The rpoD gene functions as a multicopy suppressor for mutations in the chaperones, CbpA, DnaJ and DnaK, in *Escherichia coli*

Tadasu Shiozawa, Chiharu Ueguchi, Takeshi Mizuno*

Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan

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Abstract

The CbpA protein is an analog of the DnaJ molecular chaperone of *Escherichia coli*. The dnaJ^- cbpA^- double-null mutant exhibits severe defects in cell growth, namely, a very narrow temperature range for growth. To gain insight into the functions of CbpA as well as DnaJ, we isolated a multicopy suppressor gene that permits this dnaJ^- cbpA^- mutant to grow normally at low temperatures. The suppressor gene was identified as rpoD, the gene that encodes the major σ\(^70\). The biological implications of this finding are examined and discussed.

Keywords: Heat shock response; Molecular chaperone; CbpA of *E. coli*; DnaK and DnaJ chaperones; Sigma subunit

1. Introduction

The heat shock proteins (HSPs) have become central to the study of the correct folding of nascent polypeptides, assembly of protein complexes, and uptake of proteins into organelles (for a review, [1]). Some HSPs are referred to as molecular chaperones that play crucial roles even under unstressed growth conditions. For *Escherichia coli*, the analog of eukaryotic HSP70, DnaK, is a major chaperone protein whose amino acid sequence is extremely conserved during evolution [2]. DnaK functions together with other HSPs, DnaJ and GrpE (for a review, [3]). Mutations in these genes exert pleiotropic effects on such diverse processes as cell division, proteolysis, protein export, DNA replication and RNA synthesis.

The function of these HSPs was first proposed to be protection of other proteins from thermal inactivation, and reactivation of protein aggregates formed under stressful conditions [4]. However, the coordinate action of DnaK, DnaJ and GrpE was also shown in other reactions, such as DNA replication of λ phage, P1 phage and mini-F plasmid [5–7]. These HSPs are also implicated in negative regulation of the heat shock response by affecting stability of the heat shock σ-factor (σ\(^32\), the rpoH gene product) [8,9]. Collectively, the DnaK–DnaJ–GrpE chaperones function in intimate coordination with each other in a variety of cellular processes under various physiological conditions.

DnaJ analogs have also been discovered in a number of organisms, including higher eukaryotes [10]. We recently demonstrated that *E. coli* itself possesses an analog of DnaJ [11]. This protein, CbpA, consists of 297 amino acids and exhibits 39% amino acid identity plus 17% conserved substitutions with
Several lines of genetic evidence have led us to conclude that CbpA is a functional analog of DnaJ. For instance, the cbpA gene functions as a multicopy suppressor of dnaJ mutations [11–13]. However, the expression of cbpA is largely, if not absolutely, dependent on the stationary phase-specific σ 70 (or σ 5, the rpoS gene product) [13], whereas that of dnaJ is controlled by σ 32 [14]. These results suggested that DnaJ and CbpA may display overlapping activities, but function preferentially under different circumstances. To gain further insight into the function of CbpA as well as DnaJ, we isolated a multicopy suppressor gene for the growth defect of the dnaJ^-cbpA^- double mutant, particularly at low temperatures. The suppressor gene was identified as rpoD that encodes the major σ 70.

2. Materials and methods

2.1. Strains

*E. coli* K-12 strains, used in this study, are all derivatives of MC4100 (F^−, ΔlacU169 araD139 rpsL relA flbB thiA). The derivatives are KY1456 (dnaJ::Tn10-42) [15], CU247 (dnaJ::Tn10-42 cbpA::kan) [12], and MC4100-ΔdnaK (ΔdnaK52) [16]. These bacteria were grown in Luria broth unless otherwise indicated. When required, ampicillin (50 μg/ml) was added to the medium.

2.2. Plasmids

Plasmid pKY184 is a pBR322-derived cloning vector. Plasmid pCS12 carries the region shown in Fig. 1. Plasmid pCS12D was constructed from pCS12, by removing the region downstream of the rpoD gene (see Fig. 1). Plasmid pKRS1 carries the KpnI fragment encompassing the rpoS gene, which was isolated from plasmid pKTF101 [17]. This fragment was inserted into the KpnI site of pKY184 to yield pKRS1. Plasmid pLAW2 carries the rpoA gene, which was placed under the lac/lpp promoter [18].

2.3. Detection of proteins by immunoblotting analysis

Immunoblotting was carried out with either anti-σ 70 antiserum (provided by A. Ishihama), anti-σ 32 antiserum (provided by T. Mori) or anti-α-subunit antiserum (provided by A. Ishihama).

3. Results

3.1. Isolation of multicopy suppressors for the dnaJ^-cbpA^- double mutant

We previously constructed an insertional inactivation mutant of cbpA, in which the cbpA sequence on the chromosome was replaced by the kanamycin resistant gene (kan) [11]. This cbpA^- strain was not noticeably changed in phenotype under the laboratory conditions tested, particularly with regard to temperature sensitivity for growth. A dnaJ null mutant (dnaJ::Tn10-42) is known to exhibit a poor growth at temperatures above 42°C, while it can grow at lower temperatures [8]. However, when these mutations were combined, the resultant strain carrying the dnaJ^-cbpA^- alleles was found to exhibit severe defects in growth, namely, a very narrow temperature range for growth [12]. Here we isolated multicopy suppressors that permit the dnaJ^-cbpA^- mutant to grow at 16°C.

A bank of Sau3AI partially-digested *E. coli* genomic DNA was constructed from the dnaJ^-cbpA^- strain using a pBR322 derivative which carries the lac promoter and multicloning sites (pKY184). The dnaJ^-cbpA^- strain was transformed with this bank of plasmids, and then spread on Luria agar-plates. After the plates were incubated at 16°C for 3 days, about 50 colonies appeared on the plates (note that about 10^6 transformants were obtained at 30°C). Plasmids were isolated from each colony and re-transferred into the same strain. Among the candidates, only a few plasmids exhibited the desirable

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Fig. 1. Schematic representation of the region encompassing the rpoD gene in the *E. coli* chromosome. Arrows indicate the open reading frames. Plasmids pCS12 and pCS12D carry the 8.3-kb and 2.95 kb DNA segments, respectively, as indicated.
ability to permit the dnaJ− cbpA− mutant to grow normally at 16°C. When the insert DNAs on these plasmids were examined by restriction mapping and DNA sequencing, they were found to carry an overlapping E. coli chromosomal region. One of them was thus analyzed in detail, based on the available GenBank and EMBL databases. Such inspection revealed that this plasmid (pCS12) carries a 8.3-kb insert encompassing the well-characterized rpoD gene encoding σ70 of RNA polymerase (Fig. 1). The growth defect of the dnaJ− cbpA− mutant at the low temperature (16°C) on Luria agar-plates, but not at the high temperature (42°C), was suppressed, provided that pCS12 was introduced (data not shown).

3.2. The rpoD gene functions as a multicopy suppressor for the dnaJ− cbpA− mutant

Although the 8.3-kb DNA region in pCS12 is inferred to contain several unknown coding sequences in addition to rpoD, ileX and the 3'-portion of dnaG (Fig. 2), we suspected a priori that the rpoD gene might be the one responsible for the multicopy suppression. We constructed a derivative of pCS12 that encompasses only the rpoD gene as intact (Fig. 1, plasmid pCS12D). This plasmid exhibited the same suppression ability on plates as that observed for pCS12.

To confirm this, the ability of rpoD to support the growth of the dnaJ− cbpA− mutant was quantitatively examined at various temperatures by measuring the colony-forming units (CFU) (Fig. 3). The dnaJ− cbpA− cells exhibited a relatively poor CFU at 37°C, and a very poor CFU at both 16°C and 42°C, that is indicative of a very narrow temperature

Fig. 2. Temperature sensitivity for growth. Strains MC4100 (denoted by wild), CU247 (dnaJ::Tn10−42cbpA::kan) (denoted by dnaJ− cbpA−) and MC4100-dnaK52 (denoted by dnaK−) were transformed by each indicated plasmid, pKY184 (−rpoD), pCS12 (+rpoD), and then grown in Luria broth at 30°C. At the exponential growth phase, the cells were harvested, and then the total cytoplasmic proteins were isolated. The samples were analyzed by immunoblotting analyses with anti-σ70, anti-σ32 or anti α subunit antisera. The relative contents were quantified by Image Master (Pharmacia Co.). Note that the content in the wild-type cells was taken as 1.0, and the relative values were normalized by assuming that each cell contained a constant amount of α-subunit.

Fig. 3. Quantitative immunoblotting analysis. Strains MC4100 (denoted by wild), CU247 (dnaJ::Tn10−42cbpA::kan) (denoted by dnaJ− cbpA−) and MC4100-dnaK52 (denoted by dnaK−) were transformed by each indicated plasmid, pKY184 (−rpoD), pCS12 (+rpoD), and then grown in Luria broth at 30°C. After appropriate dilution, portions were spread on Luria agar plates and incubated at the indicated temperatures for 3 days. Numbers of colonies on the plates were counted, and plotted as the function of temperatures.
range for growth. When these mutant cells were transformed with pCS12D, the growth defect at 16°C and 37°C was fully suppressed, while that at 42°C was not. These results suggested that the rpoD gene functions as the multicopy suppressor for the growth defect of the double mutant at low and intermediate temperatures, but not at higher temperatures. It should be noted, however, that the inability of the dnaJ^-cbpA^- mutant to support lambda propagation was not suppressed by the introduction of pCS12D (data not shown).

3.3. The rpoD gene functions also as multicopy suppressor for a dnaK null mutant

We recall here the fact that the phenotypes of the dnaJ^-cbpA^- mutant are very similar to those reported for dnaK null mutants, as has been reported previously [16]. We confirmed that a dnaK null mutant, ΔdnaK52, indeed exhibits a very narrow temperature range for growth (Fig. 2). It was thus of interest to see if the multicopy rpoD gene can also suppress this growth defect of dnaK^- . The rpoD gene indeed functioned as the multicopy suppressor for the growth defect not only of dnaJ^-cbpA^- but also of dnaK^- (Fig. 2B and C).

3.4. Is the rpoD gene specific?

A question was whether or not this suppression ability of rpoD is specific. It is of interest to see if genes encoding other components of RNA polymerase can also function as multicopy suppressors. We checked this possibility for σ^32 and α-subunit of RNA polymerase. The former is known as a stationary-phase specific sigma-factor, and the latter is an essential component of the core RNA polymerase. Neither the rpoS gene nor the rpoA gene exhibited such an ability (data not shown).

3.5. Cellular contents of σ^70 and σ^32

The amounts of σ^70 in cells were examined by immunoblotting analysis with an anti-σ^70 antiserum (Fig. 3). The steady-state amounts of σ^70 in the cells, grown at 30°C, increased significantly in both the dnaJ^-cbpA^- and dnaK^- backgrounds, provided that plasmid pCS12D was introduced into the cells. A several-fold increase was observed for the dnaJ^-cbpA^- mutant, when estimated on the basis of the amounts of α-subunit of RNA polymerase which was assumed to be constant. In the wild-type background, however, such an increase was not observed, although the reason for this is not clear at present. In any case, the introduction of the multicopy plasmid pCS12D resulted in an increase of intracellular content of σ^70 in the dnaJ^-cbpA^- mutant, under which conditions the multicopy suppression was observed.

Finally, we needed to examine the level of σ^32 in the dnaJ^-cbpA^- mutant, since the DnaK, DnaJ, and GrpE chaperone proteins have been shown to be involved in negative modulation of the σ^32-dependent heat shock response [8,9]. It was postulated previously that the resultant higher intracellular content of σ^32 in dnaK, dnaJ and grpE mutants at all temperatures is partly due to a deficiency in the degradation of σ^32. The contents of σ^32 were found to be higher in the dnaJ^-cbpA^- cells than those in the wild-type (Fig. 3). The same was also confirmed for the dnaK^- mutant, as reported previously [19]. The increased levels of σ^32 in both the dnaJ^-cbpA^- and dnaK^- backgrounds were not affected, even when pCS12D was introduced into the cells.

4. Discussion

In this study, we identified the rpoD gene as a multicopy suppressor that permits the dnaJ^-cbpA^- mutant to grow at low temperatures. It was also found that the rpoD gene is capable of functioning as the multicopy suppressor for the growth defect of the dnaK^- mutant at low and intermediate temperatures. This finding can be explained by assuming that both the dnaJ^-cbpA^- and dnaK^- mutations result in defects in common cellular processes, and that they can be suppressed by common cellular factors, in this case by a presumed overproduction of σ^70. These results provided us with a clue to understanding the physiological functions of CbpA as well as DnaJ and DnaK.

While DnaJ/CbpA functions are required for normal growth at intermediate and low temperatures, this requirement appears to be circumvented by an
overproduction of $\sigma^{70}$. However, there are certain roles of DnaJ/CbpA that cannot be suppressed by the overproduction of $\sigma^{70}$. That is, the inability of the dnaJ$^{-}$ cbpA$^{-}$ mutant to support $\lambda$ phage propagation was not suppressed by the overproduction of $\sigma^{70}$ (data not shown). Furthermore, the overproduction of $\sigma^{70}$ did not permit the dnaJ$^{-}$ cbpA$^{-}$ mutant to grow at high temperatures. These results indicate that DnaJ/CbpA are required for multiple cellular processes, and also that at least some of the cellular requirements for DnaJ/CbpA at high temperatures are different from those at intermediate and low temperatures.

What is the biological implication of the findings in this study? In this respect, two recent reports by others are particularly relevant [19,20]. First, Bukau and Walker have isolated secondary mutations that suppress major cellular defects of the $\Delta$dnaK$^{52}$ mutant at intermediate and low temperatures (but not at high temperatures) [19]. Most of such suppressor mutations map within the rpoH gene and cause downregulation of expression of heat shock genes. These authors proposed that the physiologically most significant function of DnaK in the metabolism of unstressed cells is its function in heat shock gene regulation. In this study we showed that the growth defect of the $\Delta$dnaK$^{52}$ mutant at intermediate and low temperatures can be suppressed by the overproduction of $\sigma^{70}$. This suggests that both the downregulation of $\sigma^{32}$ and the overproduction of $\sigma^{70}$ have a similar consequence in the dnaK null background, implying that a balance of cellular concentrations of $\sigma^{32}$ and $\sigma^{70}$ is a crucial physiological parameter for normal growth of cells. These views are strengthened by the recent finding by Blaszczak et al. [20]. They proposed that the DnaK–DnaJ–GrpE chaperone machine modulates the heat shock response by regulating the switch between $\sigma^{70}$ and $\sigma^{32}$ assembled with RNA polymerase. Mutations in dnaK, dnaJ or grpE fail to turn off synthesis of HSPs, and result in higher levels of HSPs under physiological conditions. Blaszczak et al. suggested that the magnitude of the heat shock response depends not only on the intracellular concentration of $\sigma^{32}$, but also on the relative amount, functional state and ability of $\sigma^{70}$ to compete with $\sigma^{32}$ for binding to core RNA polymerase. Together with these, our findings are compatible with the views that the derepression of $\sigma^{32}$ function in dnaJ/cbpA or dnaK null backgrounds is deleterious for normal cell growth at low and intermediate temperatures, and that the overproduction of $\sigma^{70}$ may suppress the defect of cellular processes caused by such a presumed "sigma-imbalance", by competing with $\sigma^{32}$. Verification of this view must await further experimentation, which should include characterization of in vivo effects of the overexpression of $\sigma^{70}$ on heat-shock response as well in vitro analyses of the presumed interplay between $\sigma^{70}$ and $\sigma^{32}$.

In any event, although the DnaK–DnaJ–GrpE chaperone machine is postulated to play important roles under various physiological conditions, our results in this study support the view that CbpA also plays an important role as an analog of DnaJ under certain physiological conditions. A tight regulation of the intracellular balance of $\sigma^{70}$ and $\sigma^{32}$ may be an important parameter for normal cell growth, for which the DnaK–DnaJ/CbpA–GrpE chaperones play a crucial role. In this respect, the function(s) of CbpA is closely related to that of DnaJ, and thus CbpA is capable of partly compensating for DnaJ for cell growth. Clarification of the molecular role of CbpA in this presumed mechanism also must await further experimentation.

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References


