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Role of Peptide Chain Elongation Factor G in Guanosine 5′-Diphosphate 3′-Diphosphate Synthesis

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In a wild-type strain (*relA*$^+$) of *Escherichia coli*, starvation of amino acid led to an immediate cessation of the synthesis of stable ribonucleic acids, together with the accumulation of an unusual nucleotide, guanosine 5′-diphosphate 3′-diphosphate, commonly known as ppGpp. This compound also accumulated during heat shock. When temperature-sensitive protein synthesis elongation factor G (EF-G) was introduced into *E. coli* NF859, a *relA*$^+$ strain, the synthesis of ppGpp was reduced to approximately one-half that of wild-type EF-G$^+$ cells at a nonpermissive temperature of 40°C. Furthermore, fusidic acid, an inhibitor of protein synthesis which specifically inactivates EF-G, prevented any accumulation of ppGpp during the heat shock. We suggest that a functional EF-G protein is necessary for ppGpp accumulation under temperature shift conditions, possibly by mediating changes in the function of another protein, the *relA* gene product. However, EF-G is probably not required for the synthesis of ppGpp during the stringent response, since its inactivation did not prevent ppGpp accumulation during amino acid starvation.

In *Escherichia coli*, the synthesis of RNA is normally coupled to the availability of aminoacylated tRNA's in the cells. An elevated level of uncharged tRNA, caused by either amino acid deprivation or inactivation of an aminoacyl-tRNA synthetase, results in a major readjustment of cellular activity. This phenomenon is referred to as the stringent response and it includes, among many others, the following changes in the cells: the rate of stable RNA accumulation is greatly reduced (17, 32); synthesis of ribosomal proteins (9, 10, 11, 23, 25, 28), translational factors (7, 15, 23), and α subunit of RNA polymerase (23, 25) is severely curtailed. The transport and uptake of some precursor molecules across the membrane (12, 13, 29, 33) and the synthesis of nucleotides (5, 16, 21), lipid (24, 30), and various other cellular components are also restricted.

Biochemical and genetic studies have suggested that a gene, *relA*, mapped at 59 min on the *E. coli* genetic map is responsible for this stringent response (17, 35). It has been demonstrated that the *relA* gene codes for a ribosome-bound protein with nucleotide phosphotransferase activity that produces novel guanosine nucleotides, pppGpp and ppGpp, upon amino acid starvation (3, 8, 17). Regulatory effects of ppGpp on the synthesis of stable RNA have been suggested, although direct evidence is still lacking. ppGpp may participate in modulating the enzyme activity of RNA polymerase, thereby resulting in the gross change of the transcription pattern of the cell (36). On the other hand, in vitro experiments have yielded contradictory reports; thus, although the preferential inhibitory effect of ppGpp on stable RNA synthesis has been observed in some laboratories (cited in reference 17), it has not been observed in many other laboratories (cited in reference 17). Despite this, it is generally believed that ppGpp possesses some kind of regulatory capacity and is part of the stringent adjustment of cellular activity.

Studies on the function of guanosine polyphosphates have revealed the involvement of peptide chain initiation and elongation factors. It appears that the formation of initiation complex can be inhibited by ppGpp (37). ppGpp has also been shown to bind tightly to elongation factor Tu (4, 26), and this apparently stops the exchange between free and Tu-bound GDP during peptide chain elongation (1). Although the physiological significance of these observations is far from clear, the interaction between ppGpp and components of the protein-synthesizing system is more than just coincidence. This interaction is also displayed in the metabolism of ppGpp, where several proteins have been implicated in the conversion of pppGpp to ppGpp; elongation factor G (EF-G) can hydrolyze pppGpp in vitro, although at a lower rate than GTP (20). Atherly et al. (2, 22) and Rabbani and Srinivasan (31) have reported that an inactive

EF-G in a stringent strain of *E. coli* can result in a relaxed phenotype in regard to stable RNA and ppGpp synthesis. Furthermore, an enzyme determined by the *gpp* gene has been reported by Sommerville and Ahmed to catalyze specifically the dephosphorylation of pppGpp to ppGpp (34).

Kinetic analyses have indicated that ppGpp is converted to GDP, which is, in turn, phosphorylated to GTP, the direct precursor for pppGpp production (14). These observations may be summarized as the ppGpp cycle that is shown in Fig. 1.

We reported previously that through an unknown mechanism, heat shock triggers a transient but significant accumulation of ppGpp, whereas there is little or no effect on stable RNA synthesis (18). In those experiments, wild-type *E. coli* cells of strain NF859 were grown at the abnormally low temperature of 23°C and then shifted to a temperature in a more growth-optimal range (37 to 40°C). As a result, ppGpp accumulated at a high level, although pppGpp, the precursor of ppGpp, did not. Furthermore, the synthesis of ppGpp during this heat shock was shown to be partially *relA* dependent. The failure of pppGpp to accumulate might be due to the action of *relA* along an alternative pathway which bypasses the formation of pppGpp. Another possibility is that the pathway during heat shock is similar to that during amino acid starvation, except that catabolism of pppGpp to ppGpp occurs so fast that pppGpp cannot accumulate appreciably. Evidence to be presented supports the former possibility and evidence further suggests the involvement of EF-G in this pathway.

MATERIALS AND METHODS

The parental strain of *E. coli* used in this study was NF859 (*metB argA relA*). The EF-G(Ts) derivative of NF859 was constructed by cotransducing EF-G(Ts) (the *fus* allele) with *strA* (the *rpsL* allele) from strain G1 (*metB argA* EF-G(Ts) *strA*), using the P1 transduction procedure described by Miller (27). *E. coli* NF859 and G1 were obtained from Neils Fiil and A. Atherly, respectively. Tris-glucose minimal medium supplemented with appropriate required amino acids at 100 μg/ml and KH_2PO_4 at 0.5 mM was used throughout the study and has been described previously (18). Growth of cells was under forced aeration at a temperature indicated and monitored turbidimetrically at 720 nm in a Beckman DB spectrophotometer; an optical density (OD) of 1.0 equals about 10^9 cells or 200 μg of protein per ml. ^{32}P was purchased from New England Nuclear Corp. as carrier-free orthophosphoric acid. Common nucleotides were obtained from Sigma Chemical Co., and unusual nucleotides were from either ICN or P-L Biochemicals.

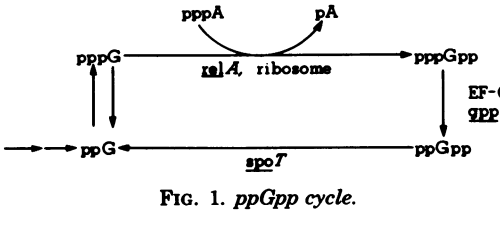

FIG. 1. *ppGpp cycle.*

Log-phase cultures at OD_{720} (OD at 720 nm) of about 0.1 were supplemented with $^{32}PO_4$ (300 μCi/ml) for at least one doubling before sampling to allow equilibration of the phosphate pools. After being extracted with 0.1 N formic acid, the samples were neutralized with Tris buffer and the cell pellet was centrifuged in a Beckman microfuge. The supernatant containing the small molecule fraction was chromatographed on PEI (polyethyleneimine)-cellulose thin-layer plates (Brinkmann Instruments Inc.) pre-eluted with 2.8% NH_4OH. Solvents used for resolution of the nucleotides were 2 M HCOOH–1.5 M LiCl in the first dimension and 1.5 M KH_2PO_4 in the second dimension. The radioactive areas of the chromatograms, identified by autoradiography using known markers, were cut out and counted in a toluene-based scintillation mixture (Liquifluor; New England Nuclear Corp.). The absolute concentration of ^{32}P in the culture was calculated from the counts obtained with samples of the culture, which were spotted directly onto PEI-cellulose thin-layer plates and dried. The pool size is expressed in nanomoles per OD_{720} unit of cells.

RESULTS AND DISCUSSION

Growth of both EF-G$^+$ and EF-G(Ts) strains of *E. coli* NF859 was monitored when the cells were shifted from 23°C to the nonpermissive temperature of 40°C. In the wild-type cells, growth proceeded at a higher rate, whereas that of the temperature-sensitive cells showed gradually as a result of the temperature-sensitive mutation and stopped completely after 8 to 10 generations (data not shown). This indicates that EF-G is only partially inactivated under these conditions and the translation process is still functional, at least for several generations. Further support for this comes from the continued incorporation of [^{3}H]leucine into protein under these temperature shift conditions (data not shown), as well as when cells were treated with a sublethal concentration of fusidic acid (see below). Establishing these facts is crucial to the interpretation of our data, since a functional translational machinery is necessary for the synthesis of pppGpp and ppGpp (6). Heat shock of cells carrying the mutant EF-G resulted in the accumulation of substantially less ppGpp in the temperature-sensitive mutant cells than in wild-type cells (Fig. 2). Inactivation of EF-G by fusidic acid led to complete and immediate prevention of ppGpp accumulation (Fig. 3). These results suggest either that EF-G is necessary for

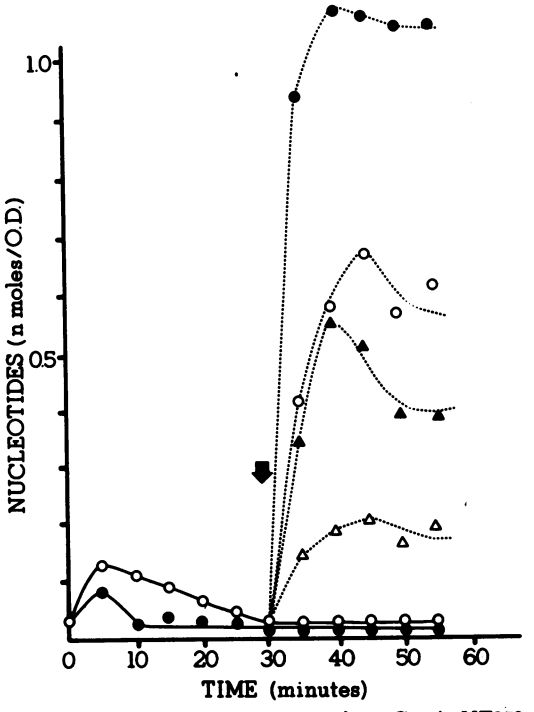

FIG. 2. *Synthesis of ppGpp and pppGpp in NF859 and NF859 [EF-G(Ts)] cells after temperature upshift and after additions of serine hydroxamate to the growth medium. Cells were grown in Tris-glucose minimal medium supplemented with required amino acids (100 μg/ml) and KH_2PO_4 (0.5 mM) at 23°C. $^{32}PO_4$ was added to 300 μCi/ml at least one doubling before sampling. Cultures of both strains were subjected to temperature upshift at time zero by shifting the culture from 23 to 40°C (solid lines). At the time indicated by the arrow, serine hydroxamate, at a concentration of 500 μg/ml, was added to parallel cultures in order to limit serine activation (dashed lines). Circles represent the level of ppGpp; triangles represent the level of pppGpp; open symbols indicate strain NF859; closed symbols indicate the strain NF859 [EF-G(Ts)].*

the synthesis of ppGpp during a thermal upshift or that it alters the kinetics of other enzymes involved in the ppGpp cycle.

Since temperature upshift itself can cause a large, but fortunately transient, accumulation of ppGpp in NF859 cells (18), the effect of a thermally inactivated EF-G on the cells' capacity to produce ppGpp after subsequent starvation for amino acid was examined in the following manner. Amino acid starvation was imposed by adding serine hydroxamate, an analog of serine, to cultures 30 min after temperature shift so that the increase of ppGpp due to temperature change was minimal, while at the same time providing ample time for the thermal inactivation of EF-G to take place. Under these conditions, the accumulation of ppGpp was affected in two ways. First, the inactivation of EF-G failed to prevent the stringent response; in fact, one of the two methods of inactivation actually allowed greater accumulation of ppGpp (Fig. 2). These data at first seem to conflict with the report of Rabbani and Srinivasan (31), who showed that inactivation of EF-G results in the "relaxed" response when cells are starved for an essential amino acid. However, simple amino acid deprivation would not be expected to produce the stringent response at a time when protein synthesis is blocked because intracellular protein degradation would provide amino acids to tRNA-activating enzymes, thereby relieving the stringent response. In our experiments, the stringent response was elicited by blocking serine activation, thus avoiding this artifact. Second, pppGpp did not accumulate during heat shock, but was produced readily in response to starvation for amino acids. This implies that heat shock uses a pathway different from that described in Fig. 1. Thermally induced accumulation of ppGpp has been shown to be partially dependent on the product of the *relA* gene (18). If, as claimed here, EF-G is also required for the reaction and if the reaction takes place in two steps, then inactivation of one of

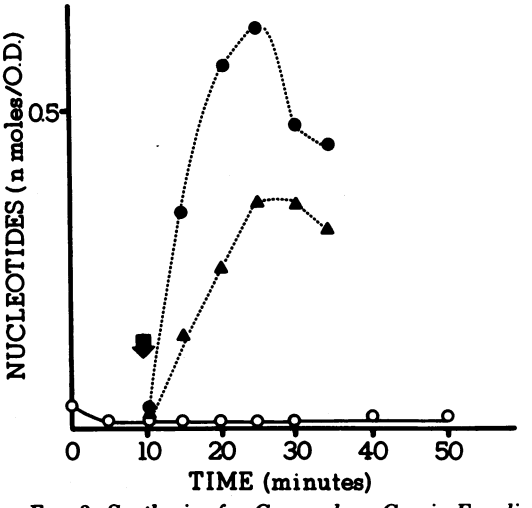

FIG. 3. *Synthesis of ppGpp and pppGpp in E. coli strain NF859 treated with fusidic acid. Cells were cultivated as described in Fig. 2. At time zero, fusidic acid was added to the culture (1 mg/ml) and ppGpp was measured at time indicated (open circles) according to the procedures described in Materials and Methods. Serine hydroxamate at 500 μg/ml was added to portions of the culture at time indicated by the arrow. Samples were then harvested, and both ppGpp (closed circles) and pppGpp (closed triangles) were determined.*

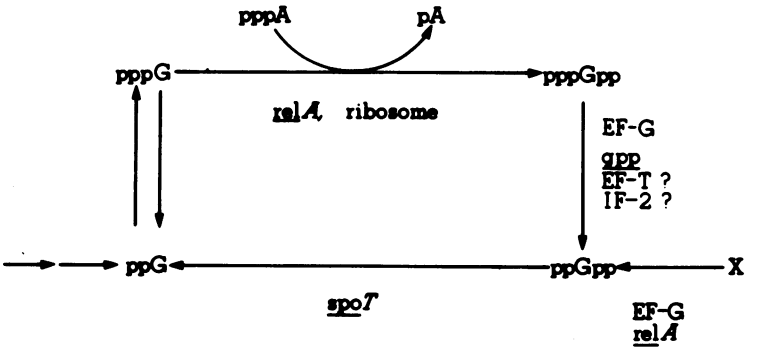

FIG. 4. *Scheme proposed for guanosine polyphosphate metabolism. This model attempts to incorporate all known in vivo and in vitro observations. Pathways for ppGpp synthesis in addition to that in Fig. 1 are included (see text).*

the two required enzymes ought to cause accumulation of precursor molecules. Since neither pppGpp nor any other novel guanosine nucleotides have been detected at any time, the reaction during thermal shock may involve only one step. If this is so, the necessary enzymes, EF-G and the *relA* gene product, may work in a cooperative fashion. EF-G provides the energy needed for peptide chain elongation by hydrolyzing GTP to GDP. Since a change in conformation of EF-G, produced by the binding of uncharged tRNA to the ribosome-mRNA complex, might affect the hydrolysis and accumulation of guanosine nucleotides, EF-G may present itself as a key enzyme in the synthesis of unusual guanosine nucleotides. If this change in the conformation of ribosomes is truly the eliciting stimulus of the stringent response, a similar change might produce ppGpp during the heat shock. Whatever the change, EF-G is apparently involved since different states of EF-G led to different intracellular levels of (p)ppGpp. Amino acid starvation after heat shock produced more pppGpp in EF-G(Ts) cells relative to that produced in wild-type cells. This strengthens the hypothesis that EF-G is one of several enzymes which hydrolyze pppGpp to ppGpp (19, 20). One problem that results from the data on the effects of amino acid starvation is that ppGpp accumulated to an even higher degree in EF-G(Ts) cells than in EF-G$^+$ cells with or without fusidic acid (compare Fig. 2 and 3); however, the mechanism of inhibition of the elongation factor protein was different in each case, and the differential accumulation can, therefore, be attributed to this.

How do *relA* gene product and peptide chain EF-G act cooperatively during heat shock, but not during amino acid starvation? One possibility is that EF-G somehow mediates the change in function of *relA* enzyme upon heat shock. Therefore, inactivation of either would prevent the accumulation of ppGpp. Conversely, it is possible that *relA* mediates the change in function of EF-G in such a way that during heat shock EF-G can form ppGpp without pppGpp as substrate. Although it is not possible to determine from the data here which possibility is more likely, either would explain why inactivation of EF-G followed by starvation fails to prevent ppGpp synthesis. What is clear is that inactivation of EF-G does not prevent ppGpp accumulation in starved cells, but does in thermally shocked cells. Therefore, we would like to amend, based on the aforementioned evidence, the model for the ppGpp cycle shown in Fig. 4.

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