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ANOMALIES IN THE GUANOSINE POLYPHOSPHATE METABOLISM: EFFECT OF THE hisU MUTATION*

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SUMMARY

The hisU1820 mutant (TA799) of Salmonella typhimurium shows a substantial increase in the levels of ppGpp (MSI) and of pppGpp (MSII) during several types of metabolic shifts. Noticeable amounts of ppGp (MSIII) are also present post-carbon/energy source downshifts and temperature up-shifts. The increased levels of these guanosine polyphosphates were observed despite the absence of the expected reduction in RNA synthesis upon a nutritional downshift. We, therefore, suggest that the hisU mutation causes an increase in the accumulation of MSI and MSII; and that ppGpp alone is not sufficient to promote restriction of RNA synthesis during a nutritional transition.

RNA synthesis is normally coupled to the availability of aminoacyl-tRNA in the cell. An elevated level of uncharged tRNA caused by either amino acid deprivation or inactivation of aminoacyl-tRNA synthetase results in a major readjustment of cellular activity; most notably, the reduction in stable RNA (ribosomal RNA and transfer RNA) accumulation (1,2). This process is referred to as the "stringent response". Substantial evidence bearing on the regulation of RNA synthesis indicates the involvement of the small metabolite, guanosine tetraphosphate (2). Guanosine tetraphosphate (ppGpp) or (MSI) reportedly functions by impeding transcription initiations at the promoters

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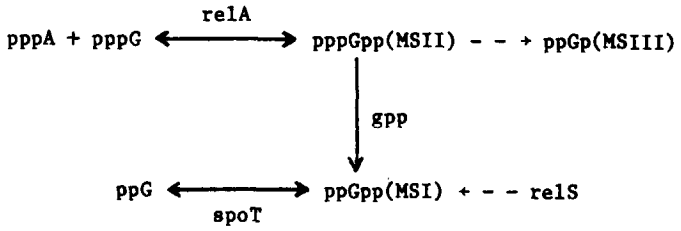

Figure 1. Pathway of guanosine polyphosphate metabolism

for stable RNAs (2). Recent evidence indicates at least two pathways for ppGpp synthesis. The major pathway is a ribosome-dependent system via activation of the stringent factor, the relA product (3). A second pathway is most likely a ribosome-independent system that is responsible for basal levels of ppGpp involving the relS gene product (4). A putative pathway for the polyphosphate guanosine cycle is presented in Figure 1.

The hisU1820 regulatory mutation of Salmonella typhimurium as expressed in TA799, has been previously shown to cause non-restricted RNA synthesis during carbon/energy source (C/E) downshifts (5). The mutation was originally isolated by Roth, Anton, and Hartman (6) while selecting for histidine derepression and has subsequently been shown to exhibit altered regulation of both isoleucine and valine biosynthesis (7). An isogenic wild-type, JC100, shows the expected stringent-type controls during C/E downshifts. Interestingly both strains are relA$^+$. In this study, we now report that this altered phenotype is observed in TA799 during C/E downshifts despite significant accumulation of ppGpp and pppGpp, indicating that ppGpp alone is not sufficient to promote restriction of RNA synthesis during C/E downshifts.

EXPERIMENTAL

Strains. The strains of Salmonella typhimurium LT2 used in this study are the hisU mutant, TA799 (hisU1820, relA$^+$) obtained from P.E. Hartman and the isogenic wild-type, JC100 (hisU$^+$, relA$^+$) which was isolated from TA799 by P22 transduction in our laboratory. Nucleotide labeling. Wild-type and mutant cells from overnight cultures were inoculated into 5 ml of the designated media in a SAF warmed to 37°C (or warmed to 23°C for the temperature upshift experiments). Cultures previously grown under one

condition (nutrient broth [NB], glucose [Glc], or 23°C) were labeled with 50-100 μCi/ml of ($^{32}PO_4$) (Amersham Corp.) before and after conditional shifts were made. Klett readings (#66 filter) were recorded, and samples were taken at pre-shift and at T = 0, 5, 15, and 30 minutes post-shift of each growth transition. Small volumes (100 μl) of the growing cultures were then extracted at various times in an equal volume of 2M formate. After the samples were allowed to extract for at least 65 minutes, the cell pellet was collected by centrifugation in a microfuge and the supernatant was frozen for subsequent analysis. In all cases, nucleotides were adsorbed in Norit by adding 50 μl of the supernatant sample to equal volumes of washed Norit. The Norit extracted supernatant which served as the control, was allowed to extract for 5 minutes at room temperature with infrequent agitation and then centrifuged and analyzed as described for the experimental samples. Thin layer chromatography. Thin layer chromatography (TLC) was carried out according to Randerath (8) with modifications from Pao and Gallant (9) for two dimensional resolution. The experiments were conducted on polyethylene-imine (PEI) plates. The first dimension solvent was 1.5 M KH_2PO_4 (used to reduce the percent of ppGpp hydrolysis by the formic acid treatment); the second dimension solvent, employed at 90° to the first, was 4 M HCOOH plus 1 M LiCl at a 2:3 ratio. When only one dimension was used, 1.5 M KH_2PO_4 was employed for its development. Autoradiography. Autoradiograms were developed on X-ray film (Kodak RP Royal X-Omat) after 16 to 24 hours of exposure in a freezer at -20°C. The position of nucleotide markers GTP, ATP, ppGpp, and pppGpp were visualized by ultraviolet absorption and circles in a dotted line. After autoradiogram development, the nucleotides of interest were removed from the X-ray paper and added to scintillation vials containing 5 ml of Econifluor and the [^{32}P] radioactivity was measured with a liquid scintillation spectrometer.

RESULTS

Concerted efforts were directed toward rigorously probing the regulatory basis of stringency of stable RNA synthesis or the lack thereof in *hisU*$^+$ and *hisU*$^-$ strains as observed during nutritional downshift transitions (5). Given that both *hisU* and *hisU*$^+$ are *relA*$^+$ and therefore not limited in supplies of tRNA, other processes affecting RNA synthesis other than the traditional stringent response had to be considered. Previous determinations of the cellular levels of guanosine polyphosphates ppGpp (MSI), pppGpp (MSII) and ppGp (MSIII) have been performed during a C/E downshift (2,10), amino acid hydroxymate inhibition (11), and temperature upshifts (12).

The results shown in Figure 2 indicate the progression of ppGpp and pppGpp levels during a glucose to succinate downshift in the *hisU* mutant and the wild type strain. The open circles representing MSII in the wild type at 0, 5, 15, and 30 minutes post-shift indicate that there is no change in the levels of this molecule post-shift, as expected. Conversely, the level of MSI

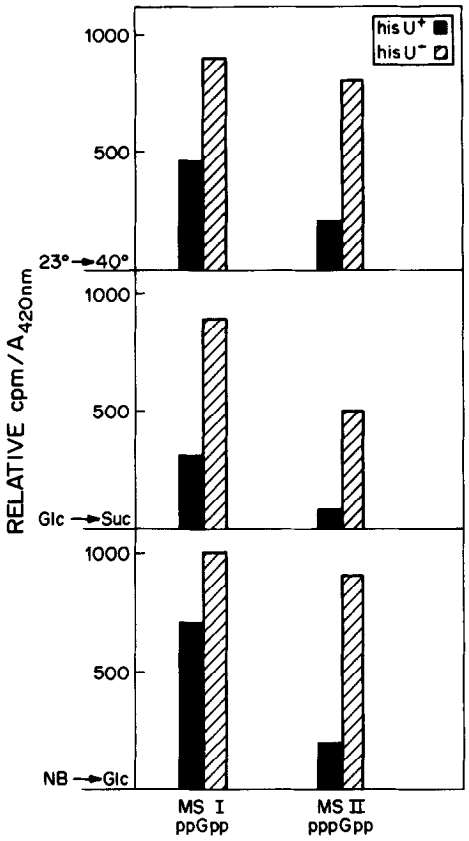

Figure 2. Levels of MSI and MSII during a Glucose → Succinate Carbon/Energy Source Downshift in JC100 and TA799

Cultures were prepared for thin layer chromatography (TLC) using [^{32}P]-labeled cells. 20 µl samples of cell free extract from T = 0, 5, 15, and 30 minutes post shift were spotted onto PEI plates along with known nucleotide markers. After one-dimensional chromatography and autoradiography development, the nucleotides of interest were removed and the radioactivity was measured in a spectrometer.

increased in the expected manner (closed circles). However, the most significant finding is the substantial increase in MSI observed in hisU post-nutritional downshift. This is especially noteworthy when one recalls that hisU exhibits relaxed control of RNA synthesis during such nutritional downshifts. Moreover in contrast to that of the wild-type strain, there is a considerable increase in MSII in this hisU mutant (Figure 2, open triangles).

Figure 3 presents a normalized comparison of the measurements of MSI and MSII at 15 minutes post-nutritional shifts during: a 23°C → 40°C temperature

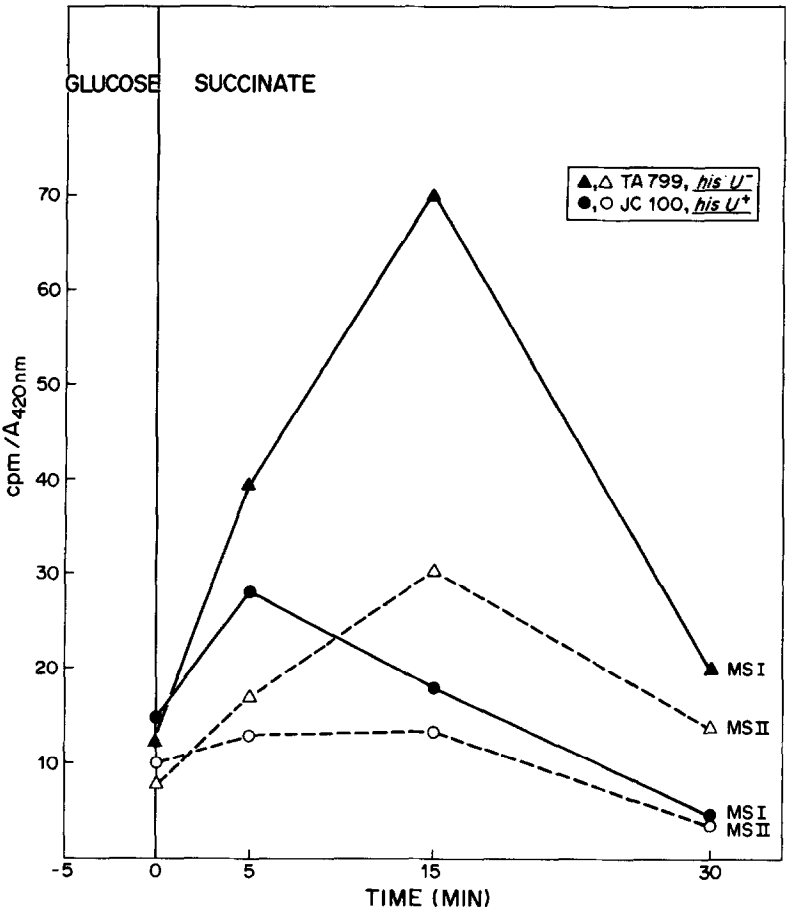

Figure 3. Relative Levels of MSI and MSII 15 Minutes Post-Metabolic Shift in JC100 and TA799

Cultures were prepared for thin layer chromatography using [^{32}P]-labeled cells and 20 µl samples taken at T = 15 minutes post-metabolic shifts were spotted and developed in two-dimensional TLC using 1.5 M K_2PO_4 in the first dimension and 2 M formate plus 1 M LiCl in the seond dimension. Radioactivity was measured as described in Figure 1. Values for MSI in nutrient broth for TA799 are arbitrarily set at 100.

upshift, which simulates the C/E downshifts in production of guanosine polyphosphates (2), but allows continued RNA synthesis, a glucose → succinate downshift, and a nutrient broth → glucose downshift. In all these nutritional shifts, the hisU mutant had substantially increased levels of MSI and MSII, and the increases of MSII in this mutant are at least four-fold above that of the wild-type strain in all instances.

DISCUSSION

These results demonstrate significant formation of MSI in both the hisU mutant and in the wild-type during nutritional downshifts and temperature upshifts. Interestingly, a significant amount of MSII is formed in the mutant during these metabolic shifts, as contrasted with the wild-type in which only MSI levels are increased subsequent to the shiftdown. Importantly, these results are the opposite of these found for the traditional spoT mutants examined under similar conditions (13,14). However, when these results are considered in relation to polyphosphate cycle, it is possible that substantial increases in the accumulation of MSI would promote an accumulation in its precursor MSII. This would imply primary increases in MSI followed by increases in MSII post shift. Our data (Figure 2) agree with this suggestion, as the levels of MSI appear to rise faster than those of MSII.

In assessing the effectiveness of ppGpp in control of RNA synthesis, it would appear that hisU is insensitive to increased levels of MSI or that hisU merely requires much higher levels of MSI to effect the expected wild-type response during a C/E downshift. However, this seems unlikely since Spadaro et al. (15) have observed RNA synthesis restriction in the absence of cellular ppGpp, the exact reciprocal of our findings. Noticeable levels of MSIII (ppGp) in both hisU and the wild-type were also revealed in this study (results not shown). Although Pao and Gallant implicate MSIII as the regulatory factor effecting stringent response during amino acid starvation (11), the results reported herein seem to suggest that this is not the case in a C/E downshift. Further complications arise when the effects of a temperature upshift (which also cause increased levels of ppGpp without RNA restriction) are considered. While the increased level of ppGpp during a temperature upshift is thought to be due to a pending amino acid starvation, the servomechanism initiating the C/E response is yet to be defined. In any case, our results suggest that ppGpp alone does not trigger that servomechanism.

We have also shown that the hisU mutant exhibits a four-fold increase in total RNA synthesis concomitant with these significant amounts of ppGpp during nutritional downshifts (unpublished data). Thus, a model for the regulatory alterations occasioned by the hisU mutation rests on the central premise that a gene coding for some element that promotes stabilization of stable RNAs is altered in this mutant. The correspondent increases in guanosine polyphosphates are, therefore, compensatory to the observed increases in RNA accumulation in the hisU mutant post metabolic transitions. Overall, our conclusions drawn from these results further implicate the involvement of the hisU gene product in a general regulatory control mechanism essential to macromolecular adjustments during changing cellular environments. Thus, continued studies of regulatory mutants such as hisU towards a more comprehensive analysis of the mechanisms by which macromolecules interact to maintain and regulate balanced growth via coupled pathways appear essential to understanding the regulatory couples between control of stable RNAs and regulation of amino acid biosynthesis.

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