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Protein Synthesis in Long-Term Stationary-Phase Cultures of *Saccharomyces cerevisiae*

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We are interested in characterizing the process of entry into and the maintenance of the stationary phase. To identify proteins that are induced during growth to stationary phase, we examined protein synthesis in long-term stationary-phase cultures using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Although the total rate of protein synthesis declined when growth ceased after the postdiauxic phase, the pattern of proteins synthesized remained similar throughout the experimental period (28 days), except at the diauxic shift. At the diauxic shift most proteins detectable by 2D-PAGE undergo a transient reduction in their relative rate of synthesis that ends when cells resume growth during the postdiauxic phase. We conclude from this that the transient repression of protein synthesis at the diauxic shift is not directly associated with stationary-phase arrest. A number of proteins that are synthesized after exponential phase have been identified by 2D-PAGE. These proteins could be divided into three temporal classes depending upon when their synthesis became detectable. One postexponential protein, designated p35, was induced later than all other proteins, and its relative rate of synthesis increased throughout stationary phase. Unlike most postexponential proteins, p35 was not regulated by heat shock or glucose repression. We also observed that a direct correlation between steady-state mRNA accumulation and protein synthesis for another postexponential protein (Ssa3p) or two closely related constitutive proteins (Ssa1p and Ssa2p) did not exist. We conclude from this result that synthesis of proteins in stationary phase is regulated by mechanisms other than the control of steady-state mRNA accumulation.

Cellular growth and proliferation are highly regulated processes, not only in multicellular organisms, in which unregulated cellular proliferation leads to neoplasia, but also in microorganisms, in which unregulated cellular proliferation may result in cell death (21). In multicellular organisms, growth factors and hormones are the primary regulators of cellular proliferation. In microorganisms, such as the budding yeast *Saccharomyces cerevisiae*, the availability of nutrients is a major factor regulating proliferation. When vertebrate cells are deprived of certain growth factors, they cease growth and enter a nonproliferating state called G_0 (2, 39). Yeast cells respond to starvation in a similar manner, by ceasing growth and entering a nonproliferating state called stationary phase, which is thought to be similar to G_0 (51).

In nature, most microorganisms exist in a stationary-phase state induced by nutrient-poor environments (32). Despite the importance of stationary phase for survival in natural environments, it remains a poorly understood period of the yeast life cycle (51). The metabolic processes that occur during starvation-induced stationary phase allow microorganisms to survive long periods without nutrients and may be important also for resumption of growth should conditions improve. Some of the physiological changes that occur when yeast cells enter stationary phase are known (33, 51), but the molecular mechanisms responsible for entry into, maintenance of, and exit from stationary phase are largely unknown. Although starvation for different nutrients elicits similar responses (19, 33), it is not known whether all stationary-phase states are identical. We are interested in further defining the process of entry into and maintenance of stationary phase.

The process of entry into stationary phase is a continuum of events in response to a nutrient-depleted environment. The best-studied system for examining entry into stationary phase in yeast involves growth of cells to saturation in rich, glucose-based medium (51). Growth in this medium is characterized by several distinct phases. After a short period of adjustment (lag phase), yeast cells begin a phase of exponential growth, with energy derived primarily from fermentation (31). Once glucose is exhausted, a transient cell-cycle arrest, the diauxic shift, occurs when cells adapt to respiratory metabolism. Following the diauxic shift, growth resumes at a much slower rate, using the products of fermentation and any other carbon sources available in the medium to provide energy. This period of slow growth has been designated the postdiauxic or slow-growth phase (51). Depending upon strain and culture conditions, postdiauxic-phase yeast cells undergo one to three doublings over a period of up to 1 week. Only after this period of postdiauxic growth does the cell number cease increasing. At this point, the culture finally enters stationary phase, which is functionally defined as the time when there is no further net increase in cell number.

Stationary-phase yeast cells are unbudded and contain an unreplicated complement of DNA, similar to cells in the G_1 phase of the mitotic cell cycle (40). However, stationary phase is distinct from the mitotic cell cycle. First, stationary-phase yeast cells that have been stimulated to proliferate take a longer time to bud than growing cells require to traverse G_1 (21, 24). A similar lag in resumption of proliferation is also observed when G_0 vertebrate cells are stimulated to divide (2, 39). Second, a yeast mutant that does not exhibit defects in the mitotic cell cycle but is conditionally defective for the resumption of proliferation from stationary phase has been isolated (18).

Recent advances toward understanding the molecular aspects of stationary phase in several different gram-negative bacteria have been made (29). A number of proteins, designated Pex (for postexponential) proteins, are consistently

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induced in *Escherichia coli* during starvation, regardless of the nutrient that is limited (35). Not all Pex proteins are induced with identical kinetics; some are synthesized relatively rapidly after the onset of starvation, while others are induced at later points in time. Additionally, some Pex proteins are synthesized transiently while others exhibit relatively prolonged periods of synthesis (35).

In yeast cells, a number of proteins that show increased synthesis after exponential phase are known (5), although relatively little is known about the kinetics of their induction or the duration of their synthesis. At least two classes of proteins, heat shock proteins and glucose-repressed proteins, exhibit increased synthesis during and after the diauxic shift (3–5, 24). However, not all heat shock or glucose-repressed proteins are induced, nor is induction of heat shock and glucose-repressed proteins a prerequisite for entry into stationary phase. Only a subset of the heat shock proteins, including the Hsp70-related protein Ssa3p (50), exhibits increased synthesis at the diauxic shift, and heat shock does not cause entry into stationary phase (17, 42), although it does result in the induction of heat shock proteins. There is also no obligatory relationship between glucose derepression and entry into stationary phase, since yeast cells are capable of exponential growth on derepressing carbon sources, when glucose-repressed proteins are synthesized (3, 4). Because earlier studies did not examine protein synthesis much later than the diauxic shift, nothing is known about the duration of synthesis of previously characterized proteins, nor is it known whether additional proteins are induced after the diauxic shift.

Despite the lack of information about protein synthesis after the diauxic shift, differential accumulation of mRNAs during growth to stationary phase has been demonstrated, suggesting that there may be several classes of proteins synthesized at various times during this process. Accumulation of most yeast mRNAs decreases dramatically during the diauxic shift, postdiauxic phase, and entry into stationary phase (13, 50, 51). However, some yeast mRNAs exhibit a transient increase in accumulation at the diauxic shift. These include the *BCY1*, *IRA1*, *IRA2*, and *YAK1* genes (15, 43, 49), which encode components of the *RAS*-cyclic AMP (cAMP) pathway involved in growth regulation (9). The mRNAs that encode most postexponentially expressed heat shock proteins also exhibit increased accumulation during the diauxic shift, postdiauxic phase, and entry into stationary phase (50, 51). Finally, some genes exhibit a complex pattern of expression. For example, the mRNA encoding Sti1p, a heat shock protein, shows a transient decrease during the diauxic shift and postdiauxic phase and increased accumulation during entry into stationary phase (37). It is not known whether synthesis of the proteins encoded by these genes parallels the accumulation of their mRNAs.

Analysis of protein synthesis during growth to stationary phase has been attempted by several laboratories. General approaches have included use of temperature-sensitive mutants, such as strains with *cdc25* or *cdc35*, that arrest in G_1 with a stationary-phase-like phenotype (24), and shifting of exponentially growing, wild-type cells to defined starvation media (24, 47, 48). The pattern of protein synthesis in cells grown to the diauxic shift in minimal medium has also been examined (3, 5). In the latter condition, the rate of protein synthesis decreases at the diauxic shift to approximately 5% of that observed in exponentially growing cells. Under these conditions, the synthesis of most major proteins is arrested while a transient induction of many heat shock proteins occurs (3, 5). The synthesis and accumulation of proteins at times substantially after the diauxic shift have not been examined.

There is a perceived difficulty in labeling yeast cells, in rich or defined medium, after the diauxic shift, when the rate of protein synthesis is known to decline substantially. Since most studies of cellular physiology and mRNA accumulation have been conducted with cells grown in rich medium, it is difficult to relate the changes in the pattern of protein synthesis in stationary-phase cells, generally grown in minimal medium, to the physiological status of the cells. In this paper, we demonstrate short-term metabolic labeling in rich medium of cells cultured for as long as 4 weeks.

We examined the pattern of proteins synthesized during the postdiauxic phase and stationary phase using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (38). The absolute rate of protein synthesis is substantially reduced after exponential growth ceases, but many of the same proteins are synthesized throughout the culture cycle. A number of proteins show increased synthesis after exponential phase. These postexponential proteins can be placed into three temporal categories. One postexponential protein, designated p35, is synthesized only during postdiauxic phase and stationary phase. p35 is neither a heat shock nor a glucose-repressed protein, indicating that it may represent a novel class of proteins synthesized during entry into stationary phase.

MATERIALS AND METHODS

Yeast strains, media, and growth conditions. For these experiments, we used the prototrophic haploid yeast strain S288C (*MATα mal gal2*) (36). Cells were grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) (45). For glucose derepression experiments, cells were transferred to YP (1% yeast extract, 2% peptone) plus 0.05% dextrose (11, 12). Unless otherwise indicated, cells were grown at 30°C in a shaking incubator at 280 rpm.

Determination of culture parameters. Culture density was determined by measuring the turbidity at 600 nm (OD_{600}). Under the conditions we used, an OD_{600} unit corresponds to approximately 2×10^7 cells ml^{-1}. Whenever cells were labeled or samples taken, cell number was determined with a hemocytometer. Viability was determined by spreading an appropriately diluted, sonicated culture onto YPD plates and then determining the number of CFU after 3 days at 30°C. Plating efficiency, which is a normalized measure of viability, was computed as CFU ml^{-1} divided by cells ml^{-1}.

To determine whether the growth of a culture was limited by carbon source (22, 33), two samples of the culture (2 ml each) were taken and 200 μl of filter-sterilized 20% glucose or distilled water was added to the sample. These samples were incubated for 24 h at 30°C with vigorous shaking, and the culture densities in each sample were compared by measuring OD_{600}.

Radioactive labeling during growth to stationary phase. For analysis of protein by 2D-PAGE, small culture samples were transferred to flasks (1 ml for cultures in exponential phase, 2 ml for cultures at the diauxic shift, in postdiauxic phase, and in early stationary phase, and 4 ml for cultures in late stationary phase), and Tran^{35}S-label (ICN) was added to a final concentration of 500 to 600 μCi ml^{-1}. Cells were incubated at the growth temperature (30°C) with vigorous shaking for 15 min (exponential-phase cultures) or for 2 h (all other phases of the culture cycle). After being labeled, the cells were collected by centrifugation at 4°C, washed once with cold distilled water, and frozen at −70°C. In order to determine steady-state protein accumulation by 2D-PAGE, several unlabeled samples of 1×10^8 to 5×10^8 cells were harvested in parallel for all

growth phases and collected in the same manner as labeled samples.

The relative efficiency of protein labeling at various points in the culture cycle was assessed by determining the incorporation of ^{35}S into acetone-precipitable material. A small sample of the culture (800 μl) was mixed rapidly with 400 μCi of Tran^{35}S-label and then incubated in a 30°C shaking water bath (250 rpm). Samples (120 μl each) were taken at the times indicated below and added to 250 μl of ice-cold NaN_3 (1 mM) to stop the incorporation. The cells were quickly pelleted by centrifugation at 4°C, washed once with ice-cold NaN_3 (1 mM), and stored at −70°C. The sampling time points were 0, 2, 5, 10, 15, and 20 min (exponential phase and diauxic shift); 0, 10, 20, 30, 60, and 90 min (postdiauxic phase); and 0, 15, 30, 60, 120, and 180 min (stationary phase).

To measure incorporation, proteins were isolated from the above samples by the same method used for 2D-PAGE, except that nuclease digestions were omitted. The amount of ^{35}S incorporated into protein was measured by liquid scintillation counting, and the rate of [^{35}S]methionine incorporation into protein was determined by performing simple linear regression (CA Cricket Graph; Computer Associates). In all cases, the square of the correlation coefficient, r^2, was greater than 0.8.

Radioactive labeling after the relief of glucose repression and after heat shock. To examine the pattern of proteins synthesized in cultures after glucose derepression (11, 12), cultures were grown in YPD with vigorous shaking at 30°C to an OD_{600} of 1, and 4 ml of culture was collected by centrifugation and washed with 4 ml of YP (without dextrose). The washed culture was divided, and the cells were pelleted by centrifugation and resuspended in 1 ml of YPD (glucose-repressed control) or 1 ml of YP plus 0.05% dextrose (glucose derepressed). The samples were added to 1 ml of the same medium, prewarmed to 30°C. After incubation at 30°C with vigorous shaking for 1 h, Tran^{35}S-label was added to a final concentration of 113 μCi ml^{-1}, and the cells were incubated an additional 15 min and collected as described above.

To examine the pattern of proteins synthesized after a heat shock, cultures were grown, centrifuged, and washed essentially as described in reference 53. Briefly, the washed culture was divided, and the pelleted cells were resuspended in 1 ml of YPD and then added to 1 ml of YPD prewarmed to 30°C (non-heat-shocked control) or 39°C (heat shocked). After incubation at the respective temperatures for 15 min, Tran^{35}S-label was added to a final concentration of 113 μCi ml^{-1} and the cells were incubated an additional 15 min before collection as described above.

Protein isolation for 2D-PAGE. Frozen cell pellets were resuspended in sonication buffer (10 mM Tris [pH 7.4], 5 mM $MgCl_2$, 50 μg of RNase A ml^{-1}) (52), and the protease inhibitors phenylmethylsulfonyl fluoride and *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK) were added to final concentrations of 1 mM and 50 μg ml^{-1}, respectively. Cells were lysed by vortexing with glass beads (average diameter, 0.5 mm) six times for 30 s, alternating with 30-s incubations on ice. The lysates were clarified by centrifugation, and the supernatant was treated with micrococcal nuclease (40 μg ml^{-1}) and DNase I (50 μg ml^{-1}). Proteins were precipitated from the supernatants by the addition of an equal volume of acetone. The precipitated pellet was resuspended in 50 μl of lysis buffer (9.5 M urea, 2% Nonidet P-40, 2% ampholines, 5% β-mercaptoethanol) (38) and stored at −70°C until use. Under these conditions, cytosolic proteins are released from cells but membrane, cytoskeleton, nuclear matrix, and chromatin proteins usually remain in the discarded pelleted fraction. Radioactivity in the resuspended precipitate was determined by liquid scintillation counting. Protein concentration from unlabeled cultures, which were prepared identically, was determined by the Bradford assay (Bio-Rad).

2D-PAGE. Isoelectric focusing was performed as described by O'Farrell (38). First-dimension tube gels contained 4% acrylamide, 9.5 M urea, 2% Nonidet P-40, and 2% ampholines (1.6% pH 5 to 7, 0.4% pH 3 to 10) (Pharmacia). Before being loaded, the gels were prerun for a total of 400 V · h. Samples in lysis buffer, containing 10^5 dpm of labeled protein or 100 μg of unlabeled protein, were loaded on the basic end of the gel and electrophoresed for 6,400 V · h. After electrophoresis, isoelectric focusing gels were equilibrated with sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris [pH 6.8], 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS) for 10 to 30 min and immediately loaded on the second dimension or frozen at −70°C until use.

Electrophoresis in the second dimension was performed as described by Laemmli (30). Discontinuous gels with 10% acrylamide resolving gels and 4.5% acrylamide stacking gels were prepared, and the equilibrated tube gels were secured onto the stacking gels. Proteins were electrophoresed at 4°C with a constant potential (90 V).

Protein was transferred from the gel to Immobilon-P (Millipore) membrane by electroblotting. The blots were exposed to X-ray film to detect newly synthesized protein or were processed with NHS-Biotin (Bio-Rad) according to the manufacturer's recommendations to detect steady-state protein accumulation. Biotinylated proteins were detected with horseradish peroxidase-conjugated avidin according to the manufacturer's recommendations. Bound horseradish peroxidase-avidin was detected with the chromogenic substrate 4-chloronaphthol.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated by a modification of the procedure used to isolate RNA from ascospores (28). Glassware was baked, and solutions were made with diethyl pyrocarbonate-treated water to inactivate ribonucleases. Cultures were grown to various densities in YPD at 30°C. Cells (10 to 30 OD_{600} units) were collected, washed once with ice-cold water, and stored at −70°C until use. After the addition of 250 μl of NaOAc buffer (50 mM sodium acetate [pH 5.0], 10 mM EDTA) and hot NaOAc-equilibrated phenol (300 μl, 65°C) to the frozen pellet, cells were lysed by vortexing with glass beads (three 30-s bursts). The samples were centrifuged for 10 min at 2,500 × *g* at 4°C, and the aqueous phase was extracted with phenol-chloroform (1:1). The aqueous phase was transferred to a new tube containing an equal volume of 2× proteinase K digestion solution (0.2 M Tris [pH 7.5], 25 mM EDTA, 0.3 M NaCl, 2% [wt/vol] SDS, 400 μg of proteinase K per ml) and incubated at 37°C for 30 min. Two additional phenol-chloroform (1:1) extractions were performed, the RNA was precipitated with 3 volumes of ethanol, and the dried pellet was dissolved in water.

Total RNA (5 μg per lane) was electrophoresed on 1% agarose gels containing 2.5% formaldehyde and blotted onto GeneScreen (NEN) according to the manufacturer's recommendation. Uniform loading and transfer were confirmed by observation of ethidium bromide-stained rRNA bands. Probes containing either the *SSA2* or *SSA3* gene (50) were labeled by the random primer method (Pharmacia). Hybridizations were carried out at 42°C, and the blots were washed at high stringency. Under these conditions, the *SSA2* probe detects both the *SSA1* and *SSA2* messages (50).

Densitometry and image manipulation. Gels were digitized with a Gateway 2000 386-based personal computer running the Image-1 program (Universal Imaging Corp., Westchester, Pa.) with a Panasonic BD-400 CCD video camera. The gels were digitized with an 8-bit resolution. Densitometry was performed

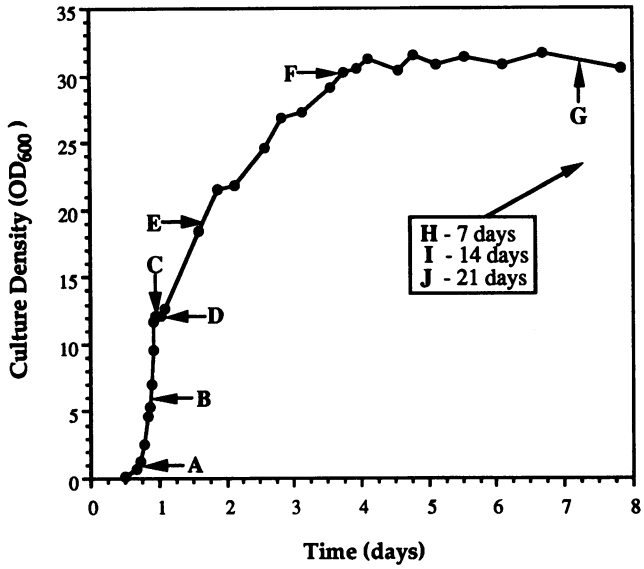


FIG. 1. Growth of wild-type yeast cells in rich, glucose-based medium at 30°C. A wild-type yeast strain (S288C) was inoculated in rich, glucose-based medium (YPD) and grown at 30°C. Culture density was monitored by measuring OD_{600}. Points in the culture cycle at which experiments were performed are indicated on the growth curve: A and B, exponential phase; C, diauxic shift; D to F, postdiauxic phase; G to J, stationary phase.

with the Image-1 program. Figures were prepared with Adobe Photoshop 2.0 (Adobe Systems Incorporated, Mountain View, Calif.) running on a Macintosh IIsi computer.

RESULTS

Wild-type yeast cells enter stationary phase after growth for approximately 5 days in rich medium. When grown in rich medium at 30°C, S288C cells exhibit typical changes in growth rate and budding index (Fig. 1). During the exponential-growth phase, approximately 50% of the cells were budded, and the culture density doubled every 80 min. When the glucose was exhausted, within 24 h of inoculation, there was a transient accumulation of unbudded cells during the diauxic shift. After the diauxic shift, cells resumed proliferation at a much slower rate and the culture density doubled once over a period of 3 days. Four days after inoculation, the fraction of budded cells declined to less than 5% and the culture density ceased increasing, indicating entry into stationary phase.

The viability of S288C cells in YPD cultures remained high throughout the experimental period (28 days). The plating efficiency of the cultures declined approximately 50% after exponential phase and then remained constant throughout the stationary-phase period of these experiments.

The growth arrest associated with stationary-phase cells grown in YPD is reported to be caused by carbon starvation (21, 22, 33). This was also the case for S288C cells grown under our conditions. Stationary-phase cultures resumed proliferation after the addition of glucose, even 28 days after inoculation, indicating that the carbon source was the growth-limiting nutrient.

Comparison of the labeling efficiencies of cells at various times in the culture cycle during growth to stationary phase. Cells grown in minimal media exhibit a substantial decrease in the rate of incorporation of L-[^{35}S]methionine into protein, relative to exponentially growing cells, and a slight delay before the rate of incorporation becomes linear (5). To establish the feasibility of labeling cells grown in rich medium and to relate the pattern of protein synthesis to the changes observed in cell physiology and gene expression in stationary-phase cells, we evaluated the relative rates of protein synthesis throughout the culture cycle when cells were grown in rich medium.

The rate of incorporation of ^{35}S-amino acids into protein at different times during the culture cycle was assayed by addition of a relatively high concentration of Tran^{35}S-label (500 μCi ml^{-1}) to culture growing in YPD. The greatest rate of incorporation occurred during exponential phase, and the rate decreased at the diauxic shift to less than 2% of that in exponential phase (Fig. 2A). During the postdiauxic phase, when growth resumes at a much slower rate, the rate of incorporation actually increased to almost 8% of the exponential-phase rate (Fig. 2A). During stationary phase, the incorporation rate declined again and continued to decrease throughout the period examined (Fig. 2A).

On a per-cell basis, the yield of protein in the lysates declined approximately 60% at the diauxic shift and remained relatively constant throughout the experimental period (data not shown). A similar decline (approximately 50%) in protein content occurs when cells are arrested by nitrogen starvation (26).

The rate of incorporation of ^{35}S into protein at different times during the culture cycle was analyzed by simple linear regression of the data (Fig. 2B). During exponential phase, the incorporation of ^{35}S into protein was linear for approximately 15 min. However, the incorporation of ^{35}S into protein was linear for at least 90 min in postdiauxic phase (data not shown) and at least 180 min in stationary phase (Fig. 2B). These results indicate that relatively long labeling times (i.e., 120 min) could be used to examine protein synthesis in stationary-phase cells and that degradation of newly synthesized proteins was not a major factor during the labeling period.

Comparison between the patterns of proteins synthesized in exponential- and stationary-phase yeast cells. Previous reports have indicated that most proteins synthesized during exponential growth in glucose-based medium are not synthesized in stationary phase and that most of the proteins synthesized by stationary-phase cells are not synthesized by exponentially growing cells (3, 5). Because these studies were conducted within 2 h of glucose exhaustion, when cells have not truly entered stationary phase, we decided to examine the pattern of protein synthesis in cells that have been glucose limited for longer periods of time. We expected additional changes in the pattern of protein synthesis since the rate of protein synthesis changed throughout growth to stationary phase (Fig. 2A and B).

Examination of the patterns of cytosolic proteins synthesized at different times in the culture cycle revealed, surprisingly, that most proteins synthesized at high rates during exponential growth (Fig. 3A and B) were also synthesized throughout stationary phase (Fig. 4). In fact, synthesis of many of the same proteins was still detectable 3 weeks after entry into stationary phase (i.e., 28 days after inoculation) (Fig. 4J). Only a few proteins were synthesized specifically or showed substantially increased synthesis relative to other proteins in stationary phase (Table 1). It is important to note that Table 1 presents the synthesis of given proteins independent of the overall changes in the rate of protein synthesis during growth. The overall rate of protein synthesis decreased substantially during growth to stationary phase and is presented in Fig. 2. Most proteins that show an increased relative rate of synthesis in stationary phase exhibit this increase prior to or during the diauxic shift (Table 1).

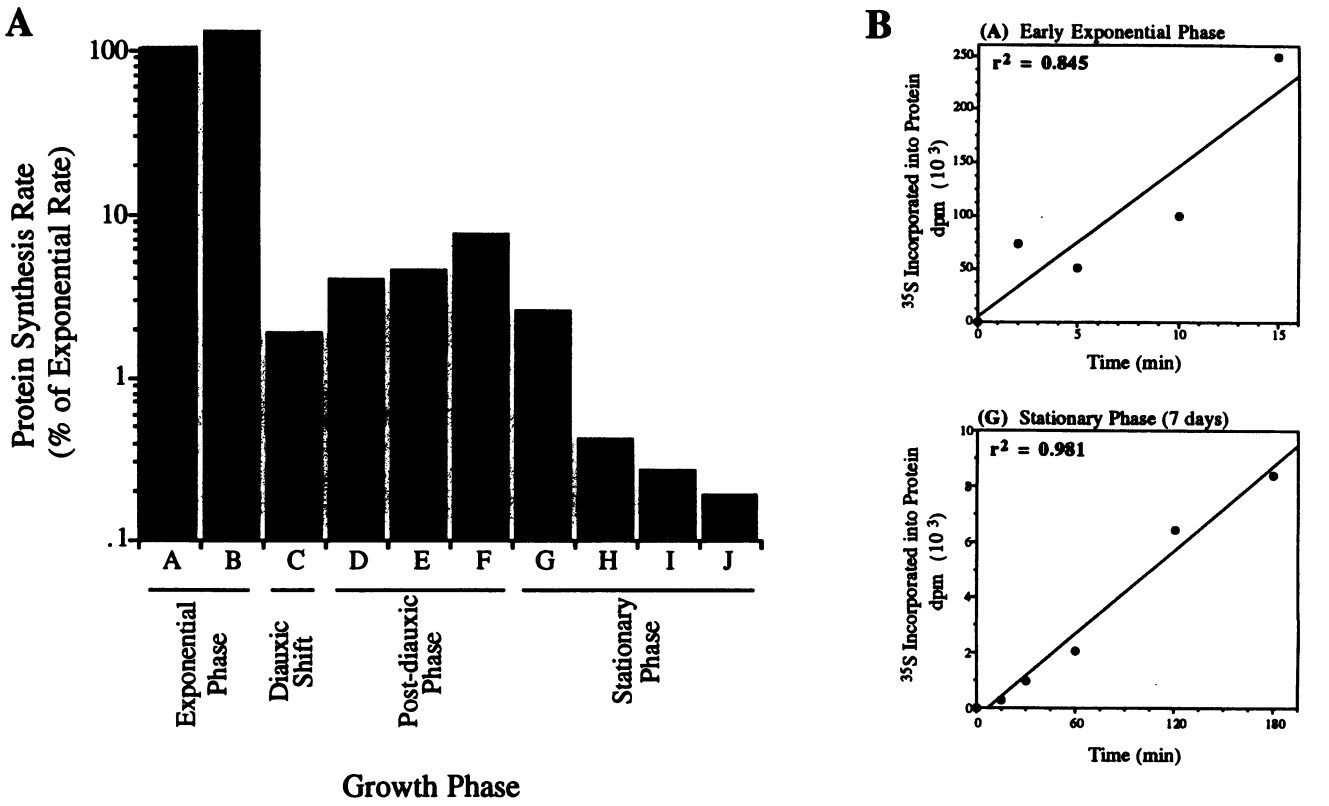


FIG. 2. Labeling efficiency of cells during growth to stationary phase in rich, glucose-based medium. Tran^{35}S-label was added to cultures at the indicated points in the culture cycle (Fig. 1). Samples for each phase of growth were taken at designated times and prepared for liquid scintillation counting as described in Materials and Methods. (A) Rate of incorporation of ^{35}S into proteins at different times during the culture cycle expressed as percent of the rate of incorporation into early-exponential-phase cells. The rate of incorporation for each phase of growth was calculated by plotting the amount of ^{35}S incorporated into acetone-precipitable material over time and calculating the slope. All points are the means of duplicate determinations, which showed a similar pattern. (B) Rate of incorporation of ^{35}S into proteins during exponential phase and stationary phase. Examples of the incorporation of ^{35}S into proteins during early exponential phase (12 h after inoculation, OD_{600} = 1) and stationary phase (7 days after inoculation, OD_{600} = 32) are shown. In all cases the square of the correlation coefficient of the incorporation of ^{35}S into protein over time was greater than 0.8.

Proteins that were synthesized during early exponential phase exhibited two patterns of relative synthesis during growth to stationary phase (Table 1, exponential proteins). Most proteins were repressed at the diauxic shift (class II), and a few proteins were synthesized at similar relative rates throughout the culture cycle (class I). This is consistent with previous reports (3, 5). The repression of protein synthesis at the diauxic shift was transient, and most exponential proteins were again detectable as early as 2 h after glucose exhaustion and rapidly attained prerepression rates of synthesis (Fig. 3 and Table 1).

Proteins that exhibited increased relative rates of synthesis after exponential phase (postexponential proteins) could be placed into three temporal classes (Table 1). Synthesis of one class of proteins (class III) was first detected late in exponential phase. The remaining two classes of proteins were first detected either during (class IV) or after (class V) the diauxic shift. The proteins in all three classes exhibited varying relative rates of synthesis during entry into stationary phase. Some showed increased relative rates of synthesis for a short period, while others were synthesized at an increased relative rate throughout postdiauxic phase and stationary phase.

We also examined unlabeled proteins by 2D-PAGE to assay steady-state accumulation throughout the culture cycle. The steady-state accumulation of most proteins during growth to stationary phase paralleled their synthesis, except at the diauxic shift (data not shown). During the diauxic shift, steady-state accumulation was similar to that observed in exponential phase (data not shown), although there was a transient arrest of protein synthesis (Fig. 3).

Synthesis of Hsp70-related Ssa proteins does not correlate with mRNA accumulation in stationary-phase cells. A number of heat shock proteins exhibit increased synthesis at the diauxic shift (3). Since mRNA accumulation also increases after the diauxic shift for many of these proteins (51), it has been presumed that their synthesis continues throughout stationary phase. We confirmed this hypothesis for the *HSP70*-related gene *SSA3* (Fig. 3 and 4). We also noticed that the synthesis of the Hsp70-related Ssa proteins did not correlate with the accumulation of their mRNAs, suggesting that synthesis of these proteins was regulated by mechanisms other than the control of steady-state mRNA accumulation.

SSA3 mRNA, which was undetectable during exponential phase, began to accumulate at the diauxic shift and increased approximately fivefold during the postdiauxic phase (Fig. 5A). During stationary phase, *SSA3* mRNA accumulation increased an additional twofold and remained at a high level throughout the remainder of the experiment (Fig. 5A). Similarly, Ssa3p was synthesized at a high rate during the diauxic shift and postdiauxic phase, but its synthesis increased only twofold

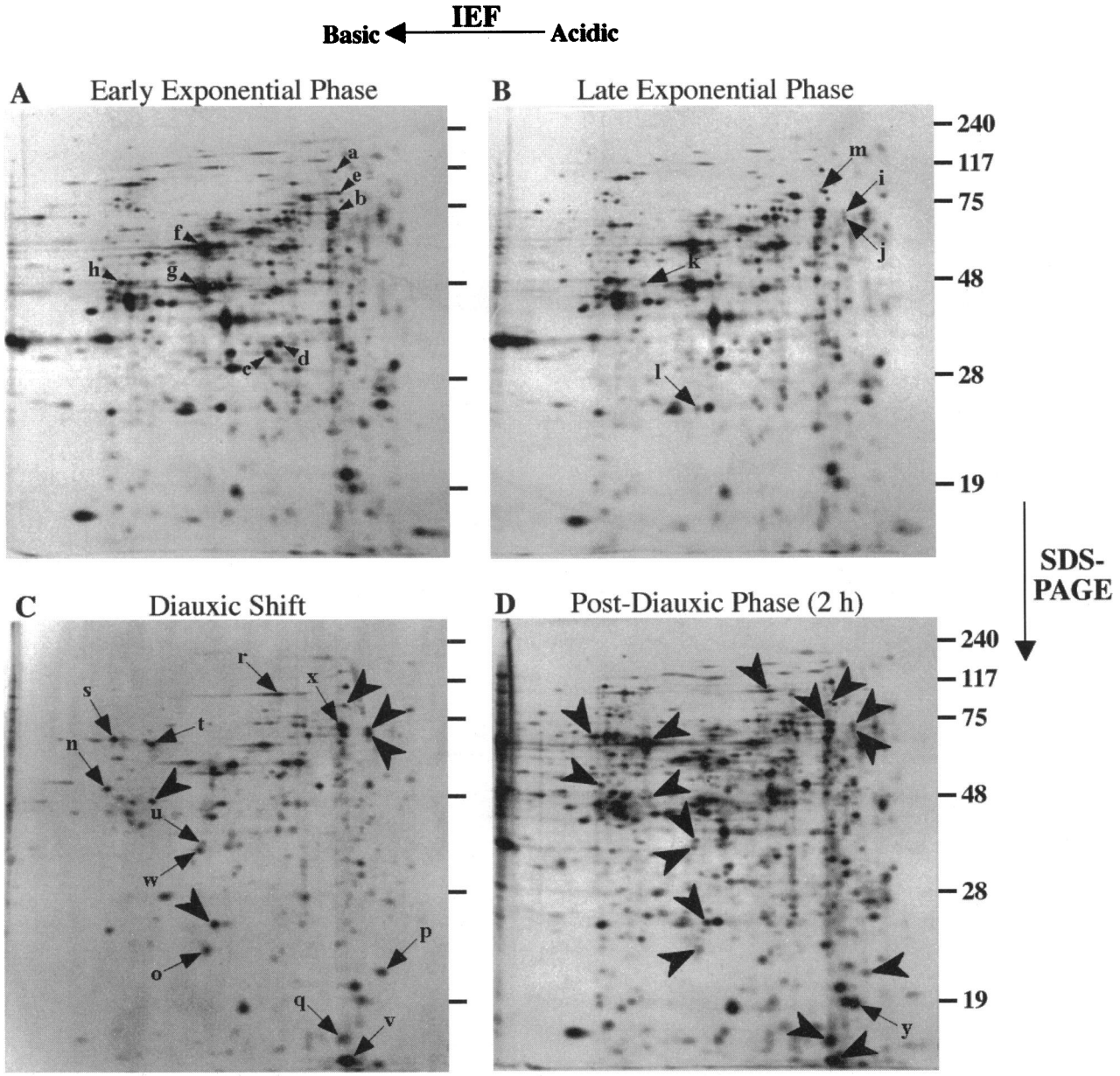


FIG. 3. Synthesis of proteins in wild-type yeast cells during growth to the diauxic shift in rich, glucose-based medium at 30°C. Proteins synthesized in wild-type yeast cells (S288C) during growth to the diauxic shift in YPD at 30°C were analyzed by 2D-PAGE. Equal amounts of labeled protein (10^5 dpm) were loaded onto each gel, and each blot was exposed to film for 10 days. (A) Early-exponential-phase cells, labeled at OD_{600} = 1; (B) late exponential phase, labeled at OD_{600} = 6; (C) diauxic shift, labeled when glucose was exhausted, at OD_{600} = 11; (D) early postdiauxic phase, labeled 2 h after glucose was exhausted, at OD_{600} = 11 (panel labels correspond to time points defined for Fig. 1). Selected exponential proteins are indicated with small arrowheads in panel A by lowercase letters, which are used in Table 1. Proteins are indicated by arrows when they are first detectable and large arrowheads subsequently. Postexponential proteins are indicated with letters that are used in Table 1. The directions of isoelectric focusing and SDS-PAGE are indicated. Numbers at right indicate positions of molecular mass standards in kilodaltons. This figure was prepared with Adobe Photoshop 2.0 as detailed in Materials and Methods.

upon entry into stationary phase and remained constant relative to total RNA throughout stationary phase (Fig. 5B). These results suggested that *SSA3* mRNA is translated more efficiently during the diauxic shift and early postdiauxic phase than in stationary phase.

In contrast to *SSA3* mRNA, accumulation of mRNA for the *HSP70*-related *SSA1* and *SSA2* genes was high during exponential growth, declined approximately sixfold at the diauxic shift, and declined an additional 10-fold during the postdiauxic phase (Fig. 5A). Throughout stationary phase, *SSA1* and *SSA2* mRNA accumulation remained constant at this low level, 60-fold lower than during exponential phase (Fig. 5A). Previous research has shown that, despite a significant decline in *SSA1* and *SSA2* mRNA accumulation, Ssa1p and Ssa2p are abundant proteins in stationary-phase cells (50). Our results demonstrated that accumulation of Ssa1p and Ssa2p in station-

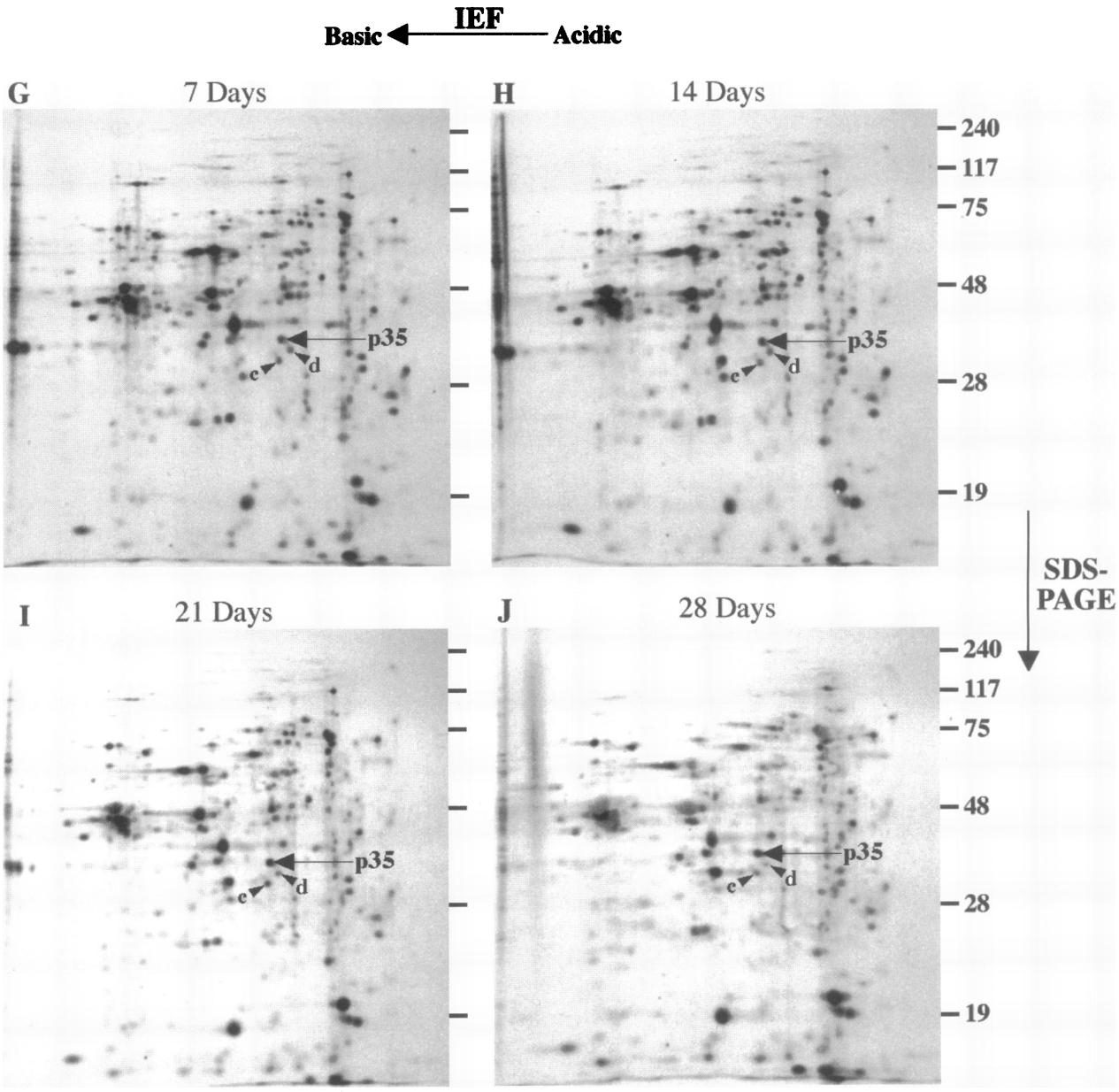


FIG. 4. Synthesis of proteins in wild-type yeast cells during stationary phase after growth in rich, glucose-based medium at 30°C. Wild-type yeast cells (S288C) were grown, and proteins were analyzed as discussed in the Fig. 3 legend. (G) Seven days after inoculation; (H) 14 days after inoculation; (I) 21 days after inoculation; (J) 28 days after inoculation (panel labels correspond to time points defined for Fig. 1). p35 is labeled and indicated with a long arrow. The positions of proteins c and d (Fig. 3A) are shown for reference. This figure was prepared with Adobe Photoshop 2.0 as detailed in Materials and Methods.

ary-phase cells was due, at least in part, to de novo protein synthesis (Fig. 5B).

Surprisingly, in stationary-phase cells the rate of Ssa1p and Ssa2p synthesis was comparable with that of Ssa3p synthesis (Fig. 5B), despite the decrease in the abundance of the *SSA1* and *SSA2* mRNA accumulation and the increase in *SSA3* mRNA abundance (Fig. 5A). The ratio of Ssa1p and Ssa2p synthesis to *SSA1* and *SSA2* mRNA accumulation increased dramatically, reflecting the decrease in *SSA1* and *SSA2* mRNA (Fig. 6). Conversely, the ratio of Ssa3p synthesis to mRNA accumulation decreased dramatically, reflecting the increase in *SSA3* mRNA (Fig. 6). We conclude from this that the relationship between protein synthesis and mRNA accumulation for *SSA1* and *SSA2* and that for *SSA3* during stationary phase is complex, probably reflecting changes in the translatability of these mRNAs.

Increased synthesis of a 35-kDa protein in long-term stationary-phase cultures. Two proteins exhibited increased synthesis after the diauxic shift (Table 1, class V). One, labeled y (Fig. 3D), showed increased synthesis 2 h after the diauxic shift. The second, labeled p35 (Fig. 4), showed increased synthesis later, during postdiauxic phase and stationary phase (Table 1). p35 was unique because it was the last protein to show increased synthesis, and its relative rate of synthesis

TABLE 1. The regulation of selected exponential and postexponential proteins during growth to stationary phase

Class and protein[a]	Mass[b]	pI[c]	Maximum observed relative synthesis[d]										Regulation[e]
			A	B	C	D	E	F	G	H	I	J	
Exponential proteins													
I													
a	117	5.1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	HS
b	70	5.1	++	+++	+++	+++	+++	+++	+++	+++	+++	++	HS
II													
c	33	6	+++	+++	−	−	++	+++	+++	+++	+++	++	N
d	34	5.9	+++	+++	−	+	+++	+++	+++	+++	++	+	N
e	82	5.1	+++	+++	−	+++	+++	+++	+++	+++	++	+	N
f	58	6.3	+++	+++	+	++	+++	+++	+++	+++	+++	++	HS
g	47	6.3	+++	+++	+	+	++	++	++	++	++	+	N
h	48	6.9	++	++	+	++	++	+++	+++	+++	++	+	HS, GR
Postexponential proteins													
III													
i	68	4.3	−	+	+++	++	−	−	−	−	−	−	HS
j	66	4.3	−	+	+++	++	−	−	+	+	+	+	HS
k	48	6.5	−	+	+++	+	+	+	+	+	+	+	HS, GR
l	26	6.3	−	+	+++	++	++	++	++	++	++	++	GR
m	82	5.1	−	+	+++	+++	+++	+++	+++	++	+	+	HS
IV													
n	52	7.1	−	−	+++	++	+	−	−	−	−	−	HS
o	23	6.3	−	−	+++	++	+	+	−	−	−	−	N
p	21	4.2	−	−	+++	++	+	+	+	+	+	+	GR
q	16	5.1	−	−	++	+++	+	+	+	+	+	+	N
r	104	6	−	−	+++	+++	++	++	++	+	+	+	HS
s	68	7.1	−	−	++	+++	++	++	++	++	++	++	ND
t	66	6.5	−	−	++	+++	++	++	++	++	++	++	HS, GR
u	36	6.3	−	−	++	+++	+++	+++	+++	++	+	+	N
v	14	5.1	−	−	+++	+++	+++	+++	+++	+++	++	++	ND
w	38	6.3	−	−	++	+++	+++	+++	+++	+++	++	++	N
x	71	5.1	−	−	++	++	+++	+++	+++	+++	+++	++	HS
V													
y	19	4.3	−	−	−	+++	+++	+++	+++	+++	++	++	N
p35	35	6	−	−	−	−	++	++	++	++	+++	+++	N

[a] Identities of known proteins: b, Ssa1p and Ssa2p (53); e, Hsc82p (44); g, Eno2p (4, 10, 34); h, Eno1p (10, 25, 34); m, Hsp82p (44); r, Hsp104p (44); x, Ssa3p (53).
[b] Observed molecular mass in kilodaltons. Predicted molecular masses of known proteins: b (Ssa1p and Ssa2p), 70 kDa; e (Hsc82p), 81 kDa; g (Eno2p), 47 kDa; h (Eno1p), 47 kDa; m (Hsp82p), 81 kDa; r (Hsp104p), 102 kDa; x (Ssa3p), 71 kDa.
[c] Observed pI, based upon comparison with control gel. Predicted isoelectric points of known proteins: b (Ssa1p), 4.84; b (Ssa2p), 4.79; e (Hsc82p), 4.61; g (Eno2p), 5.61; h (Eno1p), 6.16; m (Hsp82p), 4.69; r (Hsp104p), 5.53; x (Ssa3p), 4.89.
[d] Symbols denote the approximate percentage of the maximum observed relative synthesis for a given polypeptide: −, not detectable; +, 0 to 10%; ++, 10 to 50%; +++, 50 to 100%. Time points belong to phases of the culture cycle as follows: A and B, exponential; C, diauxic; D to F, postdiauxic; G to J, stationary.
[e] Additional regulation noted. Abbreviations: HS, heat shock protein; GR, glucose-repressed protein; N, = neither; ND, not determined.

continued to increase throughout stationary phase (Fig. 4 and Table 1).

p35, which has an approximate molecular mass of 35 kDa, exhibits a unique pattern of regulation. p35 synthesis was not detectable during exponential growth, during the diauxic shift, or 2 h after the diauxic shift (Fig. 3). p35 synthesis was first detected 16 h after the diauxic shift (Table 1), and its synthesis continued to increase into stationary phase (Fig. 4). Quantitation of p35 synthesis by scanning densitometry of the autoradiograms revealed that its synthesis increased relative to internal standards approximately twofold from day 7 to day 21. However, on the basis of total protein staining with Coomassie blue or biotin-avidin, p35 steady-state accumulation was rather low and occurred substantially after its synthesis became detectable (data not shown).

Previous reports indicate that many proteins exhibiting increased synthesis during the diauxic shift are also regulated by glucose repression and heat shock (3). To determine whether p35 was regulated by glucose repression or heat shock, Tran^{35}S-label was added to exponentially growing cultures after they were transferred to glucose-limited medium or exposed to a brief heat shock. Synthesis of p35 was examined by 2D-PAGE. Although it was possible to detect synthesis of proteins known to be induced after transfer to glucose-limited medium or heat shock, p35 synthesis was not detected (data not shown). These results suggest that p35 represents a novel type of postexponential protein that is induced at a relatively late point during growth to stationary phase.

DISCUSSION

Our experiments reveal a complex picture of induction and repression of protein synthesis throughout the yeast culture cycle. There appear to be classes of cytosolic proteins specifically synthesized as cells progress through the culture cycle during growth to stationary phase. Proteins that are synthe-

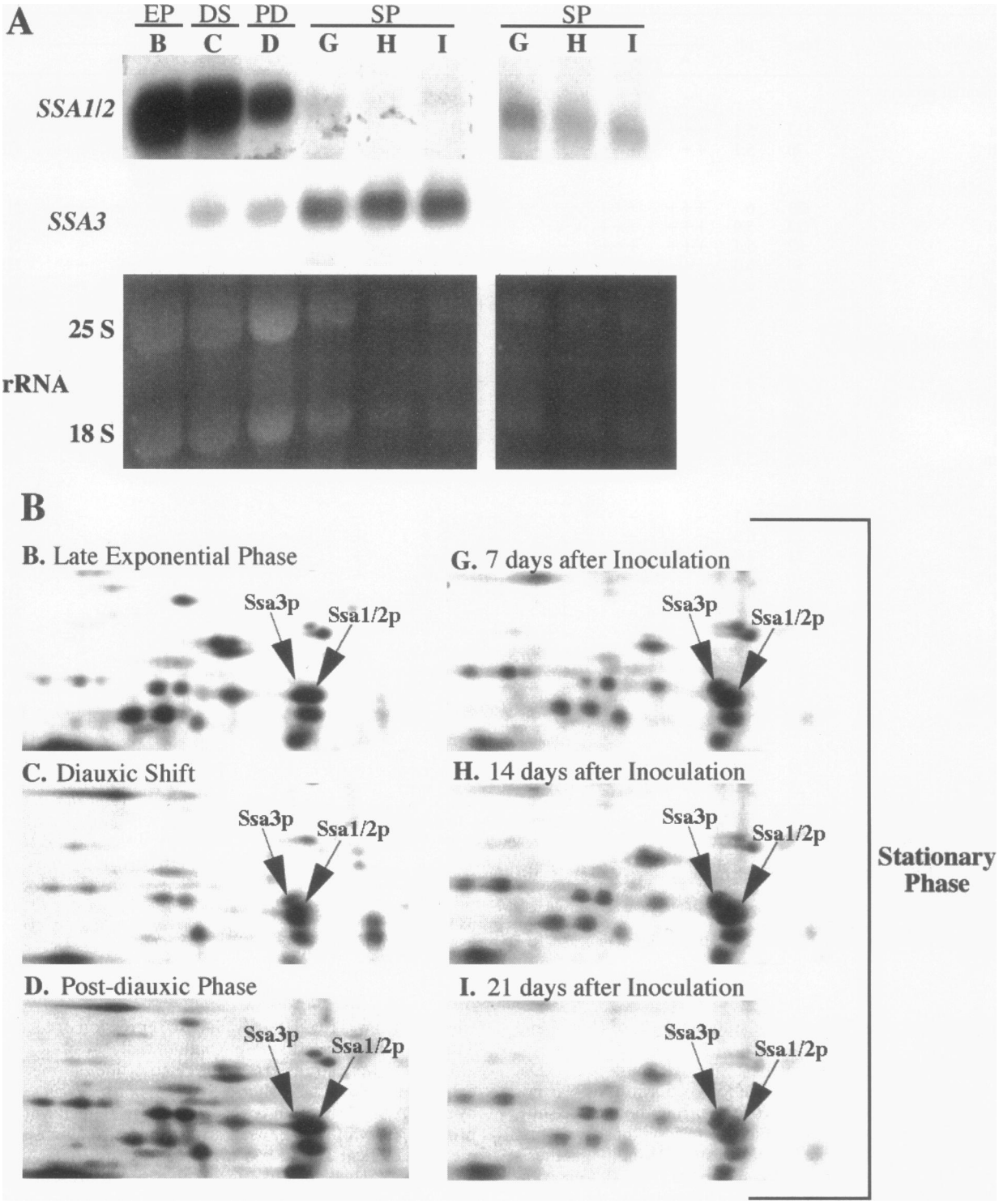
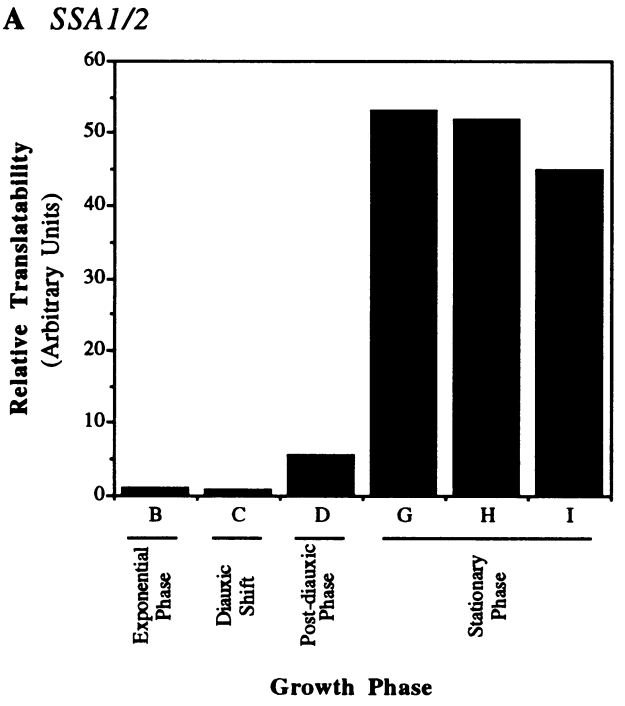


FIG. 5. Synthesis of the Hsp70-related proteins Ssa1p, Ssa2p, and Ssa3p throughout the culture cycle. Synthesis of Ssa1p and Ssa2p and of Ssa3p is compared with the accumulation of the respective mRNAs at different stages in the culture cycle. Total RNA and labeled protein were isolated from cultures at the indicated points in the culture cycle as described in Materials and Methods. (A) Accumulation of the *SSA1* and *SSA2* message and of the *SSA3* message was assayed by Northern blot analysis of total RNA (5 μg) probed with random-primer-labeled *SSA2* DNA (for *SSA1* and *SSA2*) or *SSA3* DNA (for *SSA3*). Each blot was exposed for 48 h. On the right, a 48-h exposure of a second blot probed with random-primer-labeled *SSA2* DNA with a higher (~fourfold) specific activity is shown, to demonstrate the relatively constant low level of *SSA1* and *SSA2* mRNA accumulation during stationary phase. Letters above lanes correspond to cell cycle phases as indicated in the Fig. 1 legend. Ethidium bromide-stained 18S and 25S rRNA bands are shown in the lower part of panel A. (B) Details of the region containing Ssa1p and Ssa2p (protein b in Table 1) and Ssa3p (protein x in Table 1) from the gels shown in Fig. 3 and 4. This figure was prepared with Adobe Photoshop 2.0 as detailed in Materials and Methods.



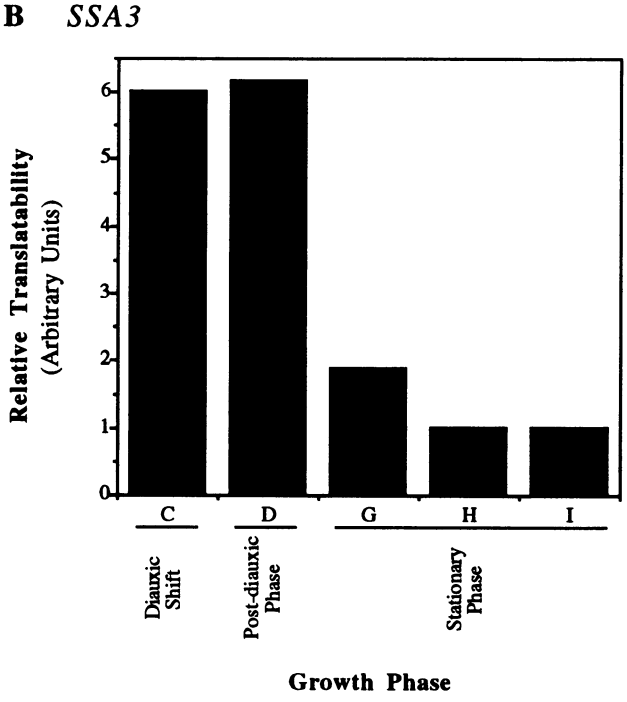


FIG. 6. Relative translatability of Hsp70 mRNA during growth to stationary phase. The relative translatability of the *SSA1* and *SSA2* message and the *SSA3* message is expressed as the ratio of the relative rate of protein synthesis and relative accumulation of the mRNAs. These values are not corrected for the absolute rate of protein synthesis. Protein synthesis and mRNA accumulation were measured by densitometry as described in Materials and Methods. (A) Relative translatability of the *SSA1* and *SSA2* mRNA; (B) relative translatability of the *SSA3* mRNA. The relative translatability of *SSA3* is not calculated for late exponential phase because *SSA3* mRNA is not detectable in late exponential phase.

sized during exponential phase (exponential proteins) can be divided into two classes: those that do not exhibit a transient repression of synthesis at the diauxic shift (class I) and those that do exhibit a transient repression of synthesis at the diauxic shift (class II). The functional significance of the transient repression of synthesis of class II proteins at the diauxic shift is unclear.

Proteins that show increased synthesis after exponential growth (postexponential proteins) can be divided into three classes: class III proteins exhibit detectable synthesis before the diauxic shift, class IV proteins are first detectable at the diauxic shift, and class V proteins are only detectable after the diauxic shift. Within these classes, significant variability with respect to the rate at which individual proteins are synthesized and the duration of their synthesis after the diauxic shift exists. As expected (3, 5, 50), many of these proteins are also regulated by heat shock or glucose repression.

We have examined one class V postexponential protein, p35, in greater detail. p35 exhibits a novel pattern of regulation. Unlike many proteins that show increased synthesis after exponential growth, it is not regulated by heat shock or glucose repression. p35 is induced later than all other proteins in these studies and is the only protein that exhibits an increase in its relative rate of synthesis during stationary phase. The functional significance of p35 induction during the postdiauxic period and stationary phase remains unknown; analysis of the nature of the p35 gene product should allow determination of its function.

Previous investigators have reported that, in *S. cerevisiae*, the rate and pattern of protein synthesis change when cells enter stationary phase (3, 5). These studies, performed in minimal medium, showed that the rate of incorporation of methionine into protein declined at the diauxic shift to 5 to 10% of that in exponential phase (5). We observed a similar decline at the diauxic shift to approximately 2% of the exponential-phase levels. The rate of incorporation increased progressively after the diauxic shift, until the cells entered stationary phase, at which point the rate decreased again. This transient repression of protein synthesis followed by a progressive increase correlates with cell growth during the postdiauxic phase, prior to entry into stationary phase.

We also found that in stationary-phase cells the incorporation of radiolabeled precursors into protein was linear over relatively long periods of time. This indicates that proteolysis does not have a major effect upon the accumulation of newly synthesized protein during the periods we used for our labeling experiments. It has been suggested that proteolysis is increased in stationary-phase cells because (i) many genes involved in the regulation of proteolysis are important to the maintenance of viability during stationary phase (51), (ii) protease activities are increased in lysates from stationary-phase cells (27), and (iii) autophagy occurs in starved cells (46). However, it is clear from our results that the overall rate of proteolysis must be comparable to the rate of protein synthesis in stationary-phase cells, since the absolute accumulation of proteins declines only 60% after exponential phase and remains constant (6) while the rate of protein synthesis declines substantially more (Fig. 2A).

Consistent with the earlier studies (3, 5), we observed that the diauxic shift was marked by a sudden alteration in the pattern of protein synthesis and that synthesis of most proteins is repressed at the diauxic shift. However, we also found that synthesis of most proteins increased again very rapidly after the diauxic shift. Previous experiments, using cells grown in minimal medium, indicated that a recovery of protein synthesis does not occur until at least 10 h after glucose exhaustion (3). We have observed that the transient growth arrest occurring at

the diauxic shift is much longer in minimal medium (ca. 10 h) than in rich medium (ca. 1 to 2 h) (6). We suggest, therefore, that the repression of protein synthesis observed by others actually occurred during the prolonged diauxic shift associated with cells grown in minimal medium and that this transient repression of most proteins occurs only during the diauxic shift.

Strikingly, the pattern of protein synthesis after the diauxic shift is remarkably similar to that observed in exponentially growing cells and this pattern of protein synthesis continues largely unaltered for up to 3 weeks in stationary phase. This suggests that the recovery of protein synthesis that occurs after the diauxic shift is not simply an effect of the resumption of growth during the postdiauxic phase. Similarities in the pattern of protein synthesis at almost all points in the culture cycle also suggest that many of these proteins provide housekeeping functions in both exponentially growing and stationary-phase cells and that a relatively small repertoire of unique proteins may be necessary for stationary-phase function or for growth.

Post-translational protein modification has been implicated in stationary-phase regulation and survival (51). The most striking evidence for protein modification is observed in Bcy1p (52), the regulatory subunit for cAMP-dependent protein kinase (A-kinase). During growth to stationary phase, cells progressively accumulate larger isoforms of Bcy1p, and this accumulation occurs at a time when inhibition of A-kinase activity is critical (52). Clearly, our results demonstrate that most newly synthesized cytosolic proteins are not posttranslationally modified in stationary phase because the relative migration was the same in protein gels from exponential and stationary-phase cultures. However, nonabundant, modified regulatory proteins, such as Bcy1p, would not be detected in this study.

The decreased transcription of most genes that occurs at the diauxic shift has already been shown to be nonessential for the cell cycle arrest or the acquisition of stationary-phase physiological characteristics. Topoisomerase I (*TOP1*) mutants do not exhibit the general repression of transcription at the diauxic shift but are phenotypically identical to isogenic wild-type strains with respect to entry into and maintenance of stationary phase (13). This has led to the suggestion that translational repression may play a major role in stationary phase. Interestingly, a number of mutants that affect translation also exhibit a temperature-sensitive cell cycle arrest phenotype that resembles entry into stationary phase (51). It is possible that some of these genes encode factors that become rate limiting for translation in stationary phase.

As reported previously, many of the proteins that show an increased relative rate of synthesis at the diauxic shift are glucose-repressed proteins and heat shock proteins (3, 5). Many of these proteins are encoded by genes that exhibit increased mRNA accumulation after the diauxic shift. This observation led to the suggestion that synthesis of proteins in stationary phase correlates with the accumulation of the mRNAs that encode them. However, on the basis of a comparison of the rate of protein synthesis and the accumulation of mRNAs encoding the Hsp70-related Ssa proteins, our results suggest that this correlation is not always observed. Synthesis of Ssa1p and Ssa2p proteins is comparable to that of Ssa3p in stationary-phase cells, despite the repression of their transcripts and the induction of the *SSA3* transcript. We believe that the continued synthesis of Ssa1p and Ssa2p (at rates comparable with that of Ssa3p) indicates that *SSA3* mRNA is translated less efficiently than are *SSA1* and *SSA2* mRNAs after exponential phase. A general repression of transcription and decline in mRNA accumulation occurs at the diauxic shift and progresses through stationary phase (13). Although this observation was consistent with the repression of most proteins that occurs at the diauxic shift, the resumed synthesis of most proteins after the diauxic shift suggests that protein synthesis during stationary phase is controlled by factors other than mRNA accumulation.

Previous results support the assertion that mRNA accumulation is not directly correlated with stationary-phase protein synthesis. For example, the *RAS2* transcript declines to nondetectable levels after the diauxic shift, but the rate of synthesis of its protein, Ras2p, is constant throughout the culture cycle (7, 8). Additionally, accumulation of *BCY1* mRNA is comparable in stationary phase and exponential phase, but accumulation of Bcy1p is approximately 10-fold higher in stationary phase than in exponential phase (49, 52). Finally, the accumulation of *RPB4* message, which encodes a subunit of RNA polymerase II (14, 54), is slightly (fourfold) lower in stationary phase than in exponential phase but the accumulation of Rpb4p remains relatively unchanged during exponential phase and stationary phase (14). Interestingly, increasing the accumulation of *RPB4* mRNA by overexpression does not cause increased Rpb4p accumulation (14). Although it has not been shown that Bcy1p or Rpb4p accumulation is regulated by protein synthesis, like that of Ras2p and that of Ssa1p and Ssa2p, these results are consistent with regulated changes in the translatability of these mRNAs.

The synthesis of a complex array of proteins during stationary phase in yeast is very different from that found in the gram-negative heterotrophic bacteria, in which most protein synthesis is arrested in stationary phase (1, 29). However, the changes in protein synthesis that occur when yeast cells enter stationary phase are very similar to those that occur when vertebrate cells are arrested in G_0. When vertebrate cells enter G_0, they continue to synthesize most of the same proteins synthesized during growth (20, 41), and few changes are evident when proteins synthesized in G_0 cells and proliferating cells are analyzed by 2D-PAGE (16). Like stationary-phase yeast cells, vertebrate cells arrested in G_0 exhibit a decrease in the rate of protein and RNA synthesis, called the pleiotypic response (23). Our results suggest that with respect to protein synthesis stationary phase in yeast cells is more similar to G_0 in vertebrate cells than stationary phase in prokaryotes.

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