of days 2, 4, and 10, respectively. Fig. 3 illustrates examples of the fluctuation in the production of some individual peptides. A schematic summary of those peptides among the 240 assayed that display large variations is presented in Table II. As can be seen, the changes in the synthesis of these individual peptides occur at various times during the transition from exponential growth into stationary phase.

DISCUSSION

In this study, we measured the extent of production of many of the major protein species in

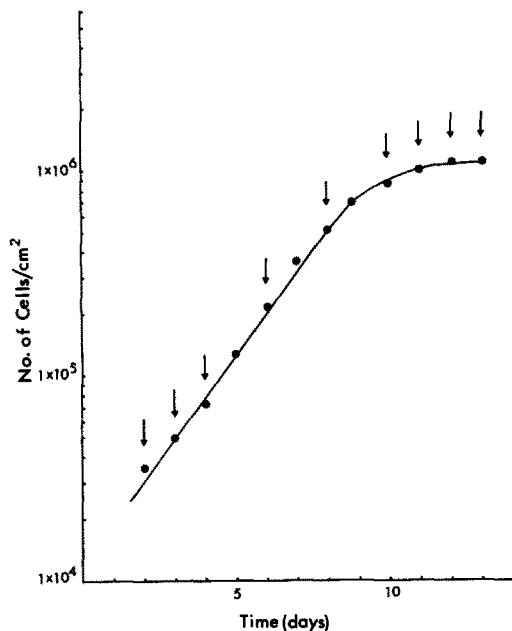


FIGURE 1 Growth cycle of Vero cells. Cells were plated at a density of 1×10^4 cells/cm². Growth medium was replaced daily. Stationary phase was reached at a density of 1×10^6 cells/cm². Arrows indicate the time-points in the growth cycle that cellular proteins were subjected to two-dimensional gel electrophoresis.

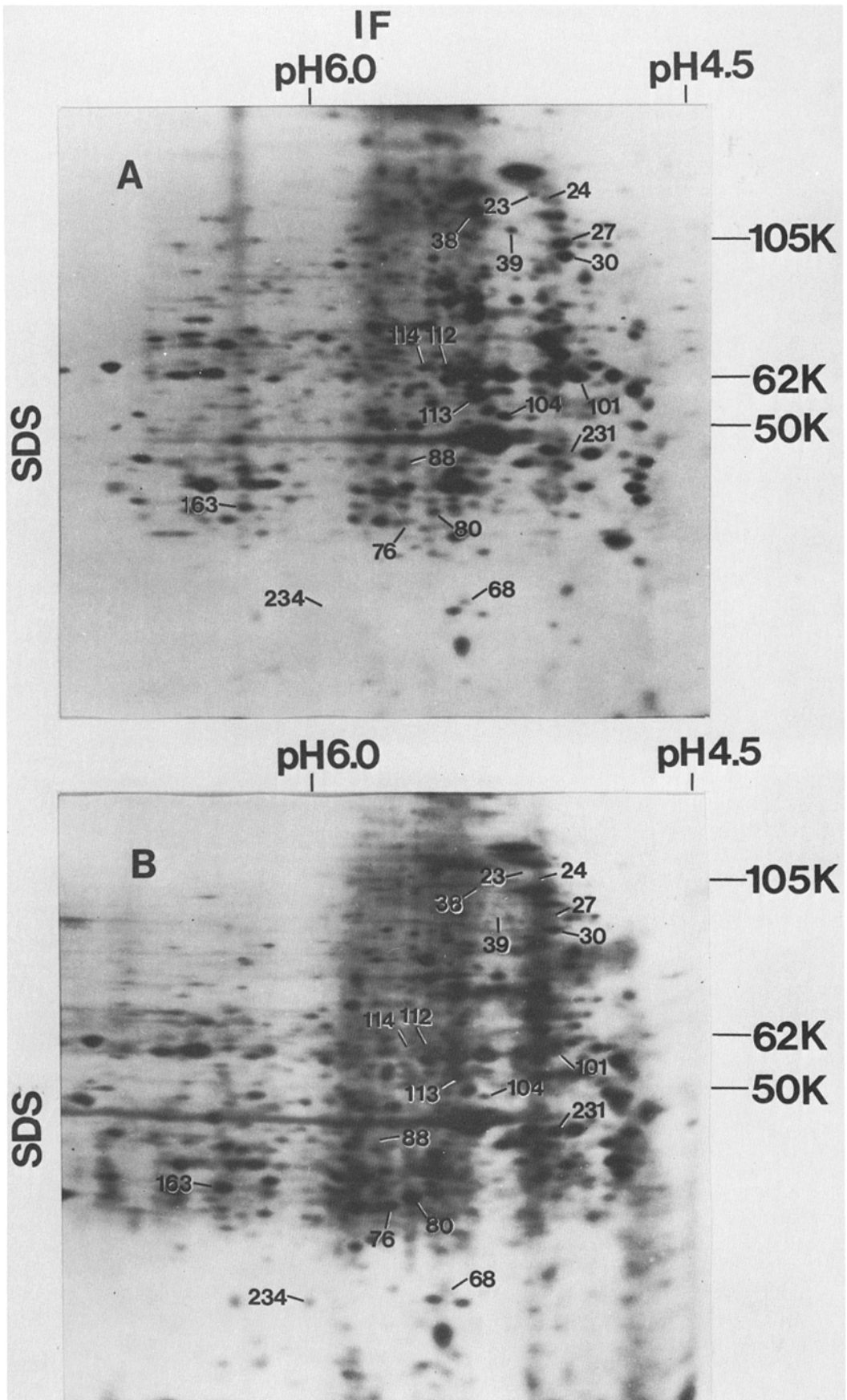
cultured cells as a function of time during growth into the stationary phase. When the patterns of newly synthesized peptides from exponential phase and stationary phase cells were compared, the relative production of certain peptides (i.e., the relative intensity of the peptide spots on an autoradiograph) was found to differ (Table I and Fig. 2). Differences of comparable magnitude have been reported by Ivarie and O'Farrell (11) who compared peptides produced in the rat hepatoma line HTC₄ plated at high or low cell densities.

We further observed that these changes in relative production occurred at various times throughout the time of the growth curve. Table II and Fig. 3 chronicle these changes for peptides differing in production by greater than threefold. Also the series of gels of peptides produced day-by-day throughout the growth curve showed many more peptides with significant daily differences than are presented in Table I. (Table I reports the averages of 3 days' production). Thus these data show that the production of many individual pep-

tides is not coordinately regulated during growth. It is not known whether these changes in production are due to differences in amount of functioning mRNA or to post-translational modifications of the products of these mRNA species. Subsequent data from this laboratory, assaying the product of *in vitro* translation of polysomal mRNA from exponential and stationary phase cells (Lee, G. T.-Y., and D. L. Engelhardt, manuscript in preparation), supports the latter hypothesis by showing many fewer differences in the amounts of functional mRNA species than would be expected from the data reported here.

These data report the relative production of individual peptides. Because the overall rate of protein synthesis in stationary phase cells is 15–25% that of exponential phase cells (4, 12), the synthesis of all peptides assayed is modulated during growth into the stationary phase. (The possible exceptions are those peptides produced in relative excess in stationary phase cells, e.g., peptides 56, 76, 80, 81, 85, 163, 231, 232, 233, and 234.) This modulation occurs in a coordinate manner for the majority of the peptides assayed in this work (within experimental error) in that the relative levels of production in exponential phase cells and stationary phase cells are approximately equal. Thus the production of individual peptides as a function of time during a growth curve is regulated by at least two separate mechanisms; one acting in a coordinated manner on all or most of the peptides assayed here and the second not acting in a coordinated manner but affecting the production of individual peptides at various specific times during the growth curve.

Stationary phase Vero cells are predominantly in the G1 phase of the cell division cycle while cells in the exponential phase under these conditions possess a bare majority of G1 cells (5, 17, and D. L. Engelhardt, unpublished data). It is possible that the observed differences in newly synthesized proteins between exponential and stationary phase cells represent normal cell division-cycle variations. However, as indicated in Table II, the cells had acquired the protein composition similar to that of cells in stationary phase for many proteins while they were still in the exponential phase (days 6 and 8) with the same proportion of cells in G1 as cells in early exponential phase (D. L. Engelhardt, unpublished data). Also, when cells in exponential growth were deprived of serum at a low cell density according to



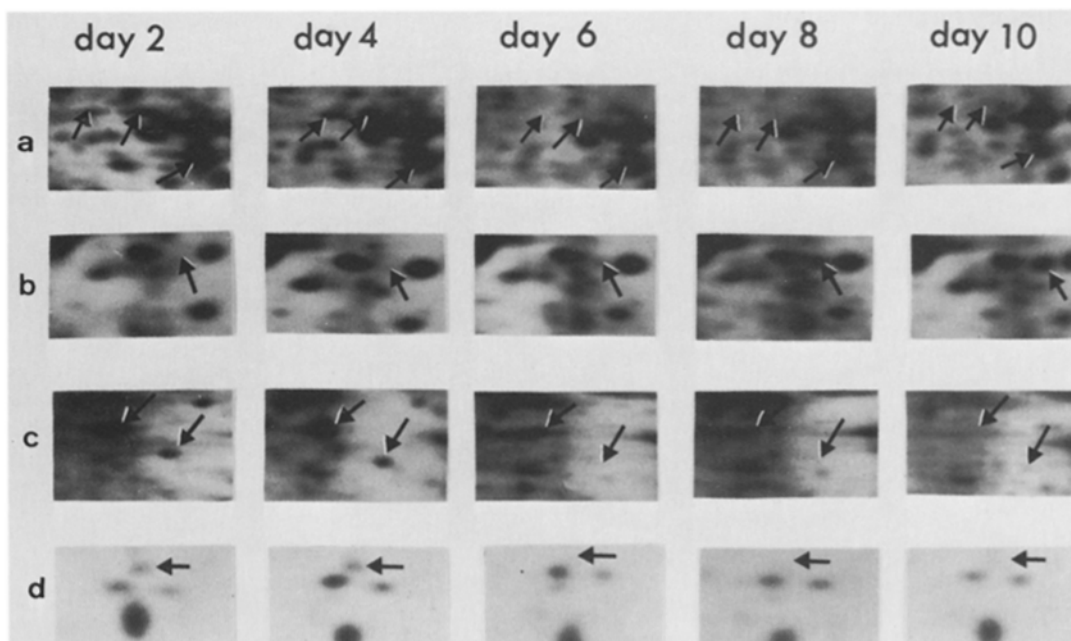


FIGURE 3 Progressive changes of cellular protein synthesis. Four separate areas of the autoradiograph are compared for cells of days 2, 4, 6, 8, and 10. Group *a*: proteins 112, 113, and 114; group *b*: protein 231; group *c*: proteins 38 and 39; Group *d*: protein 68. Arrows mark the positions of polypeptides that demonstrate large variations. All exposures are for 256 h. For day 2 cells 5.2×10^5 cpm of cellular protein was used. For day 4, 6.5×10^5 cpm; for day 6, 6.0×10^5 cpm, for day 8, 6.1×10^5 cpm; and for day 10, 4.1×10^5 cpm.

the procedure of Hassell and Engelhardt (7), cell proliferation was retarded with the great majority of cells becoming entrapped in G1 (D. L. Engelhardt, unpublished data). Under these conditions the pattern of cellular proteins made in the serum-deprived cells was identical to that in the undeprived controls (data not shown). From these two lines of reasoning, we conclude that the characteristic differences that we have observed between exponential and stationary phase cells are not due to division-cycle specified alterations.

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FIGURE 2 Cellular protein synthetic patterns of Vero cells. Cells were pulse labeled with [35 S]methionine and subjected to two-dimensional gel electrophoresis as described in Materials and Methods. 3 H-amino acid labeled Adenovirus 5 was used to provide molecular weight markers. (A) Day 4 cells, 6.5×10^5 cpm of cellular protein was used; (B) Day 10 cells, 4.1×10^5 cpm of cellular protein was used. Exposure time was 270 h. Each spot was identified with an arbitrary number. The numbers of major peptides that demonstrate significant variation are indicated.

TABLE II
Schematic Representation of the Variations in the Relative Production of Individual Peptides as Cells Grow into the Stationary Phase

Peptide no.	Day*										
	2	3	4	5	6	7	8	9	10	11	12
23	_____										
24	_____										
27	_____										
30	_____										
38	_____										
39	_____										
68	_____										
76	_____										
80	_____										
88	_____										
101	_____										
104	_____										
112	_____										
113	_____										
114	_____										
163	_____										
231	_____										
232	_____										
233	_____										
234	_____										

No data was obtained for days 5, 7, and 9. Each line ends at the approximate time when the production of the numbered peptide was intermediate between exponential and stationary phase levels.

* Days represent the day at which [³⁵S]methionine was added (See Fig. 1.)

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