Role of the *rel* Gene Product in the Control of Cyclic Adenosine 3',5'-Monophosphate Accumulation

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The presence of a *relA* mutant allele affects the kinetics of cyclic adenosine 3',5'-monophosphate accumulation during downshift from glucose to succinate. The nucleotide accumulates at the normal rate early in the downshift transition but continues to accumulate for a longer time in the *relA* mutant, leading to a two- to threefold excess by the end of the diauxic lag. Evidence is presented that this effect occurs independently of the accumulation of ppGpp.

Two unusual nucleotides, cyclic adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-bis-pyrophosphate (ppGpp) exercise control over a wide variety of metabolic and transcriptional processes in bacteria (4, 5, 12, 14). These regulatory nucleotides are not closely related metabolically, but physiological factors governing their abundance show an interesting parallelism. The levels of both nucleotides show a rough inverse correlation with growth rate on different media (3, 10, 15) and increase dramatically during glucose starvation or downshift from glucose to a poorer carbon and energy source (3, 9, 10, 11, 16; present results). Moreover, both nucleotides also accumulate during a shift from aerobic to anaerobic growth (6; H. Rickenberg, personal communication).

These parallels raise the possibility of some interconnection between the control systems that adjust the levels of the two nucleotides in response to metabolic downshift. Here we report genetic evidence of such interconnection. The product of the *relA* gene carries out the ribosome-associated synthesis of ppGpp (1), and mutation in this gene has previously been shown to affect the kinetics of ppGpp accumulation during downshift (8, 10). In a preliminary survey, we found abnormally high levels of cAMP in a *relA* mutant at the end of the period of diauxic lag after glucose-to-succinate downshift. In a series of six replicate experiments, the average cAMP level at the end of downshift was 2.1 ± 0.28 nmol per absorbancy (at 720 nm [A_{720}]) unit in the mutant, as opposed to 0.89 ± 0.16 in its isogenic *rel*+ partner.

Figure 1 shows the kinetics of cAMP production in this pair of strains during the course of the downshift transition. At first, both strains accumulate the nucleotide at the same initial rate of about 0.04 nmol/A_{720} unit/min. However, this high rate persists considerably longer in the *rel* mutant than in the wild-type strain,
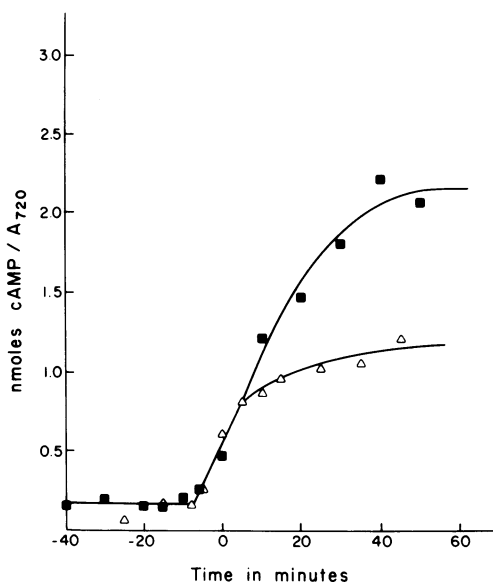

FIG. 1. *Accumulation of cAMP during glucose-succinate downshift. Cultures of NF161 (argA metB) and of NF162, its isogenic relA1 sibling, were grown in $^{32}PO_4$-labeled medium containing limiting glucose (0.02%) and excess succinate (0.2%), as described previously (10). Total cAMP levels in the culture were assayed in formic acid extracts (10) by thin-layer chromatography on cellulose layers. The plates were first developed in isopropanol-ammonium hydroxide-water (70:15:15, vol/vol), in which cAMP displays an R_f of about 0.5, but all linear nucleotides barely leave the origin (2). The plates were then cut off about 6 mm behind the position of marker cAMP and developed a second time in n-butanol–acetic acid–water (50:25:25, vol/vol) (7). The combination of these two solvent systems produced a well-resolved radioactive spot that was coincident with marker cAMP and was virtually absent in Norite-adsorbed extracts. (The two solvent systems can be used in a two-dimensional separation to obtain still better resolution, which is useful for the determination of low levels of cAMP.) As a control for the specificity of the*

leading to the observed excess in the plateau level finally attained.

For comparison, the accumulation of ppGpp in both strains is shown in Fig. 2. As previously reported (10), ppGpp accumulates much more rapidly at first in the rel^+ strain than in the mutant. Moreover, ppGpp reaches a peak value at about the same time that cAMP production is curtailed. Therefore, it might be postulated
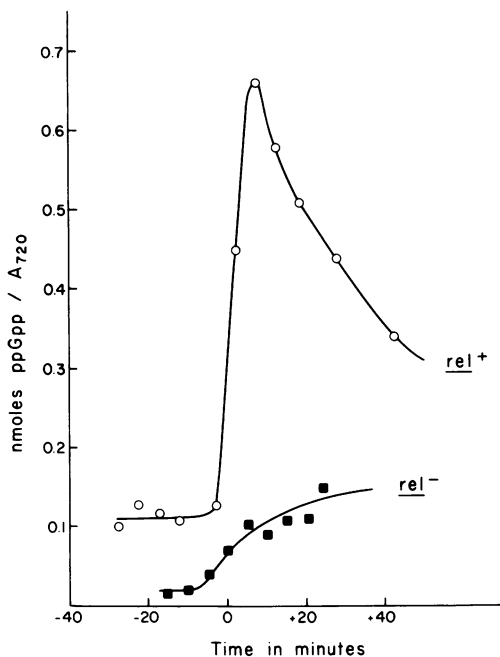

FIG. 2. *Accumulation of ppGpp during glucose-succinate downshift. Cultures of both strains were downshifted as described in the legend of Fig. 1, and ppGpp was measured as described previously (10).*

method, we subjected the cya mutant strain 5336 (13) and its cya$^+$ parent to similar downshift and chromatographic analysis. The cya mutant yielded 3% as much radioactivity in the cAMP spot as the parental strain, in good agreement with the reported 3.5% residual activity of adenyl cyclase (13). In all experiments, the labeled cultures were equilibrated with $^{32}PO_4$ for at least half a doubling before the first sample was removed (well before downshift), and each cAMP measurement was corrected for counting background and nonspecific radioactivity by means of a Norite supernatant blank chromatographed together with the experimental samples. The abscissa is time relative to the sharp break in the growth curve characteristic of downshift. The ordinate is nanomoles of cAMP per milliliter of bacteria at an optical density of 1.0 at 720 nm, measured in a Beckman DB spectrophotometer, which corresponds to approximately 10^9 cells or 200 µg of protein. Symbols: △, NF161 (rel$^+$); ■, NF162 (rel$^-$).

that ppGpp directly or indirectly modulates the production of cAMP. However, this proposition is weakened by the fact that ppGpp falls markedly after reaching its peak value in the rel^+ strain, but there is no resumption of cAMP production during this period.

A more decisive test of the proposition is shown in Fig. 3. Part of a glucose culture of the rel^+ strain was first subjected to isoleucine starvation, which triggers ppGpp accumulation to a level three times higher than the downshift peak value, and then transferred to succinate medium; a control portion of the culture was transferred to succinate medium without prior amino acid starvation. If high levels of ppGpp inhibit cAMP accumulation, this inhibition should be evident in the cells that were amino acid starved before downshift. On the contrary, Fig. 3 shows that cAMP accumulated with identical kinetics in both amino acid-starved and control cells. Identical results were obtained in a duplicate experiment.
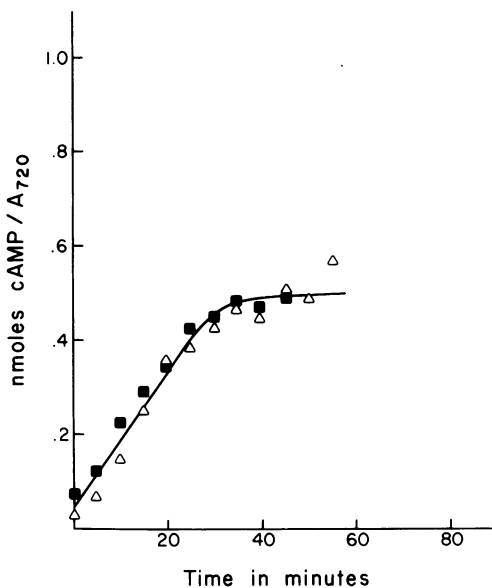

FIG. 3. *A culture of NF161 (rel$^+$), growing exponentially on 0.2% glucose, was labeled with $^{32}PO_4$ for one doubling. A portion of the culture was then subjected to isoleucine starvation through the addition of valine (400 µg/ml). After 15 min, it was rapidly filtered, washed with minimal medium at the same specific activity, and suspended in medium, at the same specific activity, containing 0.2% succinate as the sole carbon source, 100 µg of valine per ml, and 100 µg of isoleucine per ml. A control portion of the primary culture was transferred in the same way but without the period of valine pretreatment. Symbols: ■, valine pretreated; △, control.*

We conclude that a high level of ppGpp does not by itself affect the accumulation of cAMP during downshift, either directly or indirectly. Nonetheless, Fig. 1 shows that some aspect of the *relA* function does modulate the accumulation of cAMP during the course of the downshift transition. The unexpected implication of these results is therefore that the *relA* gene product carries out some function during downshift other than that of ppGpp synthesis. The nature of this function is quite unknown, and the way in which it affects adenyl cyclase activity may well be indirect, such as through the formation of a regulatory nucleotide other than ppGpp.

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