JOURNAL OF BACTERIOLOGY, May 1979, p. 352-360
0021-9193/79/05-0352/09$02.00/0

Vol. 138, No. 2

Individual Proteins Are Synthesized Continuously Throughout the *Escherichia coli* Cell Cycle

JOSEPH F. LUTKENHAUS,† BRIAN A. MOORE,‡ MILLICENT MASTERS,* AND WILLIAM D. DONACHIE

Department of Molecular Biology, King's Buildings, Edinburgh EH9 3JR, Scotland

Received for publication 4 January 1979

The pattern of synthesis of about 750 individual polypeptides was followed throughout the cell cycle of *Escherichia coli* B/r. Samples taken at different times in the cell cycle exhibited the same pattern of protein synthesis. No protein could be identified that was synthesized at different rates during different parts of the cell cycle.

The cell cycle of every organism is characterized by the occurrence of periodic events. In *Escherichia coli* these events include the initiation of chromosomal DNA synthesis, nucleoid segregation, and septation (see ref. 6 for review). The mechanisms controlling the timing of these periodic events, however, are not understood. It is possible that the occurrence of periodic events requires the synthesis of specific proteins made just before the events concerned take place. If discontinuously synthesized proteins are common, for this or indeed for any other reason, it follows that the pattern of protein synthesis during the cell cycle should change in a characteristic fashion. Such sequential changes in patterns of protein synthesis have been observed during sporulation (13) and spore outgrowth (11, 12, 22, 23), as well as in other differentiating systems (see ref. 17). We have therefore examined the pattern of synthesis of proteins during the cell cycle of *E. coli*, to see whether variations in the rate of synthesis of individual proteins could be detected.

The basic procedure was to pulse-label proteins at different stages in the cell cycle and then to separate the individual proteins by the two-dimensional (2-D) gel system of O'Farrell (20). This procedure allows the resolution of up to 1,000 individual proteins. Both synchronously dividing and asynchronous cultures were used. Before analysis the asynchronous cultures were fractionated into size classes by using a zonal rotor.

MATERIALS AND METHODS

Bacterial strain. *E. coli* B/r ATCC 12407 was used in this work.

† Present address: Department of Microbiology, University of Connecticut, Medical Health Center, Farmington, CT 06032.

‡ Present address: Pfizer Ltd., Sandwich, Kent, U.K.

Growth conditions. Cells were grown at 37°C on a New Brunswick gyratory shaker in a minimal salts medium (Helmstetter 1967) with 0.2% glycerol as the carbon source. Cell growth was followed as optical density at 540 nm. Cell number was estimated with a Coulter Electronic Particle Counter (model A) using a 30-μm-orifice tube. Cell length distributions were measured on photographs of cells lying on thin agar films (24), using phase-contrast microscopy.

Inhibition of bacterial growth. Specific inhibitors of cell division, protein, and DNA synthesis were used at the following concentrations: benzylpenicillin BP (sodium salt) at 60 U/ml, chloramphenicol at 200 μg/ml, nalidixic acid at 20 μg/ml. Formaldehyde at 0.4% was added to cells prior to photomicrography or cell number estimations. Cells fixed in this way can be stored for several days at 4°C without change in particle counts or size distribution.

Enzyme induction and assay. β-Galactosidase was induced by the addition of 10^{-3} M isopropyl-β-D-thiogalactopyranoside for 6 min, and induction was terminated by the addition of chloramphenicol. The enzyme activity was assayed according to Loomis and Magasanik (14).

Radioisotope incorporation. The rate of DNA synthesis was measured by the incorporation of [*methyl*-^{3}H]thymidine (50 Ci/mmol; The Radiochemical Centre, Amersham, England) into trichloroacetic acid-precipitable material during a 90-s pulse. [^{3}H]-thymidine was used at 5 μCi/ml. Incorporation was terminated either by addition of 5% cold trichloroacetic acid or, where necessary, by addition of nalidixic acid. For measuring incorporation of isotope, samples of up to 50 μl of labeled cells were applied to 3MM filter paper disks (2.4-cm diameter, Whatman), which were immediately washed twice with cold 5% trichloroacetic acid (containing 100 μg of unlabeled thymidine per ml) for 30 min and once in cold 80% ethanol. The disks were dried and counted in 5 ml of a 5% solution of butyl-PBD [2-(4'-tert-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole; Ciba, Cambridge, England] in toluene and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Proteins were labeled with [^{35}S]methionine (385 Ci/mmol) at 20 μCi/ml plus 0.05 μg of unlabeled methio-

nine per ml. A 3-min pulse was followed by a 2-min chase with unlabeled methionine at 200 μg/ml. Incorporation was terminated by adding chloramphenicol and placing the samples on ice.

Electrophoresis. Crude cell lysates were resolved into individual polypeptides by the 2-D electrophoretic procedure described by O'Farrell (20), with minor modifications. Cell or membrane pellets were resuspended in SDS sample buffer (0.0625 M Tris-hydrochloride, pH 6.8; 2% sodium dodecyl sulfate [SDS]; 20% glycerol; and 5% β-mercaptoethanol). The samples were then immediately heated in a boiling water bath for 4 min and cooled on ice. An equal volume of lysis buffer A (20) was added, followed by solid urea to give a final concentration of 9 M. The samples were then applied to the gel.

Fractionation of asynchronous populations into size classes. The procedure used was similar to that employed by Beck and Park (3). One-liter cultures of cells were grown for 15 to 20 generations, until they reached an optical density at 540 nm of 0.1 (about 10^8 cells/ml). Before the cells were harvested, the desired induction and labeling regimes were performed and the appropriate antibiotics were added to stop further protein synthesis, DNA synthesis, and cell division during the fractionation procedure. Cells were then rapidly chilled, concentrated by centrifugation or, more rapidly, by filtration (through a Millipore 142-mm-diameter, 0.45-μm-pore size filter), and suspended in 25 to 30 ml of cold 3% sucrose dissolved in the growth medium containing the appropriate antibiotics. This suspension was briefly sonicated (2 × 1 s at setting 6; MSE sonicator, MSE, London) to disrupt any large clumps of cells.

A 5 to 15% sucrose gradient (prepared in the growth medium with antibiotics) was loaded into an A-type, low-speed, zonal rotor (MSE, London) spinning at 400 to 600 rpm at 4°C. The gradient was delivered from a gradient device consisting of a conical flask containing 1 liter of 5% sucrose. The flask was tightly sealed with a rubber stopper, through which two tubes passed. One tube allowed sucrose to be pumped out of the flask, into which heavy sucrose from a reservoir was siphoned through the second tube. The reservoir contained 1,300 ml of 20% sucrose. A magnetic stirrer in the conical flask allowed the sucrose solutions to be mixed. The gradient obtained in this way was linear with respect to rotor radius (but not with respect to rotor volume). The gradient was delivered from the gradient device by means of a Watson-Marlow peristaltic pump.

The cell suspension was applied to the top of the gradient, followed by an overlay of 50 to 100 ml of 1% sucrose. The rotor was then accelerated to 2,500 to 3,000 rpm, which achieved the desired separation in 20 to 40 min. (The rotor is constructed of transparent Perspex, and the course of separation of the cells can be seen directly with the aid of a light source placed beneath the rotor in the centrifuge chamber.) Subsequently the rotor was decelerated, without braking, to 400 to 600 rpm, and the gradient was displaced slowly (about 50 ml/min) by pumping 30% sucrose to the rotor outer edge. Samples of 20 ml were collected in tubes in ice.

Preparation of synchronous cultures. Exponentially growing cells were fractionated in the zonal rotor as described above, except that the separation was carried out at 20°C in a 3 to 8% sucrose gradient (in growth medium, without antibiotics) that was linear with respect to rotor volume. These gradients were made with a conventional two-chambered gradient maker. Under these conditions, fractionation was achieved in 10 to 20 min. Samples of 20 ml were collected as before (except that the gradient was displaced at about 100 ml/min), and a fraction containing a suitable size fraction of cells was inoculated into 150 to 500 ml of prewarmed growth medium. The resulting culture usually had a starting density of about 2×10^7 cells per ml.

Samples were withdrawn at intervals from the culture and pulse-chased with radioisotope or induced for enzyme synthesis as described above.

Preparation of cell membranes. Bacterial membranes were prepared from sonicated cells by differential centrifugation, as described previously (15). All manipulations were carried out at 4°C. Cells were pelleted by centrifugation at 6,000 × *g* for 5 min, washed once in 10 mM Tris-hydrochloride buffer (pH 7.8) containing 5 mM EDTA and 1 mM β-mercaptoethanol, and finally resuspended in the same buffer. Cells were lysed by sonication (MSE sonicator, setting 6, 3 × 15-s bursts), and unbroken cells and debris were pelleted at 12,000 × *g* for 10 min. The supernatant was then centrifuged for 40 to 60 min at 100,000 × *g* in a Beckman L2-65B preparative ultracentrifuge. The membrane pellet was washed in the same buffer, centrifuged again, and finally suspended in 20 to 200 μl of SDS sample buffer. The membrane proteins were heated in this buffer at 100°C for 4 min and then analyzed by SDS-polyacrylamide electrophoresis (15).

Radioactive counting. Areas of polyacrylamide gels to be counted were cut out and placed in scintillation vials together with a water-tolerant scintillant containing a tissue solubilizer. This scintillant contained: 720 ml of toluene, 2.88 g of PPO (2,5-diphenyloxazole; Sigma), 80.8 mg of POPOP [1,4-bis-(2-(4-methyl-5-phenyloxazolyl))-benzene, Packard], 7.2 ml of 1% SDS and 80 ml of Soluene-350 (Packard). The radioactivity was counted in a Packard Tri-Carb Spectrometer.

Autoradiography. Gels for autoradiography were dried (15) and exposed to Kodak Blue Brand medical X-ray film, BB54, for several days before developing.

RESULTS

Synchronously dividing cultures. Synchronous cultures of *E. coli* B/r (ATCC 12407) were obtained by selecting small cells from an asynchronous exponential population by centrifugation in a sucrose gradient (18). The original procedure, in which cells were centrifuged through gradients in test tubes, was improved upon by the use of a zonal rotor to select the small cells used to start the synchronous cultures (1, 3). Cell numbers and rate of DNA synthesis were monitored during growth of synchronous cultures (Fig. 1). In some experiments it was also shown that the rate of induced synthesis of

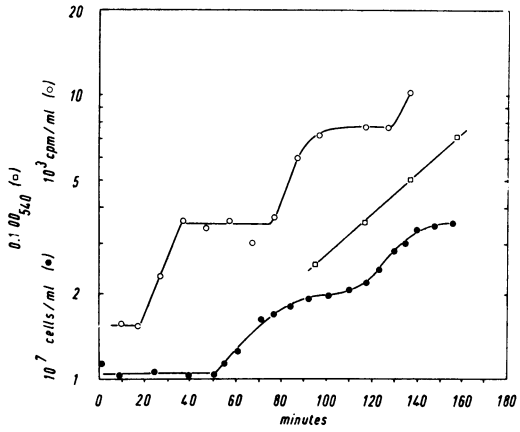

FIG. 1. *DNA synthesis, cell growth, and cell separation in a synchronous population of E. coli B/r. An asynchronous population in exponential growth was separated into size classes by centrifugation in a sucrose gradient in a zonal rotor, as described in the text, and a fraction containing small cells was used to inoculate this culture. The figure shows optical density at 540 nm (OD_{540})* (□), *cell number* (●), *and rate of DNA synthesis* (○). *Samples from this culture were removed at 5-min intervals, pulsed with [^{35}S]-methionine for 3 min, chased with unlabeled methionine for 2 min, and then used to prepare 2-D autoradiograms of total cell protein as described in the text.*

various enzymes (β-galactosidase, tryptophanase, and D-serine deaminase) doubled at the times in the synchronous cell cycle expected for the duplication of their corresponding structural genes (data not shown) (7). To study the variation in the rate of protein synthesis during the cell cycle, synchronous cultures were established, and, at 5-min intervals, samples from the cultures were pulse-labeled with [^{35}S]methionine for 3 min. Each sample was then chased for 2 min with [^{32}S]methionine, to allow all nascent polypeptides to be completed, and processed in the 2-D gel system as described above. Figure 2 shows autoradiograms of the separated proteins labeled in this way at three different times during the cell cycle. Autoradiograms of the proteins labeled in the remaining intervals of the cycle are not shown because they were all very similar or identical. Approximately 750 resolved spots can be counted in such autoradiograms. Visual comparison of the full series of autoradiograms failed to reveal the periodic synthesis of any protein. Occasionally a spot was found to be missing from a particular autoradiogram, but no spot was found to be systematically missing from a sequence of autoradiograms such that one could conclude that it was synthesized preferentially during a particular part of the cell cycle.

A more quantitative estimate of the variation in the rates of synthesis of individual proteins during the cell cycle was obtained by direct measurement of the radioactivity of individual spots at various times throughout the cell cycle. Thirty spots, some of which had produced faint and some intense autoradiographic images and none of which overlapped with other spots, were selected for measurement in this way. The amount of label in the spots measured varied between 0.005 and 4.5% of the total radioactivity applied to the gels. The radioactivity in each of the spots was measured at three different times in the cell cycle. The average percent standard deviation in radioactivity of individual spots, each measured relative to the average for that spot, was 15%. These results confirm, for the 30 proteins selected, the results obtained by visual inspection; i.e., that the relative rate of synthesis of individual proteins does not vary significantly during the cell cycle.

Asynchronous exponential cultures separated with a zonal rotor. Since it is possible that the selection procedure used to obtain the synchronous cultures could alter the physiology of the cells in such a way as to suppress a normal sequence of synthesis of proteins, similar experiments were performed with asynchronous cultures. These cultures, after labeling or other treatment, were applied to a sucrose gradient in a zonal rotor, and the cells were fractionated into different size classes. Gradients used contained the appropriate antibiotics to prevent cell growth or macromolecular synthesis during the separation. Figures 3 and 4 demonstrate the effectiveness of the zonal rotor separation in fractionating a population of cells into age classes. Figure 3 shows the length distributions of cells in selected fractions from the zonal rotor. The smallest cells, which can be used to start synchronous cultures, are extremely homogeneous; the larger cell fractions are less so. However, the degree of separation obtained is sufficiently good so that the modal cell lengths of the separated cells vary over a twofold range (Fig. 3C). Figure 4A shows the percentage of dividing cells and the mean cell size in successive fractions from the rotor. Figure 4B shows the rate of DNA synthesis and the rate of β-galactosidase synthesis in successive fractions of a population that had been pulse-labeled with [^{3}H]thymidine and pulse-induced with isopropyl-β-D-thiogalactopyranoside before separation on the rotor. The stepwise increases in rates of DNA replication and β-galactosidase synthesis are clearly demonstrated. Similar results have been obtained after tryptophanase and D-serine deaminase induction (B. Moore, Ph.D. thesis, University of

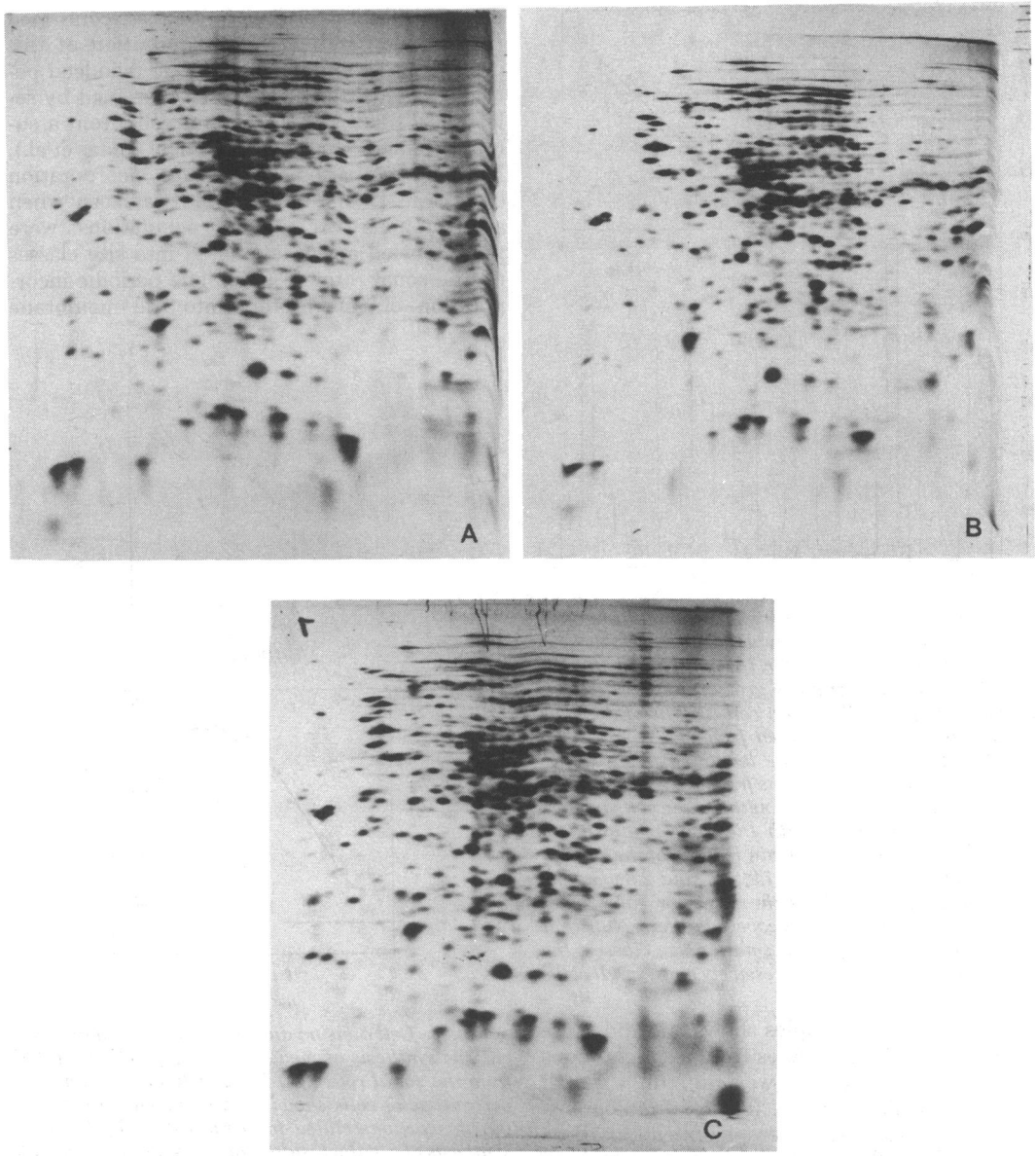

FIG. 2. *Autoradiograms of ^{35}S-labeled proteins obtained by pulse-labeling samples from the synchronous culture shown in Fig. 1. Pulsed at: (A) 10 min; (B) 30 min; (C) 60 min. The samples were applied to the upper right-hand corner of the gels so that basic proteins appear at the right, acid at the left, large at the top, and small at the bottom.*

Edinburgh, Edinburgh, 1976). The order obtained for the times of increase in rates of induced synthesis of all three enzymes, in relation to the times of increase in rate of DNA synthesis, correspond to the order and times of replication of the structural genes for these three enzymes (7). Thus it is clearly possible to use asynchronous cultures separated into age classes with a zonal rotor to demonstrate differences in the rate of synthesis of proteins during the cell cycle.

We therefore pulse-labeled exponential asynchronous cultures with [^{35}S]methionine and chased as described for the synchronous culture. Proteins were prepared from each size class of cells after separation of the labeled culture in the zonal rotor. They were then separated on gels, and autoradiograms were prepared as before. A comparison of the autoradiograms ob-

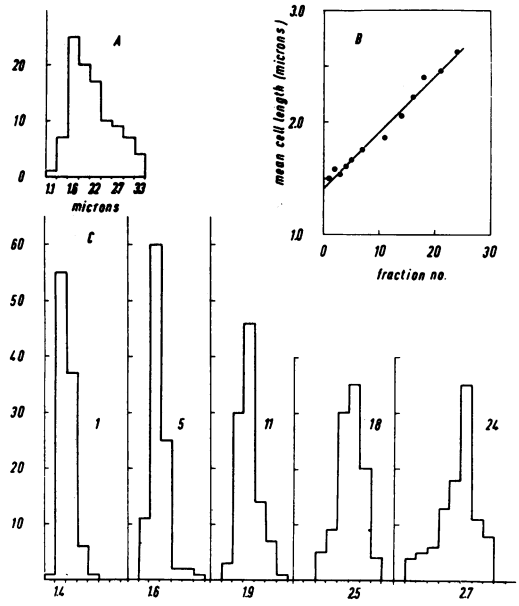

FIG. 3. *Separation of an exponential culture of E. coli B/r into size classes by using sucrose gradient fractionation in a zonal rotor. (A) Length distribution of cells in an exponentially growing unfractionated population of B/r. (B) Mean cell lengths of the cells in successive fractions eluted from the zonal rotor. (C) Histograms showing the length distributions of cells in five different fractions from the rotor (no. 1, 5, 11, 18, and 24). The modal lengths of the cells in each fraction are shown beneath the appropriate histogram; the corresponding mean lengths of the cells in each fraction were 1.5, 1.7, 1.9, 2.4, and 2.6 μm. The horizontal length scale is the same for each histogram, but the range of sizes covered differs. Note that fractionation is best for the smaller size classes; the later fractions contain progressively more small cells.*

tained from these samples showed, as did those prepared at different times from a synchronous culture, that each spot was present at equal intensity in all fractions. The autoradiograms obtained in this way were identical to those obtained from the synchronous culture and are therefore not shown. However, Fig. 5 shows preparations of membrane proteins from these fractions (and from an asynchronous control) to show that in this subset of proteins also there is no detectable change in the pattern of synthesis throughout the cell cycle (in contrast to previous reports; see below). Thus we have found no evidence of periodic synthesis for any protein during the cell cycle of *E. coli*.

We may, however, ask at what level we could detect a variation in rate of synthesis by visual inspection. We have tried to answer this as follows. Gudas et al. (9) and Churchward and Holland (5) have described the periodic incorporation of newly-synthesized protein (D) into the outer membranes of *E. coli*. We have confirmed (Fig. 6 and 7) that the incorporation of this protein into the outer membrane is indeed periodic in synchronous cultures (obtained by selecting a small size fraction of cells from a sucrose gradient, as was done also by Gudas et al.). However, no periodic membrane incorporation of protein D or other protein was seen when asynchronous exponential populations were pulse-labeled and fractionated into size classes on the zonal rotor (Fig. 5). The periodic incorporation of this protein into the membrane

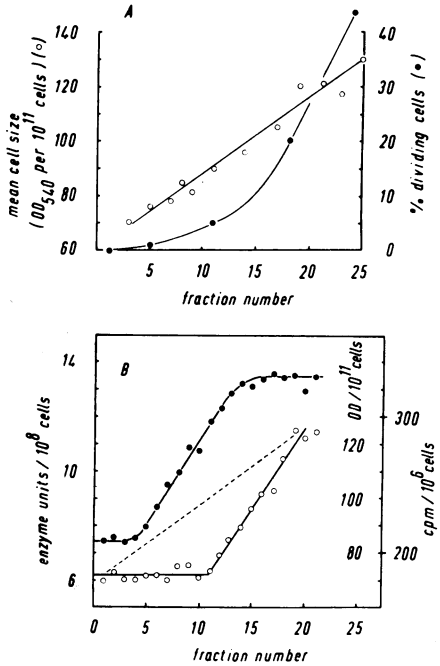

FIG. 4. *Cell division and rate of DNA and induced enzyme synthesis as measured using fractions eluted from the zonal rotor. (A) Mean cell size* (○) *and the percentage of cells showing visible signs of division* (●) *in fractions eluted from the rotor. (B) Rates of synthesis of β-galactosidase and DNA during the cell cycle of E. coli B/r. Enzyme synthesis was induced in an asynchronous exponential culture by the addition of 10^{-3} M isopropyl-β-D-thiogalactoside, and induction was terminated by the addition of 200 μg of chloramphenicol per ml 6 min later. During the last 90 s of the induction, [^{3}H]thymidine was added to the culture (at 25 Ci/mmol; 5 μCi/ml). This incorporation was terminated by the addition of nalidixic acid (20 μg/ml). Penicillin G was added at 60 IU/ml to prevent further cell division. The cells were then separated in a sucrose gradient in the zonal rotor. Enzyme activity, acid-insoluble radioactivity, optical density (OD) (at 540 nm), and cell number were measured in each fraction. The dashed line represents optical density of 10^{11} cells; the upper curve* (●) *is enzyme units per 10^8 cells; and the lower curve* (○) *is counts per minute per 10^6 cells.*

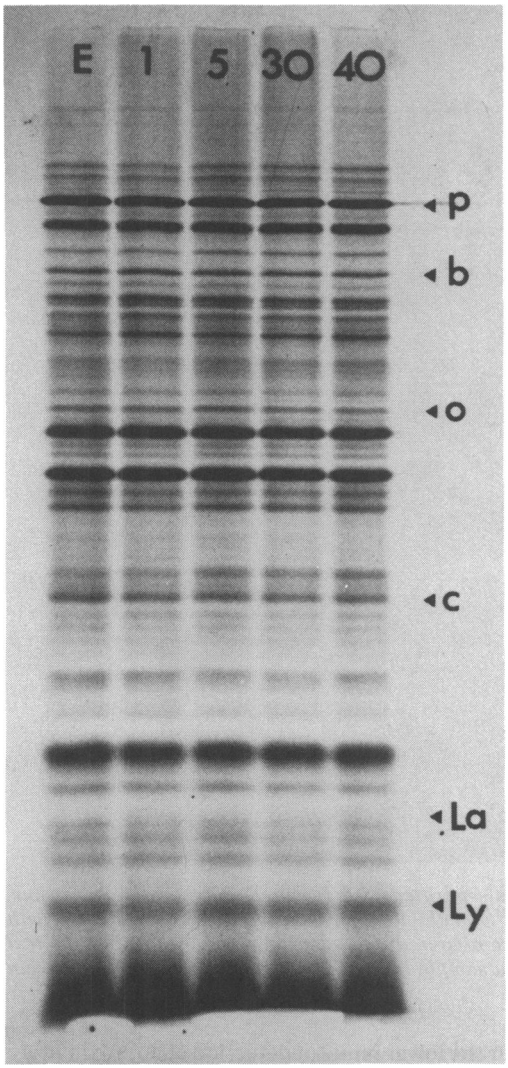

FIG. 5. *Synthesis of membrane proteins during the cell cycle. An exponential culture of E. coli B/r was pulse-labeled with [^{35}S]methionine and chased with unlabeled methionine as described in the text. The cells were fractionated in the zonal rotor, and total cell membranes were prepared from selected fractions and analyzed by gel electrophoresis and autoradiography (above). The percentage of dividing cells in the fraction studied is indicated at the top of each gel track (cf. Fig. 4A); E denotes the exponential population. The positions of the standard protein markers are indicated (molecular weight): p, phosphorylase A (92,000); b, bovine serum albumin (68,000); o, ovalbumin (42,000); c, carbonic anhydrase (29,000); La, β-lactoglobulin (18,500); Ly, lysozyme (14,000). Protein D would be expected to appear at the position of the dark band below p.*

therefore appears to be an artifact resulting from the selection procedure used to obtain the synchronous culture. This conclusion is in accord with the finding of Boyd and Holland (4) that periodic incorporation of this protein into the outer membrane can also be induced by filtration of the cells (a normal step in the selection procedure of small cells to start synchronous cultures) and is not at all related to the cell cycle. Nevertheless, the change in rate of synthesis of protein D that occurs in synchronous cultures can be used to allow us to determine the difference in amount of incorporated label necessary for visual detection. A synchronous culture was prepared and pulse-labeled at intervals with [^{35}S]methionine, and the membrane proteins were prepared and separated as before. Figure 6 shows an example of a 2-D gel prepared from one of these samples. A spot of the correct molecular weight and expected intensity for protein D was identified. This spot could easily be seen by visual inspection to vary in intensity during the cell cycle. The protein D spots were cut out of the gels, and the amount of radioactivity was determined for each time point. The results of these measurements are presented in Fig. 7. We could easily see, by visual inspection, the twofold change in rate of synthesis of this protein, represented by the samples measured at 60 and 80 min. We can therefore conclude that, if the rate of synthesis of a significant number of proteins varied by as much as twofold during the cell cycle, it should have been possible to identify at least some of them by visual inspection of gels such as those presented in Fig. 2.

DISCUSSION

The cell cycle of *E. coli* must be one of the simplest to be found in any organism. The morphological events of the cycle consist of a continuous increase in cell length, accompanied by nucleoid enlargement and division, and terminated by the formation of a central septum. The simplicity of this cycle perhaps makes *E. coli* the most appropriate organism in which to investigate the regulation of periodic cell cycle events. It is therefore striking that, although we have been able to observe the synthesis of approximately 750 individual proteins during the cell cycle, we have found the synthesis of none of them to be periodic. This observation is in marked contrast to results obtained with the stalked bacterium *Caulobacter crescentus* (19), in which the synthesis of a number of proteins appears to take place only at defined periods in the cell cycle. However, our results are similar to those obtained by Elliott and McLaughlin (8), who observed no variations in the rate of synthesis of individual proteins during the cell cycle of the budding yeast, *Saccharomyces cerevisiae*. Since yeast are eucaryotes and exhibit life cycles

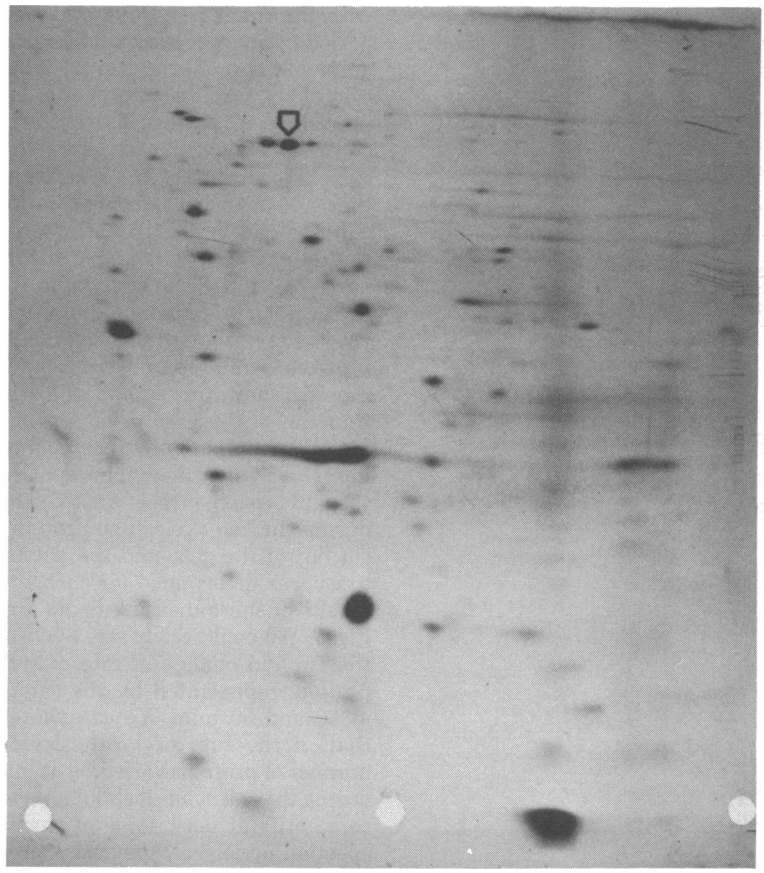

FIG. 6. *An autoradiogram of total membrane proteins separated by 2-D gel electrophoresis. At various times in the cell cycle, samples were pulse-labeled with [^{35}S]methionine for 3 min and chased for 2 min with unlabeled methionine (100 μg/ml). Total membranes were prepared and separated as described in the text and in the legend to Fig. 2. This autoradiogram is from a sample labeled at 45 min. The position of protein D is indicated by the arrow.*

of considerably more complexity than that of *E. coli*, their result is even more remarkable than our own.

Our data, unfortunately, are insufficient to rule out the possibility that some proteins in *E. coli* are synthesized periodically. This is due to the technical limitations of the 2-D gel system in our hands. First, some spots, particularly those formed by large proteins with neutral pK values, tend to streak and overlap, rendering it difficult to discern how the rates of synthesis of such proteins vary. Second, proteins that are present in low numbers of molecules per cell are difficult to detect. Attempts to increase the number of visible spots by increasing the exposure time of the autoradiograms failed because of the problem of audioradiographic spreading. The limits of detection of proteins in the 2-D gels have been estimated by direct measurement of the radioactivity of faint spots, giving estimates for the lower limits of detection of approximately 80 molecules per cell for a protein of 80,000 daltons and approximately 1,000 molecules per cell for a protein of 20,000 daltons. (Lower-molecular-weight proteins are harder to detect because of their relatively greater spreading during electrophoresis through the gel.) The range of molecular weights of proteins seen on the autoradiograms is approximately 10,000 to 200,000. Third, the proteins resolved in this gel system are limited to those with isoelectric points between 3.5 (as defined by ribosomal proteins L7 and L12) and approximately 8.5. Thus basic proteins, such as most ribosomal proteins, are missing from our analysis. Finally, proteins that lack methionine would of course not be detected.

Nearly 650 genes were listed in the last edition of the *E. coli* genetic map (2). Of these, 58 represent rRNA, tRNA, or promoter sites (coding for no proteins); 32 are genes for regulatory

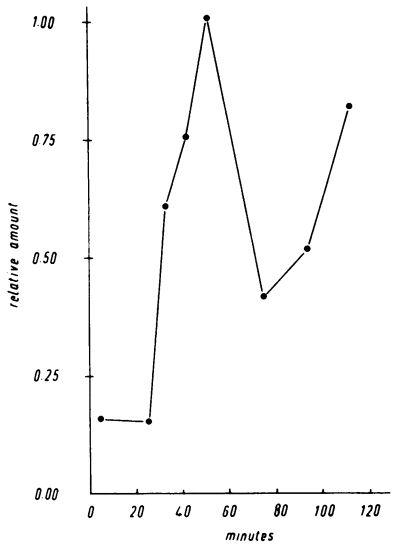

FIG. 7. *Incorporation of protein D into the membrane in a synchronous culture of E. coli B/r. The spot corresponding to protein D was cut out of each gel (Fig. 6), and the amount of radioactivity was determined.*

proteins (present in very low numbers per cell); 46 code for inducible enzymes of carbohydrate catabolism (which would not be induced in the culture medium used in our experiments); and 50 code for ribosomal proteins (which do not enter the gels), leaving about 460 which might be expected to produce proteins resolvable in our gels. The number actually seen is closer to 750, which is evidence that not all *E. coli* genes have yet been mapped. It is difficult to estimate how many genes there actually are on the *E. coli* chromosome. The molecular weight of the chromosome is about 2.5×10^9, and, if the average weight of an *E. coli* protein is taken as 50,000, this will be sufficient for about 2,500 genes. However, Bachmann et al. (2) have pointed out that the known genes are not randomly distributed around the genetic map but are clustered into regions of high gene density. Masters (16) has shown that such gene-dense regions are transduced with much higher efficiency than gene-poor regions, thus confirming that this arrangement is not due to chance but may represent some underlying structural or functional organization. These observations make it possible that the observed distribution of known genes reflects the true distribution of all genes and suggests that about one half of the chromosomal DNA may have no coding function. The estimated number of genes in *E. coli* might thus be reduced to about 1,200 to 1,300, which is very close to the maximum number of *E. coli* proteins that have been resolved by the O'Farrell system (20). However, Hahn et al. (10) have concluded from their RNA/DNA hybridization experiments that there may be a sufficient number of mRNA species present in *E. coli* to code for about 2,300 different proteins, which would imply that the large majority of proteins are not being detected by us. However, the estimate of Hahn et al. of the numbers per cell of a very large proportion of these presumed mRNA's is so low as to suggest that the proteins for which they would be coding could not be present in numbers as high even as one per cell. Thus such proteins could not possibly have any role to play in the timing of periodic events in the cell cycle. It also cannot be excluded that the relatively abundant class of "rare" mRNA species might not consist of genuine messages, but might result from errors in the transcription system (e.g., from mistakes in recognition of start and stop signals). It is therefore at least a possibility that we have been able to monitor the syntheses of the large majority of all significant gene products throughout the cell cycle.

The most interesting class of proteins that are not represented in our analysis must be those, such as repressors and other regulatory proteins, that are present in low numbers per cell. However, there are reasons for supposing that none of these is likely to be synthesized periodically. The first reason is that most regulatory proteins have their effects via the synthesis of the much larger numbers of the molecules of the proteins they control. Periodic production of regulatory proteins would therefore be expected to be amplified and detectable as the periodic production of some relatively common protein. There is also a statistical argument against the idea that a cell can produce small numbers of particular proteins in relatively fixed numbers at relatively fixed times. The transcriptional and translational steps in gene expression are the results of collisions between reacting molecules and, as such, are subject to statistical fluctuations in frequency which will be large when the number of collisions is low. Even if the initial event triggering this period of gene expression were quite precisely timed (as for example the replication of a particular stretch of DNA), it is not easy to see how a very small average number of molecules per cell of a particular protein could be produced without a very large spread in the number of molecules produced in individual cells (21).

Our experiments therefore provide no evidence for a "developmental program" involving sequential gene expression in the regulation of the cell cycle of *E. coli*. What then does trigger

the periodic events of the cell cycle? One possibility is that, although most proteins are synthesized continuously, their actual activities vary during the cycle, either because of variations in the concentrations of inhibitors or activators or because they are only in the correct spatial locations to interact with their substrates at certain times. Variation in carboxypeptidase II activity during the cell cycle of *E. coli* was found by Beck and Park (3) when they assayed the enzyme in toluenized samples of exponential populations which had been separated into size classes by zonal rotor centrifugation. When sonic extracts of similar samples were assayed, this periodic variation in activity disappeared. This is an especially interesting enzyme because of its role in cell wall (peptidoglycan) synthesis and its possible function in cell division. Thus it cannot be excluded that variations in enzyme activities, rather than amounts, are important in controlling the cell cycle. Finally, there remains the possibility that cell cycle events are triggered by events that involve neither the periodic synthesis nor activation of controlling enzymes, such as the attainment of critical cell volumes per se, or by the attainment within the cell of critical amounts of certain key proteins. If there are such cell cycle-controlling proteins (and we shall present evidence elsewhere that they do exist; Vicente, Lutkenhaus, Begg, Otsuji, Salmond, and Donachie, manuscript in preparation), then it seems likely from the present work that they are produced continuously throughout the cell cycle.

ACKNOWLEDGMENTS

We thank the Medical Research Council, U.K., for financial support. B. A. Moore was an MRC scholar during this work.

LITERATURE CITED

1. **Andresdottir, V., and M. Masters.** 1978. Evidence that F' *lac* replicates asynchronously during the cell cycle of *Escherichia coli* B/r. Mol. Gen. Genet. **163**:205-212.
2. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116-166.
3. **Beck, B. D., and J. T. Park.** 1976. Activity of three murein hydrolases during the cell division cycle of *Escherichia coli* K-12 as measured in toluene-treated cells. J. Bacteriol. **126**:1250-1260.
4. **Boyd, A., and I. B. Holland.** 1977. Protein d, an iron-transport protein induced by filtration of cultures of *E. coli*. FEBS Lett. **76**:20-24.
5. **Churchward, G. G., and I. B. Holland.** 1976. Envelope synthesis during the cell cycle of *Escherichia coli* B/r. J. Mol. Biol. **105**:245-261.
6. **Donachie, W. D.** 1979. The cell cycle of *Escherichia coli*, p. 11-37. *In* J. H. Parish (ed.), The developmental biology of prokaryotes. Blackwell Scientific Publications, Oxford.
7. **Donachie, W. D., and M. Masters.** 1969. Temporal control of gene expression in bacteria, p. 37-76. *In* G. M. Padilla, G. L. Whitson, and J. L. Cameron (ed.), The cell cycle. Academic Press, New York.
8. **Elliott, S. G., and C. S. McLaughlin.** 1978. Rate of macromolecular synthesis through the cell cycle of the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **75**:4384-4388.
9. **Gudas, L. J., R. James, and A. B. Pardee.** 1976. Evidence for the involvement of an outer membrane protein in DNA initiation. J. Biol. Chem. **251**:3470-3479.
10. **Hahn, W. E., D. E. Pettijohn, and J. Van Ness.** 1977. One strand equivalent of the *Escherichia coli* genome is transcribed: complexity and abundance classes of mRNA. Science **197**:582-585.
11. **Hansen, J. N., G. Spiegelman, and H. O. Halvorson.** 1970. Bacterial spore outgrowth: its regulation. Science **168**:1291-1298.
12. **Kennet, R. H., and N. Sueoka.** 1971. Gene expression during outgrowth of *Bacillus subtilis* spores. The relationship between gene order on the chromosome and temporal sequence of enzyme synthesis. J. Mol. Biol. **60**:31-44.
13. **Linn, T., and R. Losick.** 1976. The program of protein synthesis during sporulation in *Bacillus subtilis*. Cell **8**: 103-114.
14. **Loomis, W. F., and B. Magasanik.** 1964. The relation of catabolite repression to the induction system for β-galactosidase in *Escherichia coli*. J. Mol. Biol. **8**:417-426.
15. **Lutkenhaus, J. F.** 1977. The role of a major outer membrane protein in *Escherichia coli*. J. Bacteriol. **131**: 631-637.
16. **Masters, M.** 1977. The frequency of transduction of the genes of *Escherichia coli* as a function of chromosomal position: preferential transduction of the origin of replication. Mol. Gen. Genet. **155**:197-202.
17. **Mitchison, J. M.** 1971. The biology of the cell cycle. Cambridge University Press, Cambridge.
18. **Mitchison, J. M., and W. S. Vincent.** 1965. Preparation of synchronous cell cultures by sedimentation. Nature (London) **205**:987-989.
19. **Newton, A.** 1977. Regulation of flagellin synthesis in the cell cycle of *Caulobacter*: dependence on DNA replication. Cell **12**:393-400.
20. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007-4021.
21. **Sompayrac, L., and O. Maaløe.** 1973. Autorepressor model for control of DNA replication. Nature (London) New Biol. **241**:133-135.
22. **Steinberg, W., and H. O. Halvorson.** 1968. Timing of enzyme synthesis during outgrowth of spores of *Bacillus cereus*. I. Ordered enzyme synthesis. J. Bacteriol. **95**:469-478.
23. **Steinberg, W., and H. O. Halvorson.** 1968. Timing of enzyme synthesis during outgrowth of spores of *Bacillus cereus*. II. Relationship between ordered enzyme synthesis and deoxyribonucleic acid replication. J. Bacteriol. **95**:479-489.
24. **Teather, R. M., J. F. Collins, and W. D. Donachie.** 1974. Quantal behavior of a diffusible factor which initiates septum formation at potential division sites in *Escherichia coli*. J. Bacteriol. **118**:407-413.