TRANSCRIPTIONAL REGULATION OF THE *GLTA* AND *TLC* GENES IN *RICKETTSIA PROWAZEKII* GROWING IN A RESPIRATION-DEFICIENT HOST CELL

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Summary. – The regulation of the citrate synthase (*gltA*) and ATP/ADP translocase (*tlc*) genes of the obligate intracellular bacterium, *Rickettsia prowazekii*, was analyzed in rickettsia-infected respiration-deficient G14 cells. The level of the *gltA* mRNAII and the *tlc* mRNA was much lower in the total RNA isolated from the infected G14 cells grown in 1 g/l glucose (low glucose, GL) medium than in that from infected G14 cells grown in 4.5 g/l glucose (high glucose, GH) medium. However, the level of the *gltA* mRNAI relative to 16 S rRNA was the same in GL and GH media. An increase in the level of the *gltA* mRNAII and the *tlc* mRNA could be observed as early as 2 hrs after shifting from GL to GH medium. We conclude that, under these experimental conditions, the *tlc* promoter and the *gltA* promoter P2, but not *gltA* promoter P1, were transcriptionally regulated.

Key words: *Rickettsia prowazekii*; *glt*A gene; *tl*C gene; transcriptional regulation; G14 cells

Introduction

Rickettsia prowazekii, the etiological agent of epidemic typhus, is an obligate intracellular bacterium that grows slowly with a generation time of about 10 hrs directly in the cytoplasm of eukaryotic host cells (reviewed in Winkler, 1990, 1995). Although the eukaryotic cell has developed a variety of mechanisms to maintain a constant internal milieu, in those instances when the host cytoplasm does change, rickettsiae can adapt. Citrate synthase is posttranslationally regulated by the ATP concentration (Phibbs and Winkler, 1982), and ribonucleotide reductase is allosterically regulated (Cai *et al.*, 1991).

Evidence for the transcriptional regulation is more recent. We demonstrated that the level of mRNA transcribed from the *tlc* gene for the ATP/ADP translocase decreases as the L929 host cell fills with rickettsiae in the course of infection (Cai and Winkler, 1996). Concomitantly, the level of mRNAII for citrate synthase, transcribed from P2, one of two promoters in the *gltA* gene (Cai *et al.*, 1995), increases. The level of mRNAI, transcribed from the other *gltA* promoter, the house-keeping promoter, remains constant. The rate of specific mRNA degradation is constant indicating that the regulation is transcriptional. The *tlc* and *gltA* genes were chosen as the target genes to investigate transcriptional regulation in *R. prowazekii* because these genes encode alternative means for this parasite to obtain energy. The ATP/ADP translocase of *R. prowazekii* is an obligate exchange transport system designed for the acquisition of energy, not adenylate, and the citrate synthase catalyzes the condensation of acetyl-CoA with oxaloacetate as the first step of the tricarboxylic acid (TCA) cycle. In addition, these genes and their products are amongst the best characterized in rickettsiae (Winkler, 1976; Phibbs and Winkler, 1982; Wood *et al.*, 1983, 1987; Winkler and Daugherty, 1984; Krause *et al.*, 1985; Williamson *et al.*, 1989; Plano *et al.*, 1990).

The previous study (Cai and Winkler, 1996) of transcriptional regulation in rickettsiae as the infection burden in-

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Abbreviations: DMEM = Dulbecco's Modified Eagle's Medium; GH = high glucose; GL = low glucose; *gltA* = citrate synthase; SDS = sodium dodecyl sulphate; TCA = tricarboxylic acid; *tlc* = ATP/ADP translocase

creases in L929 cells was exquisitely physiologic, but was not amenable to a search for kinetics for regulation or its biochemical signals. In the present study, the respiration-deficient G14 cells, which are solely dependent on glycolysis for their energy production (DeFrancesco *et al.*, 1976; Breen and Scheffler, 1979), were used. We reasoned that we could alter the cytoplasmic milieu of these host cells by changing the concentration of glucose in their growth medium, and that these alterations might affect transcription of the rickettsial *gltA* and *tlc* genes.

Materials and Methods

Enzymes and reagents. Restriction enzymes and modifying enzymes were obtained from GIBCO-BRL Life Technologies, Inc., Gaithersburg, MD. Recombinant RNasin ribonuclease inhibitor, and T3 and T7 RNA polymerase were purchased from Promega Corporation, Madison, WI. [alpha-^{32}P]UTP (6,500 Ci/mmole) was obtained from ICN Biomedicals, Inc., Irvine, CA. Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Mediatech, Washington, DC. Unless indicated elsewhere, the chemical reagents used in this study were purchased from Sigma Chemical Co., St. Louis, MO.

Infection of G14 cells with rickettsiae. G14 cells are respiration-deficient Chinese hamster cells with a defect in NADH-coenzyme Q reductase (DeFrancesco *et al.*, 1976; Breen and Scheffler, 1979), and are solely dependent on glycolysis for their energy production. G14 cells were grown as monolayers in a humidified atmosphere of 10% CO_2 in air at 34°C in DMEM supplemented with 10% fetal calf serum, and asparagine (396 mg/ml). G14 cells, suspended at a concentration of 10^7 cells/ml in Hanks balanced salt solution supplemented with 5 mmol/l L-glutamic acid (monopotassium salt) and 0.1% gelatin (HBSSGG), were infected with purified, yolk-sac grown *R. prowazekii* Madrid E at a multiplicity of infection of 150 to 200 rickettsiae per cell to achieve an initial infection of 10 to 20 rickettsiae per infected cell, and another portion was mock-infected. After incubation at 34°C for 1 hr, the infected cells were washed twice, suspended in DMEM supplemented with 10% fetal calf serum, and seeded into 8 tissue culture plates. After incubating the infected G14 cells for 72 hrs, the growth medium was changed to either the GL (low glucose) or the GH (high glucose) medium. Except for the concentration of glucose, GH and GL media had the same nutrient concentrations. After changing the medium, infected G14 cells were incubated overnight at which time the percentage infected cells in both GH and GL conditions was about 60%, and the cells contained 50 to 90 of rickettsiae per infected cell. Culturing of the infected G14 cells was in the absence of eucaryotic protein synthesis inhibitor, emetine, which was toxic to these cells.

Isolation of total RNA and ribonuclease protection assay. Isolation of purified rickettsiae before extraction of mRNA results in the lost of labile mRNA which has a half-life of about 15 mins (Winkler, 1987; Cai and Winkler, 1993). Therefore, total RNA, a mixture of host cell and rickettsial RNA, was isolated with the hot phenol method as described previously (Cai and Winkler, 1993, 1996). After ethanol precipitation, the RNA pellets were stored in absolute ethanol at -20°C. When used, RNA pellets were dissolved in 0.5% sodium dodecyl sulphate (SDS), and RNA concentrations were determined by measuring the absorbance at 260 nm.

Antisense RNA probes labeled with [alpha-^{32}P]UTP were generated by an *in vitro* run-off transcription with T3 or T7 RNA polymerase; 16 S-probe for the rickettsial 16 S rRNA, TLC-probe for the rickettsial *tlc* mRNA, and CS-probe for the rickettsial *gltA* mRNAs were generated using pHW40, pHW50 and pHW52 as templates, respectively, as described previously (Cai and Winkler, 1993, 1996). Ribonuclease protection assays were performed as described previously (Cai and Winkler, 1993). The density of each protected band on a film was determined by using a laser scanning densitometer (model SLR-TRFF-DNA, Biomedical Instruments, Inc., Fullerton, CA). The linearity of the measured band density was established as previously described (Cai and Winkler, 1996).

Results and Discussion

Determination of the levels of the gltA mRNAs or tlc mRNA in rickettsia-infected G14 cells grown under various conditions

After overnight incubation of the infected G14 cells in either GH or GL medium, GH and GL RNA preparations were isolated and analyzed by the ribonuclease protection assay. Rickettsial 16 S rRNA was used to normalize the recovery of rickettsial RNA and the growth of rickettsiae. All three mRNA species (*gltA* mRNAI, *gltA* mRNAII and *tlc* mRNA) were easily detected in GH RNA preparations (Fig. 1). However, in GL RNA preparations, although the *gltA* mRNAI could be easily detected, the levels of the *gltA* mRNAII and *tlc* mRNA were at the lower limit of detection. The results of three experiments (Table 1) indicated that: (a) the level of the *gltA* mRNAII in GL RNA preparations was 16 to 65 times lower than that in GH RNA preparations, (b) the level of the *tlc* mRNA in GL RNA preparations was at least 67 times lower than that in GH RNA preparations, and (c) the level of the *gltA* mRNAI was not very different between GH and GL RNA preparations (0.8 to 1.2-fold). These results confirm our previous suggestion that both the *gltA* promoter P2 and *tlc* promoter are transcriptionally regulated, but that the *gltA* promoter P1 is a "house-keeping" promoter (Cai and Winkler, 1996). Because of the very low level of *gltA* mRNAII and *tlc* mRNA in GL RNA preparations, the rickettsial mRNA stability under these two growth conditions was not compared.

In the rickettsia-infected L929 cell system (Cai and Winkler, 1996), the direction of regulation of the *tlc* gene was opposite to that of the *gltA* gene: expression of the *tlc* gene decreased in the heavily rickettsia-infected cells, while the expression of the *gltA* gene increased. However, in the cur-

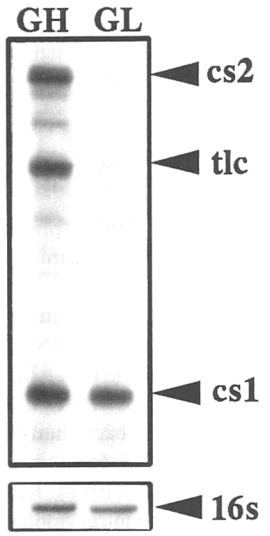

Fig. 1
Levels of *gltA* mRNAs and *tlc* mRNA in GH and GL RNA preparations
RNA samples were isolated from rickettsia-infected G14 cells grown overnight in GH or GL medium, and analyzed by the ribonuclease protection assay. The autoradiograms for the *tlc* and *gltA* samples were exposed overnight, and the *rrs* (16 S RNA) sample was exposed for 10 mins. Bands that represent *tlc* mRNA, *gltA* mRNAI, *gltA* mRNAII and 16 S rRNA are indicated as *tlc, cs1, cs2* and 16s, respectively. The lengths of protected probe fragments for *tlc, cs1, cs2* and 16s are about 200, 120, 258 and 525 nt, respectively.

Table 1. Levels of *tlc* mRNA, *gltA* mRNA I and *gltA* mRNA II in GH and GL conditions

mRNA	Exp. No.	Density[a] GH	Density[a] GL	GH/GL ratio	Average of GH/GL ratio ± SD
gltA mRNA II	1	109.8	6.8	16.1	42.3 ± 20.0
	2	104.9	2.3	45.6	
	3	26.1	0.4	65.3	
tlc mRNA	1	91.9	0	NA	67.6[b]
	2	86.8	1.5	57.9	
	3	30.9	0.4	77.3	
gltA mRNA I	1	82.4	94.2	0.9	1.0 ± 0.2
	2	51.8	64.7	0.8	
	3	36.8	29.7	1.2	

[a]Density was expressed after normalizing the density of each mRNA band to that of the 16 S rRNA within each experiment.
[b]SD was not calculated. NA = not applicable.

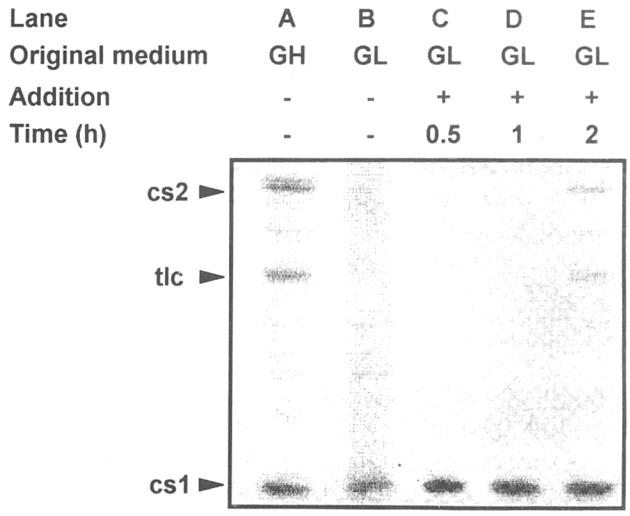

Fig. 2
Time course study: changing the expression of the *gltA* and *tlc* mRNAs in rickettsia-infected G14 cells from the GL to the GH pattern
Rickettsia-infected G14 cells were grown in GH (lane A) or GL medium (lane B), and the total RNA was isolated. The total RNA (used in lane C, D and E) was isolated at 0.5, 1, and 2 hrs after addition of the nutrient mixture (containing glucose, sodium pyruvate, amino acids, vitamins, and $NaHCO_3$) to the overnight-growth GL medium. The *gltA* mRNAI (*cs1*), *gltA* mRNAII (*cs2*) and *tlc* mRNA (*tlc*) were analyzed by the ribonuclease protection assay. The overnight-growth GH or GL medium is indicated as original medium; the addition or non-addition of the nutrient mixture is indicated as (+) or (-).

rent rickettsia-infected G14 cell system, the *tlc* mRNA and *gltA* mRNAII were regulated in the same direction, i.e., both dramatically decreased in GL conditions. These results strongly suggest that there is more than one mechanism involved in transcriptional regulation of the *tlc* and *gltA* genes.

Time course studies and the effect of nutrients on changing the expression of the gltA and tlc mRNAs in the infected G14 cells

We analyzed the *gltA* mRNA and the *tlc* mRNA levels in the total RNA isolated at 2, 4, 6, 8 and 24 hrs after changing the overnight-growth GH medium to fresh GL medium. The GL RNA pattern was not established until 24 hrs after lowering the glucose concentration in the medium (data not shown). These results suggest that rickettsiae in host cells grown in GH medium need a relatively long time to deplete the relevant nutrients in their cytoplasm after switching to GL conditions. However, when the nutrient shift was in the opposite direction, i.e. fresh GH medium was exchanged for the overnight-growth GL medium, the expression of *gltA* mRNAII and *tlc* mRNA increased within 2 hrs (Fig. 2). The addition to the overnight GL medium of a a 0.1 volume of a 10 X DMEM nutrient mixture (containing glucose, sodium pyruvate, 15 amino acids, 8 vitamins, and $NaHCO_3$, but not other inorganic salts) gave the same effect. These results suggest that rickettsiae in host cells grown in GL medium need just a relatively short time to replenish the relevant nutrients in their cytoplasm after switching to GH conditions.

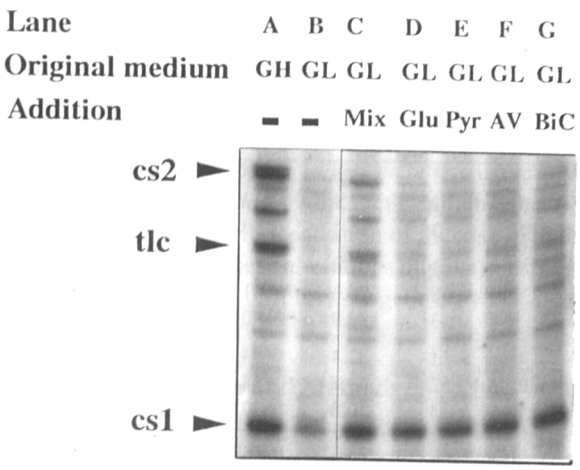

Fig. 3
Effect of nutrient addition on the expression of the *gltA* and *tlc* genes

Rickettsia-infected G14 cells were grown overnight in GH (lane A) or GL medium (lane B), and the total RNA was isolated. The total RNA (used in lanes C, D, E, F, and G) was isolated at 2 hrs after adding either the nutrient mixture (Mix, lane C), glucose (Glu, lane D), sodium pyruvate (Pyr, lane E), amino acids plus vitamins (AV, lane F) or NaHCO3 (BiC, lane G) to the overnight-growth GL medium and analyzed by the ribonuclease protection assay.

Effect of nutrients in growth medium on the expression of the gltA and tlc genes

We next attempted to determine what nutrient or nutrient group in this nutrient mixture was responsible for the change from the GL to the GH RNA pattern in 2 hrs. We added glucose, sodium pyruvate, mixture of amino acids and vitamins, or $NaHCO_3$, separately to the overnight-growth GL medium, incubated the cells for 2 hrs, isolated the total RNA, and analyzed the relative amount of the *gltA* and the *tlc* mRNAs. However, only the complete mixture was effective; no single nutrient or subgroup of nutrients was able to convert the GL to the GH pattern within 2 hrs (Fig. 3). The rapid response upon the addition of the nutrient mixture, but not the individual nutrient(s), indicates the complexity of the changes in the cytoplasm of the host cell (and/or rickettsia) that triggered the regulation of these rickettsial genes. The host cell cytoplasm is a compartment that complicates the study of regulation in rickettsiae: one may simply change the medium used to cultivate the host cells to effect changes in rickettsial transcription, but this action undoubtedly results in undefined changes in the host cell cytoplasm, that, in turn, modify the rickettsial cytoplasm.

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