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Function of a Relaxed-Like State following Temperature Downshifts in *Escherichia coli*

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Temperature downshifts of *Escherichia coli* throughout its growth range resulted in transient growth inhibition and a cold shock response consisting of transient induction of several proteins, repression of heat shock proteins, and, despite the growth lag, continued synthesis of proteins involved in transcription and translation. The paradoxical synthesis of the latter proteins, which are normally repressed when growth is arrested, was explored further. First, by means of a nutritional downshift, a natural stringent response was induced in wild-type cells immediately prior to a shift from 37 to 10°C. These cells displayed decreased synthesis of transcriptional and translational proteins and decreased induction of cold shock proteins; also, adaptation for growth at 10°C was delayed, even after restoration of the nutrient supplementation. Next, the contribution of guanosine 5′-triphosphate-3′-diphosphate and guanosine 5′-diphosphate-3′-diphosphate, collectively abbreviated (p)ppGpp, to the alteration in cold shock response was studied with the aid of a mutant strain in which overproduction of these nucleotides can be artificially induced. Induction of (p)ppGpp synthesis immediately prior to shifting this strain from 37 to 10°C produced results differing only in a few details from those described above for nutritional downshift of the wild-type strain. Finally, shifting a *relA spoT* mutant, which cannot synthesize (p)ppGpp, from 24 to 10°C resulted in a greater induction of the cold shock proteins, increased synthesis of transcriptional and translational proteins, decreased synthesis of a major heat shock protein, and faster adaptation to growth than for the wild-type strain. Our results indicate that the previously reported decrease in the (p)ppGpp level following temperature downshift plays a physiological role in the regulation of gene expression and adaptation for growth at low temperature.

When the temperature of a culture of *Escherichia coli* in exponential growth in rich medium is abruptly decreased from 37 to 10°C, growth stops for several hours before a new rate of growth is established (11). Protein synthesis is greatly decreased during the lag, and the pattern of proteins synthesized is unique. Some proteins are induced (termed cold shock proteins) or continue to be made at preshift differential rates; others (including some heat shock proteins) are severely repressed (11). Because some of the identified cold shock proteins participate in transcription or translation (11) or are on operons containing other transcriptional and translational genes (11) and because cold temperature has been reported to restrict growth by inhibiting translation (1), the cold shock response has been interpreted as an adaptive response to facilitate expression of genes involved in translation.

The pattern of protein synthesis in *E. coli* following temperature shifts to lower temperatures that decrease growth rate is reminiscent of the pattern following nutrient shifts that increase growth rate. For this reason, we directed attention to a global control system in which (p)ppGpp (guanosine 5′-triphosphate-3′-diphosphate and guanosine 5′-diphosphate-3′-diphosphate) has been implicated as a regulator. *E. coli* responds to amino acid starvation by increasing (p)ppGpp levels via an *relA* gene-dependent mechanism and responds to a carbon source downshift by increasing (p)ppGpp levels in a *spoT* gene-dependent mechanism (15). The *relA* mechanism involves activation of (p)ppGpp synthetase I by high uncharged/charged tRNA ratios (21, 22). The *spoT* gene product degrades (p)ppGpp, but the rate of degradation is slowed during carbon source downshift (2). Furthermore, SpoT appears to function as a synthetase [(p)ppGpp synthetase II] of (p)ppGpp even in an *relA* deletion strain (10, 15, 33). The physiological mechanism responsible for determining the distribution of synthetic or degradative functions of SpoT is not known. The increase in the level of (p)ppGpp under both nutrient stress conditions results in a coordinate decrease in the rate of synthesis of RNA and ribosomal proteins (for reviews, see references 2 and 24). *E. coli* responds to the shortage of charged tRNA and to the poorer carbon source by decreasing the synthesis of translational components which are in functional excess and by increasing the synthesis of enzymes of biosynthetic and fueling pathways (17).

The basal level of (p)ppGpp is maintained during balanced growth by complex contributions from RelA- and SpoT-mediated synthesis [(p)ppGpp synthetases I and II] as well as SpoT-mediated degradation (for reviews, see references 2 and 14). Although not as extensively characterized as the nutritional responses, changes in the (p)ppGpp level also occur as a function of temperature (13, 20, 23). Shifting *E. coli* from 23 to 40°C results in an immediate temperature-dependent increase in the synthesis of RNA followed by an increase in the (p)ppGpp level and a corresponding decrease in the rate of synthesis of RNA (13). Conversely, a downshift from 40 to 23°C results in an immediate decrease in the synthesis of RNA followed by a decrease in the (p)ppGpp

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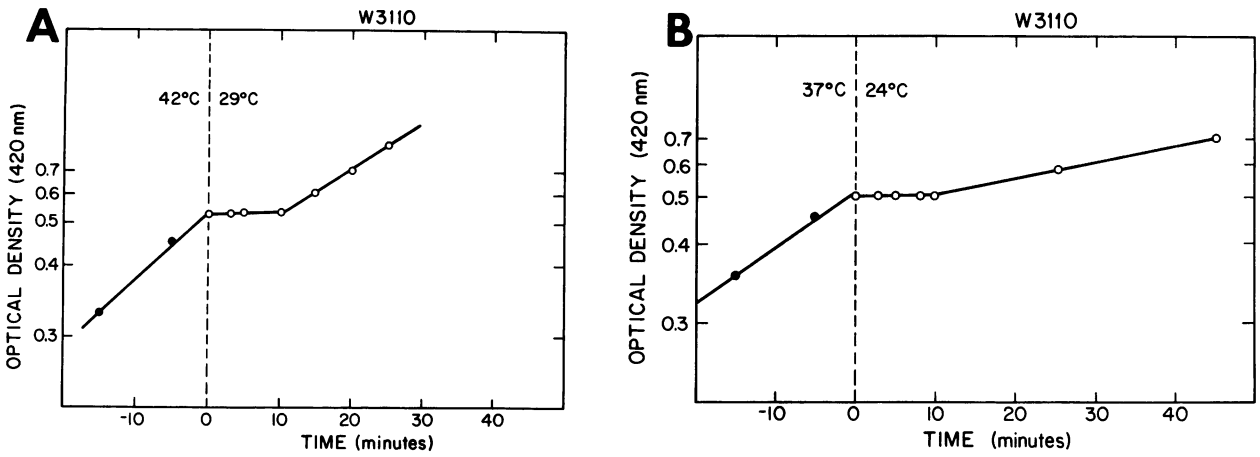


FIG. 1. Growth of strain W3110 in glucose-MOPS rich medium. (A) Shift from 42 to 29°C. (B) Shift from 37 to 24°C.

level and a corresponding increase in the rate of synthesis of RNA (13). Furthermore, shifting *E. coli* from 41°C to any lower temperature results in a decrease in the (p)ppGpp level; the greater the drop in temperature, the greater the decrease in the (p)ppGpp level (20). It has been suggested that the level of (p)ppGpp as a function of temperature shifts has a role in regulating RNA synthesis in *E. coli* (13).

Here we report findings of studies of a wild-type strain and of two strains altered in (p)ppGpp metabolism: one incapable of producing (p)ppGpp (33) and one that can be induced to overproduce (p)ppGpp with isopropyl-β-D-thiogalactopyranoside (IPTG) (26). Our results indicate that the induction of the cold shock response is negatively affected by (p)ppGpp levels and that there is an interplay between the stringent and cold shock response networks during temperature downshifts.

MATERIALS AND METHODS

Bacterial strains. *E. coli* W3110 was used as the wild-type strain in these studies. Its derivative, CF1946, contains deletions in both the *relA* and *spoT* genes, *relA251* and *spoT207*, respectively. Strain W3110/pSM11 contains a plasmid, pSM11, with a truncated *relA* gene driven by an IPTG-inducible *p-tac* promoter (26).

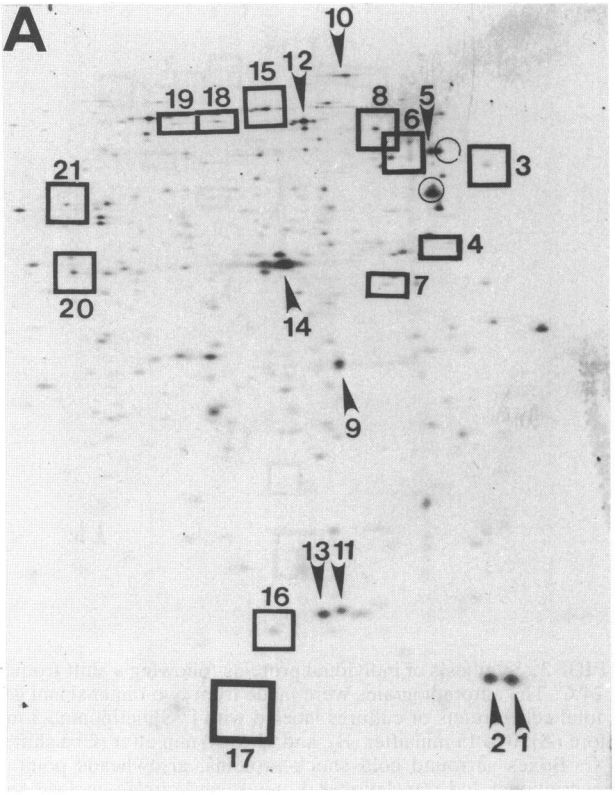
Media and bacterial growth. All experiments employed rich medium composed of defined morpholinepropanesulfonic acid (MOPS) medium (16) supplemented with 0.4% glucose, 20 amino acids, 5 vitamins, and 4 bases (31). The concentration of carrier methionine was 0.02 mM when labeling was done with [^{35}S]methionine. For experiments with strain W3110/pSM11, ampicillin (50 μg/ml) was incorporated in the medium. Cells were grown aerobically in rotary-action water bath shakers; growth was monitored spectrophotometrically at an optical density of 420 nm.

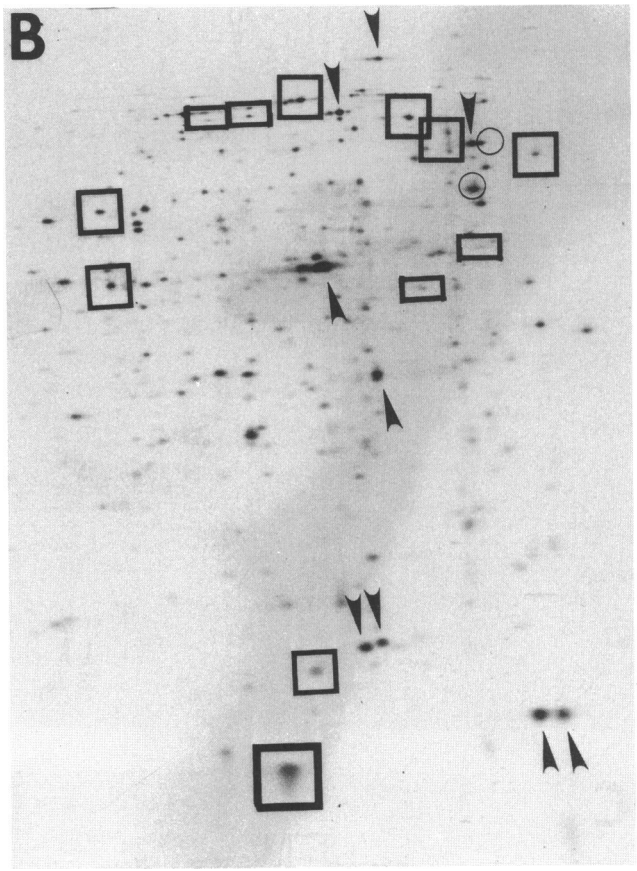
Radioactive labeling of cultures for autoradiography. Steady-state cultures (25-ml volume) of bacterial strains were grown at the preshift temperature to an optical density at 420 nm of approximately 0.30 and then shifted to the postshift temperature. For temperature shifts to 10°C, a 1-ml portion of the culture was labeled with [^{35}S]methionine (12.5 Ci/mmol; 250 μCi/ml) at the times indicated. For other experiments, a 1-ml portion of the culture was labeled with [^{35}S]methionine (5 Ci/mmol; 100 μCi/ml) at various times. For steady-state labeling of proteins, a 1-ml portion of the culture was labeled for several generations. Extracts were prepared and processed by two-dimensional polyacrylamide gel electrophoresis (19).

RESULTS

Cold shock response is induced following shifts from 37 to 24°C and from 42 to 29°C. The 40°C range of temperature over which *E. coli* can grow is conveniently considered to consist of three subranges: a central normal range from 20 to 37°C, within which the Arrhenius temperature characteristic of growth rate is constant; a low range (below 20°C), within which the temperature characteristic approaches infinity as the temperature is lowered toward 8.5°C; and a high range (above 37°C), within which the temperature characteristic approaches negative infinity as the temperature is raised toward the maximum possible for growth (9). To determine whether the cold shock response is induced by any downshift in temperature or whether a shift to some temperature in the low range is necessary, cultures of *E. coli* W3110 were shifted from 37°C (normal range) to 24°C (normal range) and from 42°C (high range) to 29°C (normal range). Samples were labeled 5 min prior to the shift and 0 to 15 and 15 to 30 min after the shift. Extracts were made of each sample, and portions with equal amounts of radioactivity were processed by two-dimensional electrophoresis.

Shifting a culture from 37 to 24°C or from 42 to 29°C resulted in a 10-min lag period (Fig. 1). We can estimate the magnitude of the cold shock response by the behavior of protein F10.6 (Fig. 2, spot 17), the most dramatically induced (200-fold) protein following a shift from 37 to 10°C; other cold shock proteins are induced along with F10.6, but the range of their inductions is from 2-fold to 10-fold (11). Judging from the labeling intensity of protein F10.6 in Fig. 2B and 3B, a mild cold shock response occurred during the lag periods at 24 and 29°C (cold shock proteins are boxed). After the lag period at 24°C, but not at 29°C, the rate of synthesis of F10.6 (spot 17) continued to increase. Shifts from 42 to 36°C and from 36 to 29°C resulted in a small induction of protein F10.6 (unpublished observation). Although there does not seem to be a threshold temperature for induction, the 13°C downshifts from higher temperatures (42 and 37°C) resulted in milder induction of the response than the downshift from 24 to 10°C (see Fig. 11), suggesting that there may be some special feature of low temperature. Also,





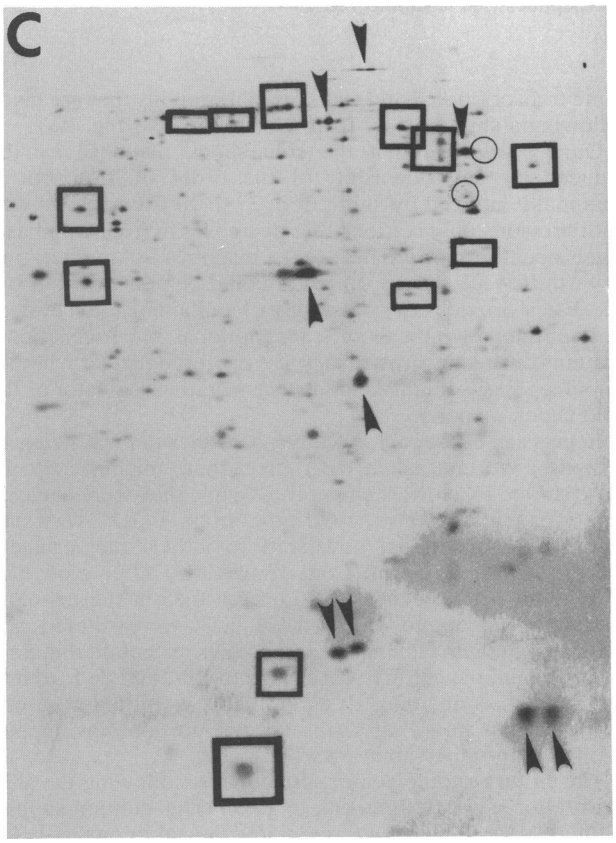
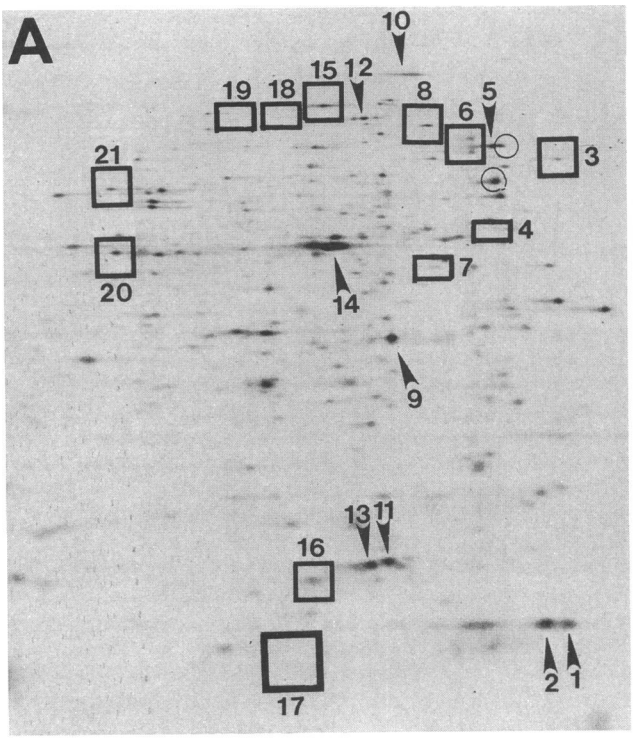
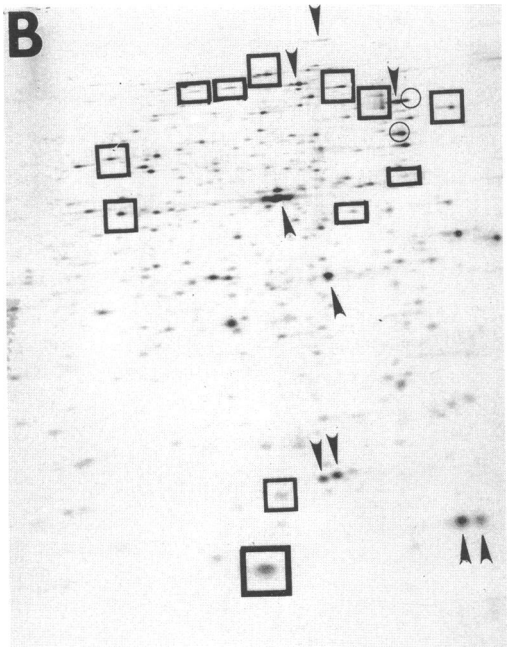


FIG. 2. Synthesis of individual proteins following a shift from 42 to 29°C. The autoradiograms were made from two-dimensional gels of total cell extracts of cultures labeled with [^{35}S]methionine 5 min before (A), 0 to 15 min after (B), and 15 to 30 min after (C) a shift to 29°C. Boxes surround cold shock proteins, arrowheads point to transcriptional and translational proteins, and circles enclose heat shock proteins DnaK and GroEL. The identities of the numbered polypeptides are as follows: spot 1, ribosomal protein L7; spot 2, ribosomal protein L12; spot 3, NusA; spot 4, B46.5; spot 5, ribosomal protein S1; spot 6, dihydrolipoamide acetyltransferase subunit of pyruvate dehydrogenase; spot 7, RecA; spot 8, polynucleotide phosphorylase; spot 9, elongation factor Ts; spot 10, beta subunit of RNA polymerase; spots 11 and 13, ribosomal protein S6; spot 12, elongation factor G; spot 14, elongation factor Tu; spot 15, pyruvate dehydrogenase; spot 16, H-NS; spot 17, F10.6; spot 18, F84.0; spot 19, G74.0; spot 20, G41.2; spot 21, G55.0.

the 27°C shift from 37 to 10°C resulted in the strongest induction of the cold shock response, showing that the magnitude of the temperature shift does affect the magnitude of the response; in particular, initiation factor 2 and the dihydrolipoamide acetyltransferase subunit of pyruvate dehydrogenase are induced following the shift from 37 to 10°C (11) but not by smaller downshifts. Finally, maximum induction occurs 2 h following a shift from 37 to 10°C (11), but the response to smaller decreases in temperature is mounted more quickly (15 to 30 min). The difference in timing probably is related to the severe inhibition of protein synthesis that follows a shift to 10°C.

It has been reported that shifting *E. coli* from 37 to 24°C results in repression of heat shock proteins (29). The autoradiograms in Fig. 2C and 3C reveal that the two 13°C downshifts resulted in repression in synthesis of two major





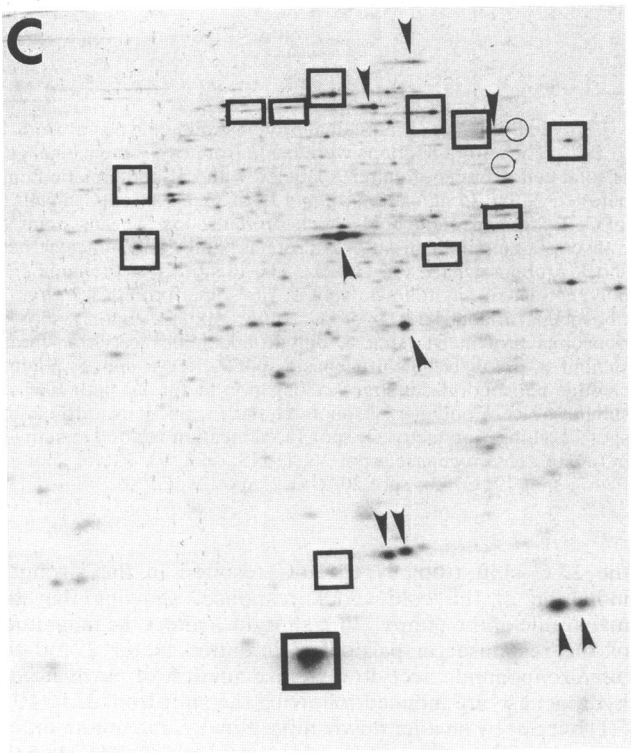


FIG. 3. Synthesis of individual proteins following a shift from 37 to 24°C. The autoradiograms were made from two-dimensional gels of total cell extracts of cultures labeled with [^{35}S]methionine 5 min before (A), 0 to 15 min after (B), and 15 to 30 min after (C) a shift to 24°C. Boxes surround cold shock proteins, arrowheads point to transcriptional and translational proteins, and circles enclose heat shock proteins DnaK and GroEL. For identifications of numbered polypeptides, see legend to Fig. 2.

heat shock proteins, with GroEL more affected than DnaK, and maximum repression occurring following the lag periods.

The same autoradiograms also revealed that both downshifts in temperature resulted in continued synthesis of the same transcriptional and translational proteins that are made following a shift from 37 to 10°C (11; see also Fig. 7A).

Our observation that the cold shock response can be induced by any downshift of the order of 13°C but is maximally induced by shifts from 37 to 10°C correlates well with previous observations that decreases in (p)ppGpp basal levels occur proportionately to the magnitude of temperature downshifts (20). This correlation could be coincidental, but because it provides the possibility of explaining the paradoxical continued synthesis of transcriptional and translational proteins during the growth lags, we explored further whether (p)ppGpp levels might function as a negative regulator of the cold shock response.

Repression of the cold shock response by a natural stringent response. We first asked what effect there might be if cells were shifted to a lower temperature while they were engaged in a stringent response. Wild-type strain W3110 was subjected to a nutritional downshift to induce the stringent response prior to a shift from 37 to 10°C. After growth at 37°C to an optical density (420 nm) of 0.25 in rich medium containing 20 amino acids, these cells were filtered and placed in medium lacking all amino acids except methionine. After 10 min, the culture was shifted to 10°C and divided into two, and the remaining 19 amino acids were added to one portion. Four hours later, the second portion was supplemented with the 19-amino-acid mixture.

The culture supplemented immediately following the shift grew after a 4-h lag period (Fig. 4). The culture supplemented after 4 h at 10°C exhibited an additional 4-h lag before growth could occur at that temperature (Fig. 4). To learn the patterns of protein synthesis in these two cultures,

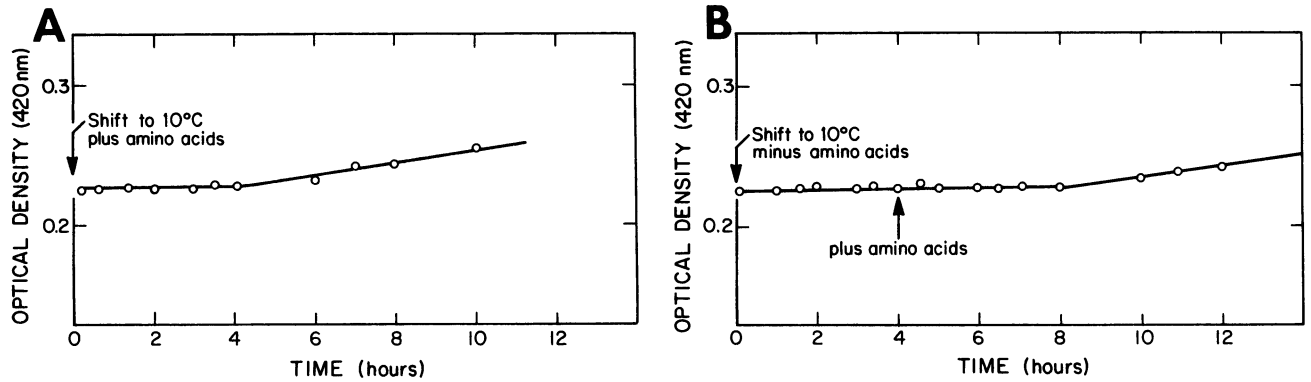


FIG. 4. (A) Growth of strain W3110 in glucose-MOPS medium plus 20 amino acids following a shift from 37 to 10°C. (B) Growth of strain W3110 in glucose-MOPS medium minus all amino acids except methionine after a shift to 10°C followed by the addition of the 19 amino acids 4 h later.

separate portions of each were labeled with [^{35}S]methionine from 0 to 120 min after the shift to 10°C. Extracts of each sample were made, and portions with equal numbers of cells were processed by two-dimensional electrophoresis. Not surprisingly, autoradiograms of the resulting gels (Fig. 5) revealed that the nutritional downshift resulted in repression of ribosomal proteins L7/L12 (spots 1 and 2) and S1 (spot 5) and elongation factor G (spot 12). Of greater interest, some cold shock proteins were also repressed: proteins NusA (spot 3), PNP (spot 8), pyruvate dehydrogenase (spot 15), dihydrolipoamide acetyltransferase subunit of pyruvate dehydrogenase (spot 6), and F10.6 (spot 17).

The overall result is simply stated: imposition of stringency diminishes induction of cold shock proteins upon a shift to 10°C and prevents or delays growth adaptation to this temperature. We next explored whether (p)ppGpp itself, or some other aspect of nutrient limitation, was responsible.

Repression of the cold shock response by IPTG in strains containing *relA* fused to *p-tac*. Cultures of strain W3110 containing plasmid pSM11 with a *p-tac* promoter fused to a truncated *relA* gene were grown at 37°C and then shifted to 10°C with or without treatment with 400 μM IPTG for 10 min prior to the shift to 10°C. This concentration of IPTG results in overproduction of (p)ppGpp at 37°C due to a *relC*-

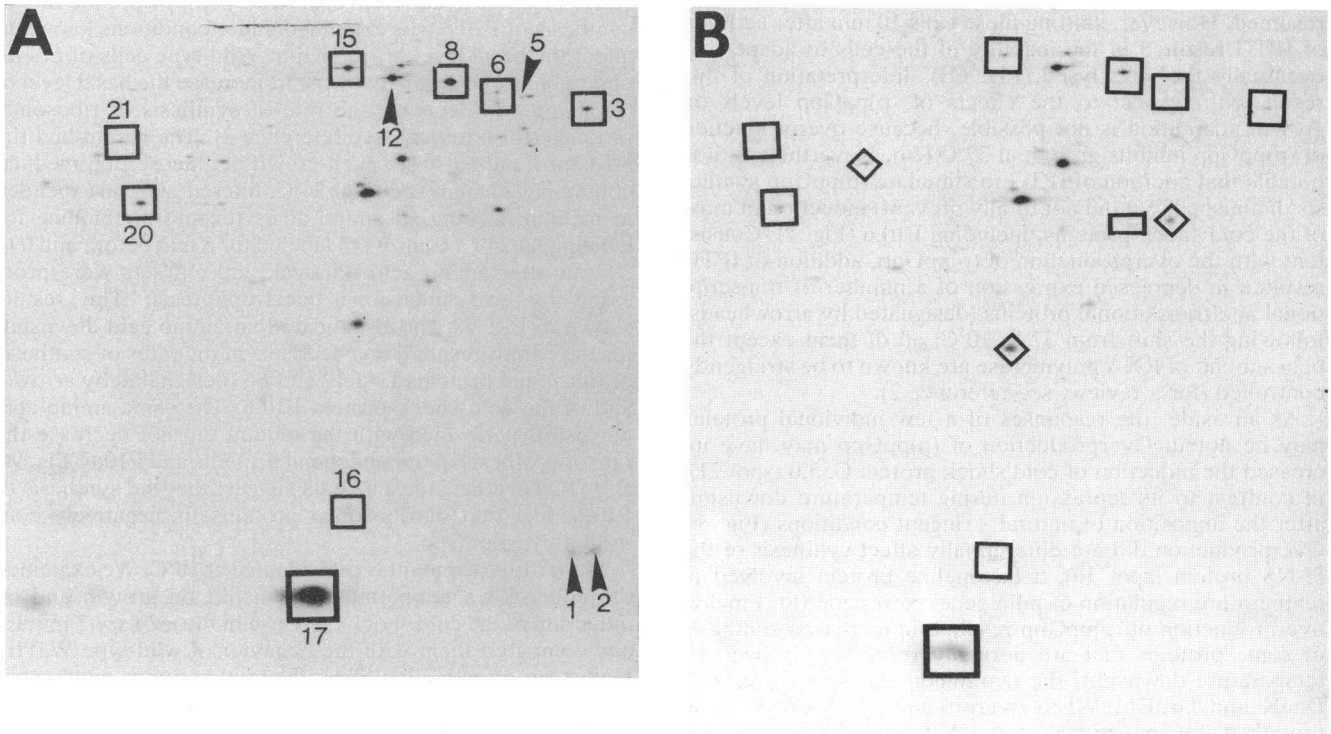


FIG. 5. Synthesis of individual proteins following a shift from 37 to 10°C. The autoradiograms were made from two-dimensional gels of total cell extracts of cultures labeled with [^{35}S]methionine 0 to 120 min after the shift to 10°C. (A) Labeled in medium containing 20 amino acids. (B) Labeled in medium lacking 19 amino acids. Boxes surround cold shock proteins, arrowheads point to transcriptional and translational proteins, and open diamonds surround non-cold shock proteins synthesized during the lag. For identities of numbered polypeptides, see legend to Fig. 2.

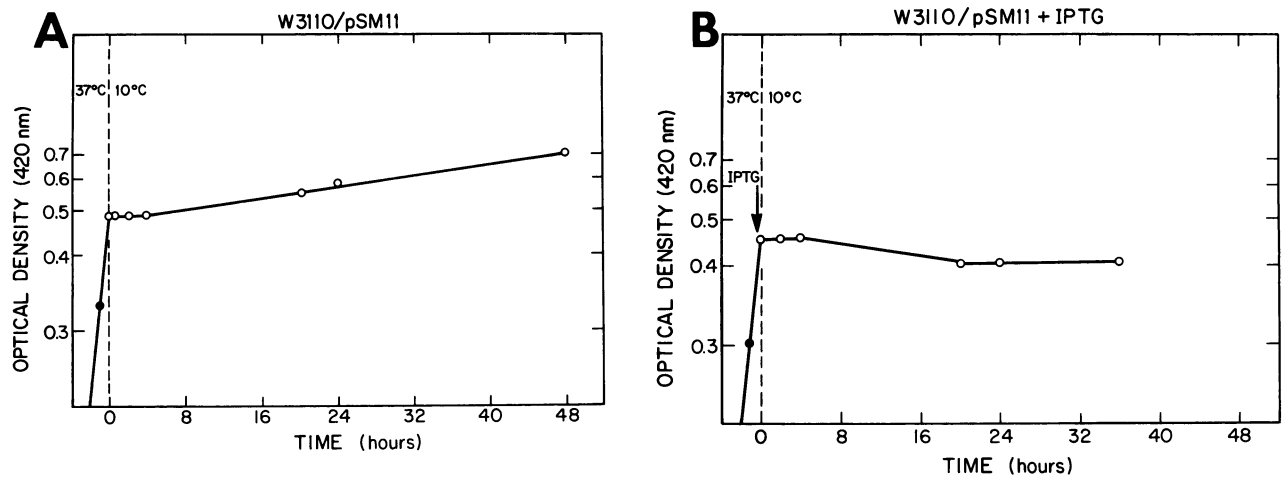


FIG. 6. Growth of strain W3110/pSM11 in glucose-MOPS rich medium without (A) and with (B) the addition of IPTG prior to the shift from 37 to 10°C.

independent (p)ppGpp-synthetic activity associated with the amino-terminal fragment of the *relA* gene (26). Samples were labeled with [^{35}S]methionine from 0 to 180 min postshift. The overproduction of (p)ppGpp at 10°C resulted in severe inhibition of protein synthesis (unpublished observation). To compare the effects of synthesis of individual proteins more readily, samples containing equal amounts of radioactivity were processed by two-dimensional electrophoresis.

As shown in Fig. 6A, shifting strain W3110/pSM11 grown without IPTG resulted in the usual 4-h lag before growth resumed. However, shifting these cells 10 min after addition of IPTG resulted in the inability of the cells to adapt, and eventually the cells lysed (Fig. 6B). Interpretation of this result with respect to the effects of (p)ppGpp levels on growth adaptation is not possible, because overproduction of (p)ppGpp inhibits growth at 37°C (26). Nevertheless, it is notable that addition of IPTG to stimulate (p)ppGpp synthesis diminished (but did not totally prevent) induction of most of the cold shock proteins, including F10.6 (Fig. 7). Consistent with the overproduction of (p)ppGpp, addition of IPTG resulted in decreased expression of a number of transcriptional and translational proteins (designated by arrowheads) following the shift from 37 to 10°C; all of them except the beta subunit of RNA polymerase are known to be stringently controlled (for a review, see reference 2).

As an aside, the responses of a few individual proteins may be noted. Overproduction of (p)ppGpp may have increased the induction of cold shock protein G55.0 (spot 21), in contrast to its repression during temperature downshift after the imposition of natural stringent conditions (Fig. 5). Overproduction did not differentially affect synthesis of the H-NS protein (spot 16), a histonelike protein involved in temperature regulation of pilin gene expression (6). Finally, overproduction of (p)ppGpp resulted in increased synthesis of some proteins that are normally repressed following a temperature downshift: the two major heat shock proteins, DnaK and GroEL. When overproduction of (p)ppGpp is provoked by nutritional downshift and cells are cold shocked, DnaK and GroEL are not derepressed (Fig. 5).

Major cold shock protein is elevated in the *relA spoT* mutant. The *relA spoT* double-deletion mutant that does not synthesize detectable (p)ppGpp was used in experiments to determine whether the lack of (p)ppGpp affects steady-state levels of the cold shock proteins. The mutant and parent were labeled with [^{35}S]methionine for several generations at 24°C. Extracts were made, and portions with equal amounts of radioactivity were processed by two-dimensional gel electrophoresis.

Visual examination of the autoradiograms shown in Fig. 8 revealed that the *relA spoT* mutant has a significantly higher steady-state level of one of the cold shock proteins, F10.6.

To explore further this indication that the synthesis of F10.6 might be negatively regulated by (p)ppGpp, the rate of synthesis of F10.6 was examined under conditions known to affect the basal level of (p)ppGpp. Wild-type cells of *E. coli* undergoing a nutritional downshift increase the basal level of (p)ppGpp and decrease the rate of synthesis of ribosomal proteins (for a review, see reference 2). The parent and the *relA spoT* mutant were each grown in glucose-rich medium containing 20 amino acids at 24°C, filtered, and resuspended in medium lacking all amino acids (except methionine for labeling) at 24°C. Cells were labeled for 5 min before and 0 to 30 min after amino acid removal, and extracts were processed by two-dimensional electrophoresis. The results shown in Fig. 9A and B indicate that amino acid downshift for the parent resulted in a decrease in the rates of synthesis of ribosomal proteins L7/L12 and S6 (designated by arrows) and of the cold shock protein F10.6. The same amino acid downshift performed with the mutant did not decrease the rate of synthesis of the ribosomal proteins and F10.6 (Fig. 9C and D). Together, these results suggest that the synthesis of F10.6, like that of ribosomal proteins, is negatively controlled by (p)ppGpp.

The *relA spoT* mutant is cold adapted at 10°C. We examined the effects of a temperature downshift on growth and on induction of the cold shock response in the *relA spoT* mutant and compared them with the behavior of wild-type W3110. Under our growth conditions, the mutant grows much more slowly than the parent at 37°C but has about the same doubling time as the parent at 24°C; therefore the downshift was from 24 to 10°C. Samples were labeled for 5 min prior to the shift and 0 to 60 min postshift.

Whereas a shift of W3110 from 37 to 10°C resulted in a 4-h lag (11, 18), a shift of W3110 from 24 to 10°C resulted in a 2-h lag (Fig. 10A). The mutant, however, grew without a lag

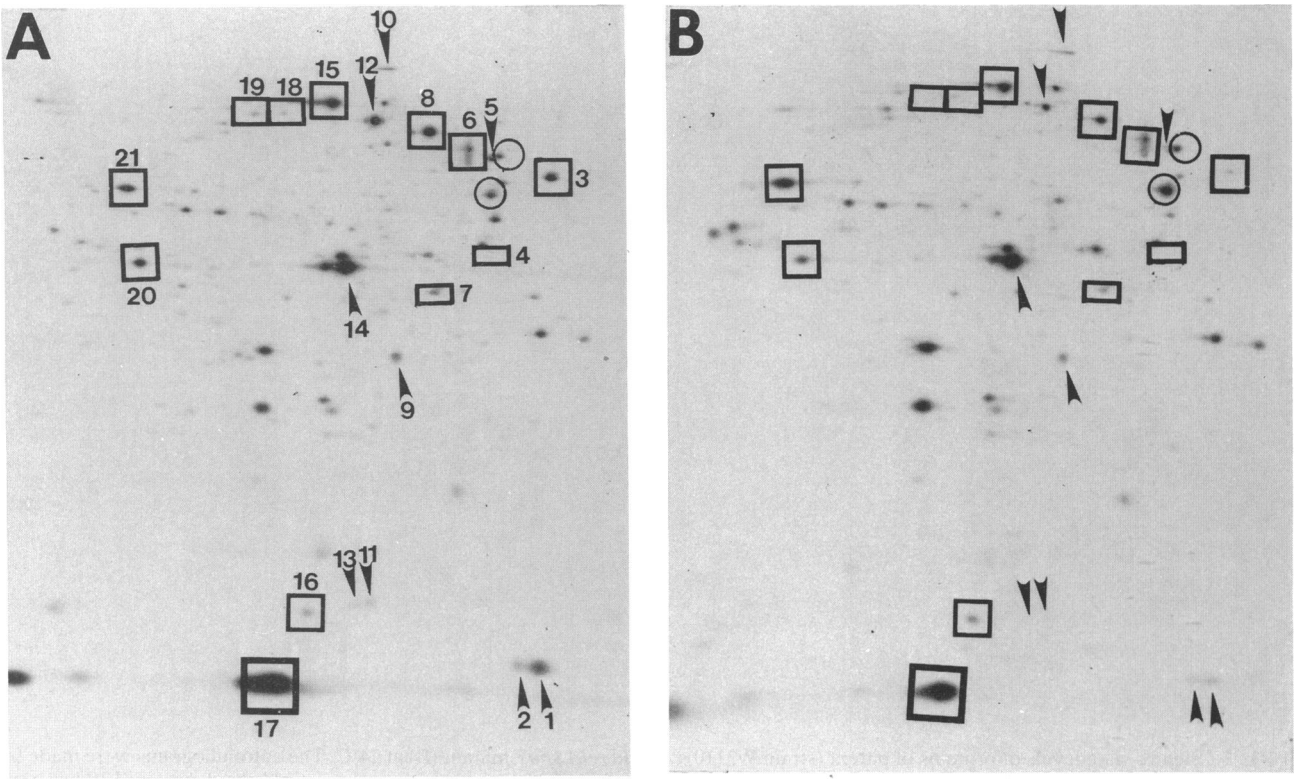


FIG. 7. Synthesis of individual proteins of strain W3110/pSM11 following a shift from 37 to 10°C in medium lacking (A) and containing (B) IPTG prior to the shift to 10°C. The autoradiograms were made from two-dimensional gels of total cell extracts of cultures labeled with [^{35}S]methionine 0 to 180 min postshift. Boxes surround cold shock proteins, arrowheads point to transcriptional and translational proteins, and circles enclose heat shock proteins DnaK and GroEL. For identities of numbered polypeptides, see legend to Fig. 2.

period (Fig. 10B), acting as though it was already adapted for growth at 10°C.

Visual inspection of the autoradiograms shown in Fig. 11 reveals that the cold shock response was induced in both the mutant and parent, but with a higher overall induction of the cold shock response in the mutant than in the parent. Of the cold shock proteins identified, there was an increased rate of synthesis of NusA (spot 3), PNP (spot 8), and RecA (spot 7). There also was a significantly higher rate of synthesis of cold shock protein F10.6 (spot 17). Further, the mutant also displayed higher rates of synthesis of transcriptional and translational proteins (ribosomal protein L7/L12 [spots 1 and 2], beta subunit of RNA polymerase [spot 10], elongation factor G [spot 12], ribosomal protein S6 [spots 11 and 13] and elongation factor Tu [spot 14]) and decreased synthesis of heat shock protein GroEL (circled). However, the synthesis of one cold shock protein, G55.0 (spot 21), was diminished in the mutant. It will be recalled that this same protein was likewise out of step with the other cold shock proteins during IPTG-induced overproduction of (p)ppGpp at 10°C (Fig. 7), though it behaved similarly to its cohorts during the imposition of natural stringent conditions during temperature downshift (Fig. 5). Apparently, the synthesis of this protein, unlike that of the other cold shock proteins, is modulated during stringency by some condition other than the levels of (p)ppGpp.

DISCUSSION

Abrupt temperature downshifts of *E. coli* from 37 to 10°C are followed by a long growth lag during which the cells (i) induce a few proteins scarcely made at high temperature, (ii) severely repress the basal rate of synthesis of heat shock proteins, and (iii) continue to synthesize many transcriptional and translational proteins that are normally repressed during nongrowth (11). The present work extends these studies to 13°C downshifts from 42, 37, and 24°C and includes studies in which (p)ppGpp levels are manipulated (i) by amino acid limitation, (ii) by IPTG induction of (p)ppGpp without nutrient limitation (26), and (iii) by using a strain genetically defective in the ability to form (p)ppGpp as a result of deletions in both the *relA* and *spoT* genes (33). The manipulations leading to increased (p)ppGpp levels are found qualitatively to have negative regulatory effects on the synthesis of cold-induced (cold shock) proteins. The (p)ppGpp-deficient strain is found to express high levels of the predominant cold shock protein, F10.6, constitutively during steady-state growth at 24°C; further, these cells behave as if they are cold adapted, continuing to grow exponentially when shifted to 10°C rather than having the 2-h lag displayed by wild-type strains with normal selective expression of cold shock proteins. Qualitative assessments of the synthesis of individual cold shock proteins suggest the existence of complex regulatory features of the cold shock response in addition to effects that can be assigned to (p)ppGpp levels.

First, we have found that the cold shock response is induced by any downshift of 13°C or more. These shifts induced cold shock proteins, repressed heat shock proteins, and allowed continued synthesis of transcriptional and translational proteins, despite a growth lag. In general, the greater

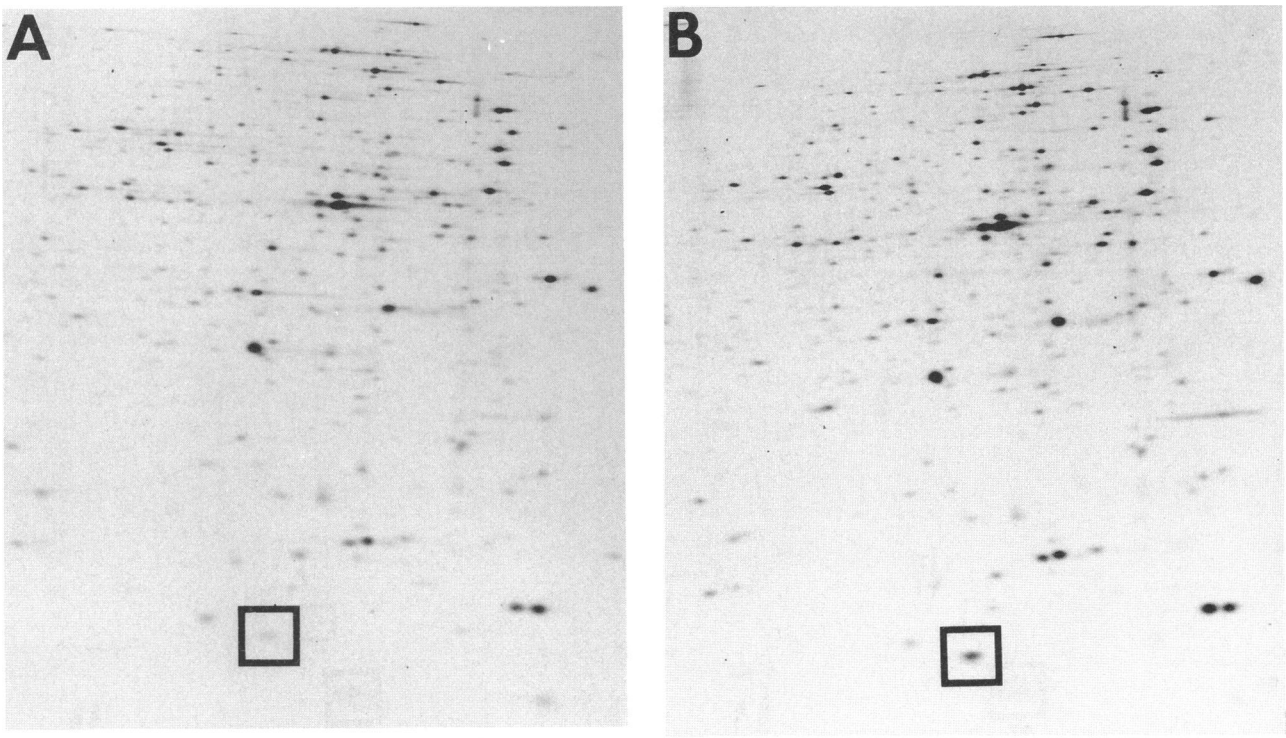


FIG. 8. Steady-state levels of proteins of parent (strain W3110) (A) and *relA spoT* mutant (B) at 24°C. The autoradiograms were made from two-dimensional gels of total cell extracts of cultures labeled with [^{35}S]methionine for several generations. Cold shock protein F10.6 is boxed.

the magnitude of the temperature shift, the more pronounced the cold shock response. Since early reports had indicated a drop in the (p)ppGpp level proportionate with the magnitude of a temperature downshift (20), we wondered whether the continued synthesis of transcriptional and translational proteins might be related to this decrease. If so, a temperature downshift would in some respects resemble a nutritional upshift, in which there occurs a decrease in the (p)ppGpp basal level and increased synthesis of translational components.

There are other reasons for considering temperature downshifts to be similar to nutritional upshifts. *E. coli* induces the cold shock response in the presence of low concentrations of inhibitors of elongation of protein synthesis that cause a decrease in the basal level of (p)ppGpp (30). The addition of kasugamycin, an inhibitor of initiation of translation that causes a decrease in the (p)ppGpp level (3), also results in the induction of the cold shock response (unpublished observation). Furthermore, it has been reported that very low temperature (<5°C) restricts growth by blocking initiation of translation (1).

Taken together, these observations suggest that a partial block in translation initiation caused by a downshift in temperature might generate the signal indicating insufficient translation machinery, lower the level of (p)ppGpp, and enhance or facilitate the cold shock response. The restriction in translation would be minor and would last only for minutes during temperature downshifts of a moderate nature but would be more severe and last for hours upon shift to 10°C. In both instances, the physiological state would resemble that following a nutritional upshift in at least one key feature, namely, that of insufficient protein-synthesizing capacity. The implication of this model is that shift to lower temperature reduces the protein-synthesizing capacity of the cell relative to the ability to synthesize aminoacyl-tRNAs, so that this growth limiting condition triggers a regulatory response more like that caused by a growth promoting condition.

We have explored this model by testing in various ways the interaction of the stringent response network, the cold shock response, and the adaptation to growth at low temperature. The stringent response has been monitored by tracking the synthesis of the several stringently controlled proteins visible on two-dimensional gels, including ribosomal proteins S1, S6, and L7/L12 and elongation factor G. The cold shock response, on the other hand, is most strikingly evident in the behavior of the major cold shock protein F10.6 (called Cs7.4 by Inouye and coworkers and encoded by the *cspA* gene [5]). The induction of transcription of this gene (5) and preferential synthesis of this small protein consumes much of the resources of the cell during the adaptation lag before growth at 10°C (11). The induction of this protein by any downshift in temperature occurs in a manner dependent on the magnitude of the temperature shift as well as on the new absolute temperature: the lower the temperature, the higher the induction (Fig. 2 and 3). Also, protein F10.6 is maintained at levels inversely proportional to temperature and is marginally detectable at high temperatures (data not shown). The level of protein F10.6 correlates most closely with the ability of cells to adapt to growth at 10°C after shifts from higher temperatures. We assume that expression of protein F10.6 can be used as a sensitive index of the cold shock response. Unfortunately, antibody to this protein which would facilitate quantitative measurement of its abundance was not available, and we have relied on the visual appearance of the labeled protein on gels.

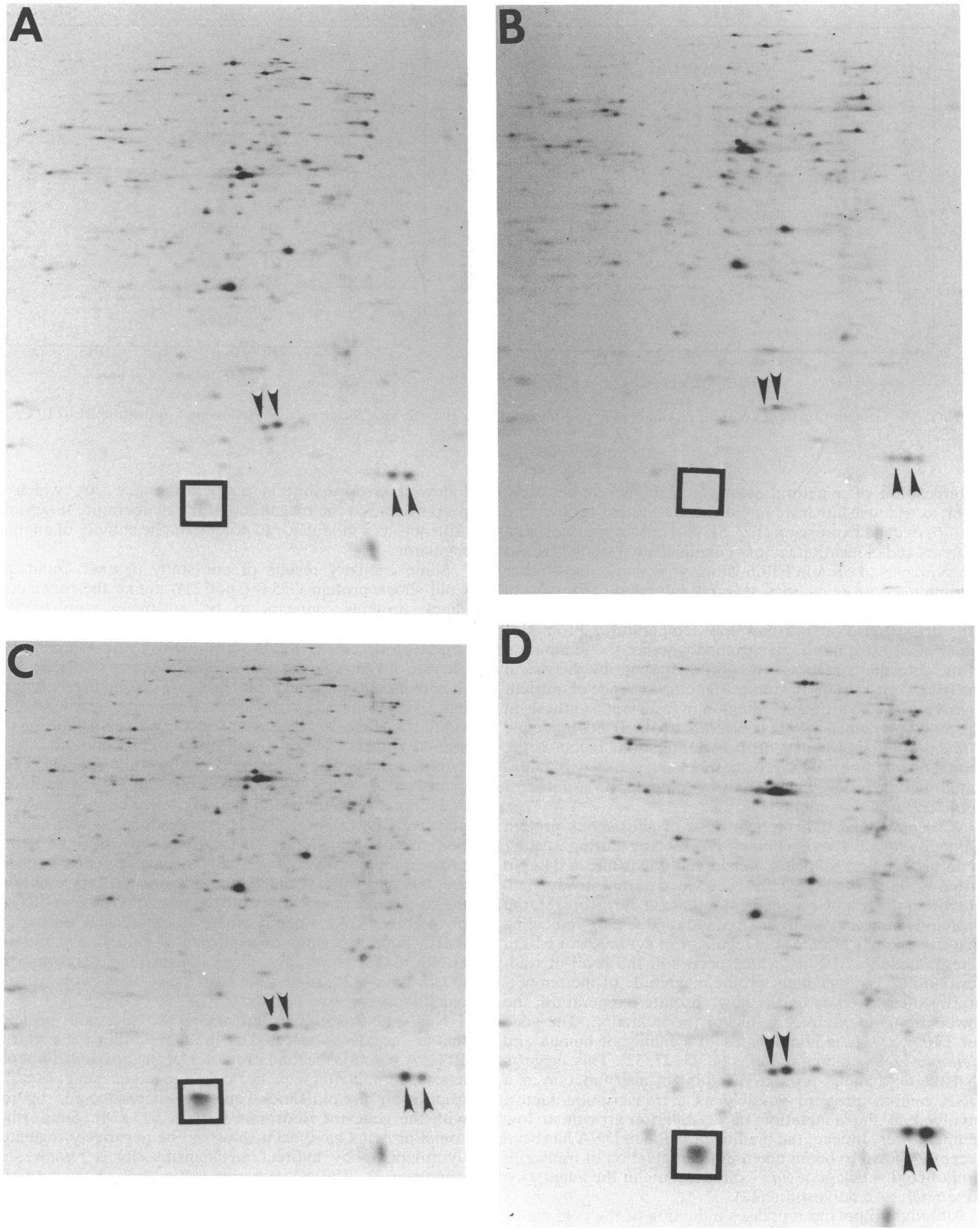


FIG. 9. Effect of amino acid removal on synthesis of individual proteins. (A and B) Wild-type strain W3110. (C and D) Mutant strain W3110 *relA spoT*. Cells were grown in glucose-MOPS rich medium at 24°C and labeled 5 min before (A and C) and 0 to 30 min following (B and D) amino acid removal. Cold shock protein F10.6 is boxed, and ribosomal proteins L7/L12 and S6 are designated by arrowheads.

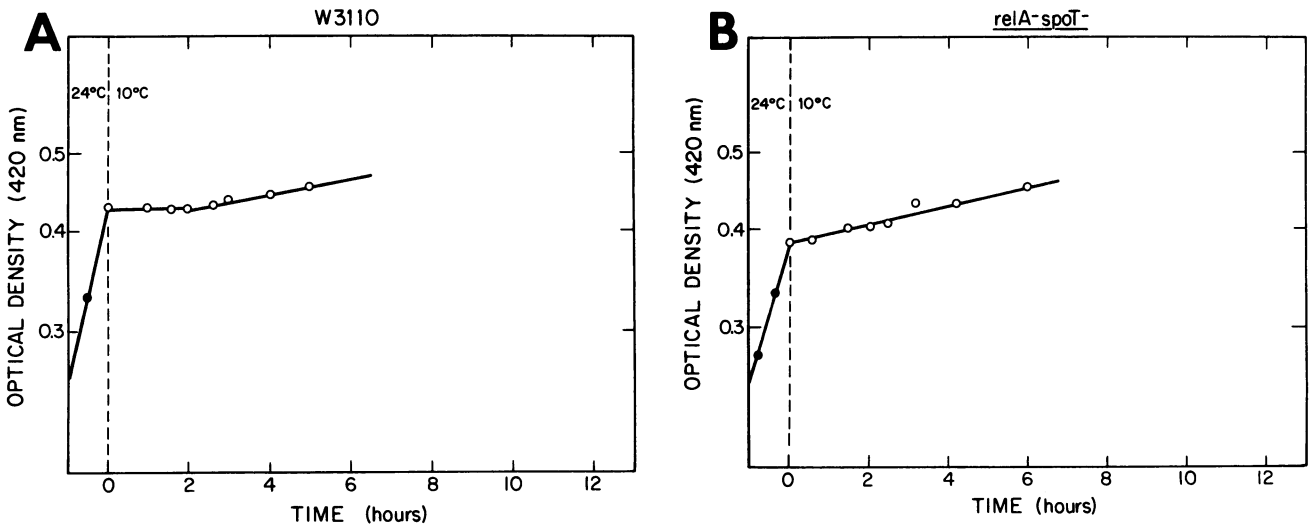


FIG. 10. Growth of parent (W3110) (A) and *relA spoT* mutant (B) in glucose-MOPS rich medium following a shift from 24 to 10°C.

Imposition of a natural stringent response immediately prior to a downshift in temperature was found to alter the pattern of gene expression (Fig. 5). Not only were ribosomal proteins and elongation factor G diminished in synthesis, but the synthesis of protein F10.6, along with that of most other cold-induced proteins, was severely inhibited. This pattern persisted for hours, even though there was considerable protein synthesis at the new low temperature. From this experiment it cannot be determined whether the diminution of the cold shock response was brought about by the rise in the (p)ppGpp level or by some other consequence of nutrient deprivation. The artificial induction of (p)ppGpp synthesis in the mutant in which *relA* is controlled by the IPTG-inducible *p-tac* promoter provided further clarification: much of the stringent inhibition of the cold shock response was duplicated merely by overproduction of (p)ppGpp in a nutrient-sufficient growth medium (Fig. 7).

We have found that the synthesis of cold shock protein F10.6 is negatively regulated by (p)ppGpp during steady-state growth and following nutritional downshift at 24°C in strain W3110. Although F10.6 is elevated during steady-state growth in the (p)ppGpp-deficient mutant at 24°C (Fig. 8), the protein is not elevated in the mutant at 30°C (30), indicating that (p)ppGpp is not the sole regulator of synthesis of F10.6. The induction of F10.6, which occurs at the level of transcription (5), is dependent on the magnitude of the temperature shift as well as on the new absolute temperature: the lower the temperature, the higher the induction. The gene for F10.6, *cspA*, is homologous to a family of human and *Xenopus* DNA binding proteins (4, 25, 27, 32). This suggests that the cold shock protein F10.6 might also function as a DNA binding protein, possibly as a transcription factor, involved in the adaptation of *E. coli* for growth at low temperatures. Indeed, the binding of F10.6 to DNA has been recently shown to be involved in the regulation of transcription of cold shock gene *hns* expressed late in the adaptation to temperature downshifts (12).

Although (p)ppGpp represses induction of the cold shock response, as shown by results with strains that both overproduce and underproduce this nucleotide, the absence of (p)ppGpp does not by itself lead to induction. Rather, the evidence suggests that the decrease in the (p)ppGpp level following a downshift in temperature (13, 20), which is proportional to the magnitude of the temperature downshift (20), acts as a modulator to potentiate the activity of another regulator.

Some ancillary results of our study deserve comment. Cold shock protein G55.0 (spot 21), unlike the other cold shock proteins, appears to be positively regulated by (p)ppGpp. Consistent with the observation that the level of (p)ppGpp decreases following a downshift in temperature, the rate of synthesis of G55.0, unlike the rates of synthesis of the other cold shock proteins, decreases immediately following a shift from 37 to 10°C (11). Furthermore, the rates of synthesis of most of the cold shock proteins peak during the second hour (following the shift to 10°C), while the rate of synthesis of G55.0 is similar to its preshift rate (11). The synthesis of another cold shock protein, H-NS (spot 16), does not seem to be specifically affected positively or negatively by (p)ppGpp but is expressed late during adaptation to cold shock (11). Because not all the cold shock proteins are negatively regulated by (p)ppGpp (Fig. 7 and 9), the data suggest that more than one regulon may comprise the cold shock response. While the rates of synthesis of most of the other cold shock proteins are decreasing during the fourth hour, the rates of synthesis of cold shock proteins H-NS, G41.2 (spot 20), and G55.0 are highest during the fourth hour (12), suggesting that these three proteins may comprise a separate regulon.

NusA, PNP, and RecA are among the cold shock proteins that are negatively affected by (p)ppGpp following a shift to 10°C. A potential second promoter of the *nusA-infB* operon has a discriminator sequence common to genes regulated by (p)ppGpp (7). Cold shock gene *pnp* shares the S15 operon with the gene for ribosomal protein S15 (28). Some ribosomal proteins have been shown to be negatively regulated by (p)ppGpp by indirect mechanisms (for a review, see reference 2). The S15 operon is located adjacent to the *nusA-infB* operon on the *E. coli* chromosome, and some cellular transcripts have been reported to include both *nusA-infB* and the S15 cluster (8).

Although there is no conclusive evidence that the cold shock response is adaptive, our growth data support this view. The strongest finding is that the (p)ppGpp-deficient

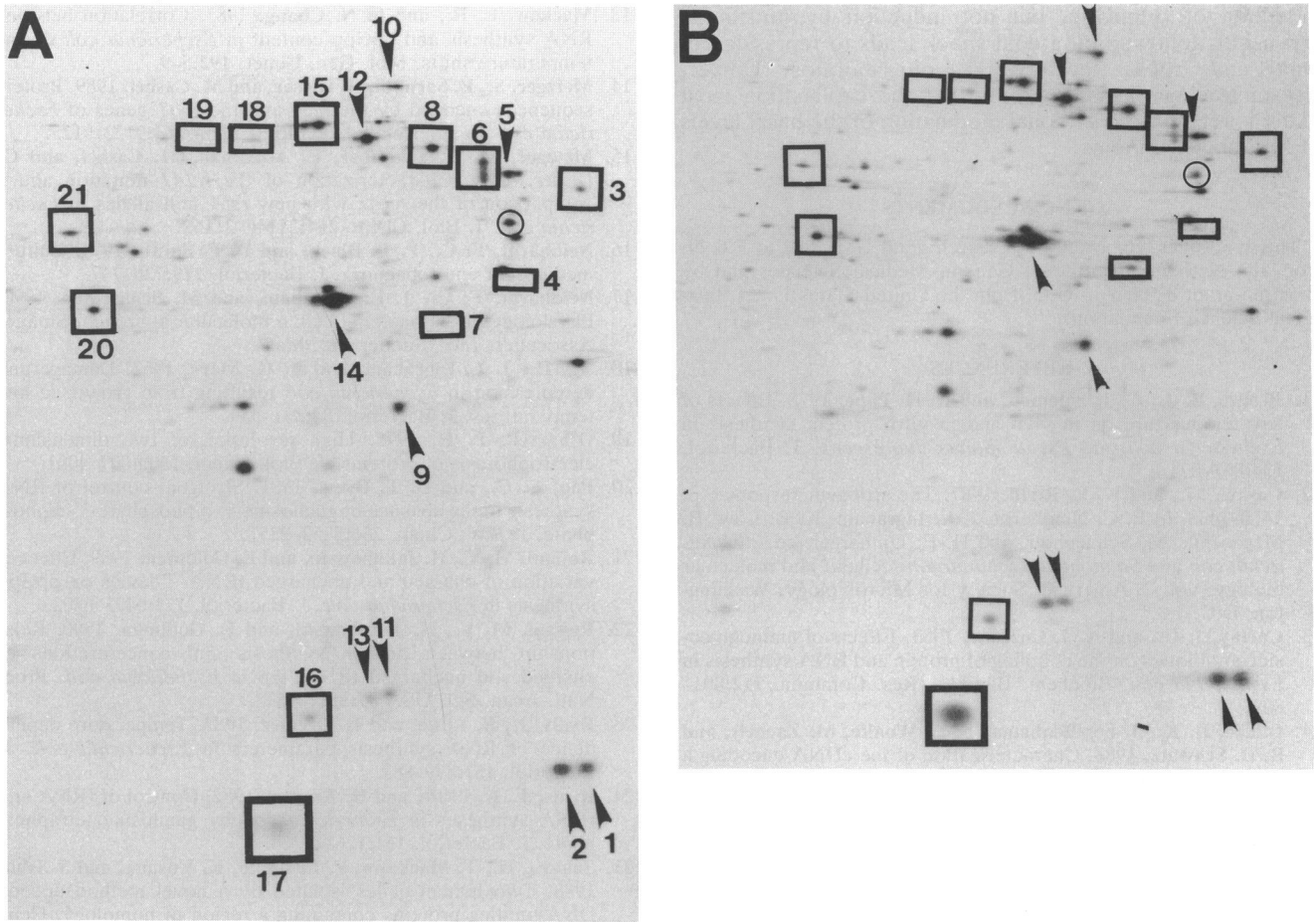


FIG. 11. Synthesis of individual proteins of parent (W3110) (A) and *relA spoT* mutant (B) following a shift from 24 to 10°C. The autoradiograms were made from two-dimensional gels of total cell extracts of cultures labeled with [^{35}S]methionine 0 to 60 min postshift. Boxes surround the cold shock proteins, arrowheads point to transcriptional and translational proteins, and a circle encloses heat shock protein GroEL. For identities of numbered polypeptides, see legend to Fig. 2.

mutant when grown at 24°C is uniquely able to traverse a shift to 10°C without the usual 2-h lag period (Fig. 10). The fact that protein F10.6 is elevated in the mutant at 24°C leads to the suspicion that high expression of F10.6 is required for cold adaptation, though of course the high level and the growth capability may both be the result of some unknown third factor affected by the (p)ppGpp level. The IPTG-mediated (p)ppGpp induction results in repression of the cold shock response, growth inhibition, and even detectable cell lysis. As noted earlier (Results), induction of (p)ppGpp inhibits growth at 37°C, so interpretation of an extended lag period at 10°C is complicated. Normally, shifting the prototrophic strain W3110 from 37 to 10°C results in a 4-h lag (11). If amino acids are removed by filtration prior to the temperature shift to 10°C and added back 4 h postshift, an additional 4-h lag ensues (Fig. 4B). When cells were labeled from 0 to 120 min during the postshift period (during the first half of the starvation period), the induction of the stringent response did result in specific repression of the cold shock response (Fig. 5). Interestingly, there was no indication from the gels that overall protein synthesis was inhibited during starvation; instead, the distribution of proteins changed in that synthesis of cold shock proteins was repressed. Therefore, the nutritional downshift arising from removal of amino acids suppressed the induction of the cold shock response and prolonged the lag period.

The evidence presented here shows that the (p)ppGpp-related stringent response interacts in a major way with the cold-induced response network of *E. coli*. But our studies indicate also an interaction with the heat shock response. Repression of basal synthesis of heat shock proteins was found with any major downshift in temperature. This repression, however, could be overcome by overproduction of (p)ppGpp. Under some physiological conditions, the heat shock proteins have been shown to be positively regulated by conditions that provoke a stringent response (for a review, see reference 2), and the repression following the downshift in temperature is at the level of transcription (29). Possibly, the decrease in the (p)ppGpp level following temperature downshift can contribute to repression of the heat shock proteins. Shifting cells to 10°C during IPTG-induced overproduction of (p)ppGpp resulted in an increase in the rate of synthesis of the heat shock proteins and in a decrease in the rate of synthesis of several cold shock proteins (Fig. 7). The cold shock response and the heat shock response thus may have an oppositional relationship—conditions that induce one repress the other—and the stringent response system modulates each of them. It is unclear why IPTG

induction of (p)ppGpp, but not induction by nutritional downshift, followed by a cold shock leads to repression of DnaK and GroEL. Recent work in our laboratory (University of Michigan) (4a) suggests that the explanation most likely is related to nutritional modulation of the basal levels of heat shock proteins.

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