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The *groESL* Operon of *Agrobacterium tumefaciens*: Evidence for Heat Shock-Dependent mRNA Cleavage

GIL SEGAL AND ELIORA Z. RON*

Department of Molecular Microbiology and Biotechnology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

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The heat shock response of the *groESL* operon of *Agrobacterium tumefaciens* was studied at the RNA level. The operon was found to be activated under heat shock conditions and transcribed as a polycistronic mRNA that contains the *groES* and *groEL* genes. After activation, the polycistronic mRNA appeared to be cleaved between the *groES* and *groEL* genes and formed two monocistronic mRNAs. The *groES* cleavage product appeared to be unstable and subjected to degradation, while the *groEL* cleavage product appeared to be stable and became the major mRNA representing the *groESL* operon after long periods of growth at a high temperature. The polycistronic mRNA containing the *groES* and *groEL* genes was the major mRNA representing the *groESL* operon at a low temperature, and it reappeared when the cells were returned to the lower growth temperature after heat shock induction. These findings indicate that the cleavage event is part of the heat shock regulation of the *groESL* operon in *A. tumefaciens*.

The heat shock response is a widespread phenomenon that has been found in all living cells examined (7). It is characterized by the induction of several proteins, some of which are highly conserved in evolution, especially the proteins encoded by the *groEL* (*hsp60*) and the *dnaK* (*hsp70*) genes (22, 39). Several of the heat shock proteins are also induced by other stress conditions such as exposure to ethanol or heavy metals and infection of eukaryotic cells (18, 21, 30).

The *groES* and *groEL* genes were first defined by mutations affecting the morphogenesis of several bacteriophages (39). The GroEL protein is the prokaryotic member of the Hsp60 family of chaperon proteins, which presumably mediate the correct folding of polypeptides. The GroEL protein is known to adopt a 14-mer structure in which two 7-mers are organized in rings (double "doughnut"), and the GroES protein is known to adopt a 7-mer structure. There is also genetic and biochemical evidence for protein-protein interactions between these two proteins (12, 39). Although the exact roles of the GroES and GroEL proteins in cell physiology are unknown, their corresponding genes cannot be deleted from the *Escherichia coli* genome, and it is most likely that they perform essential functions in the cells at all temperatures (8).

In *Agrobacterium tumefaciens* (29), like in many other bacteria (39), the *groESL* operon is under heat shock control. It contains the *groES* and *groEL* genes and is transcribed as a polycistronic mRNA. Genes encoded by polycistronic mRNA may undergo a second level of regulation after transcription, leading to differential expression of the genes in the operon. For bacteria, several strategies for the differential expression of such genes are known. For example, transcriptional pausing and attenuation regulate expression within the *E. coli trp* operon (38). Internal promoters and termination signals are additional factors affecting expression within an operon, as shown for the *rpsU-dnaG-rpoD* operon of *E. coli* (16). Another important mechanism in regulating expression of multiple genes encoded by operons is the differential stability of segments derived from the polycistronic mRNA. This mechanism has been shown for the *pufBLMX* operon of *Rhodobacter capsulata* (3), for the gene cluster encoding the *pap* pili of *E. coli* (1), and for others (15, 23).

Here we present data indicating the existence of a cleavage site in the *groESL* polycistronic mRNA of *A. tumefaciens*, located between the *groES* and the *groEL* genes. The cleavage event is observed during heat shock and generates two monocistronic mRNAs, one containing the *groES* gene and the other containing the *groEL* gene. The *groES* segment appears to be unstable and subject to degradation, while the *groEL* segment is stable and becomes the major mRNA of the operon during growth at a high temperature. The cleavage event and the appearance of the *groEL* mRNA as the major mRNA of the operon during growth at a high temperature may constitute an additional regulatory mechanism of the *groESL* operon during heat shock and during growth at a high temperature.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. tumefaciens* C58 (ATCC 33970) was used for the preparation of RNA and for conjugations. *E. coli* MC1022 [*araD139* Δ(*ara leu*)*7697* Δ(*lacZ*)M15 *galU galK strA*] (5) was used for transformations. *E. coli* 71-18 [F′ *lacI*^{q} Δ(*lacZ*)M15 *proAB*/Δ*lac-proAB thi supE*] (37) was used for RNA preparation and for M13 manipulations. *E. coli* SM-10 (*thi thr leu su*$_{III}$) (31) was used for conjugations. Plasmid pKT200 (4) was used for construction of the *E. coli* probes, and plasmid pGS-AG3a (29) was used for construction of the *A. tumefaciens* probes. The plant cloning vector pPCV-702 (11) and the cloning vector pHG-165 (32) were used for construction of the shuttle vector pGS-SV1. pPCV-702 was digested with *Eco*RI and *Hin*dIII; after the two sites were filled in, the *Hpa*II-*Hpa*II fragment containing the *lacZ′* α-complementation cassette from the cloning vector pHG-165 was cloned into it, to generate pGS-SV1 (pGS-SV1 contains the origin of replication of pPCV-702). pGS-SV1 was used as a shuttle vector between *E. coli* and *A. tumefaciens*. The cloning vector pUC18 (37) was used for the cloning stages described in the legend to Fig. 5.

Bacterial conjugation. Cultures of donor (*E. coli* SM-10) and recipient (*A. tumefaciens* C58 [ATCC 33970]) bacteria were grown in Luria broth (17) overnight. A total of 0.25 ml of each culture was mixed, and the bacteria were concentrated on a 0.22-μm-pore-size, 13-mm-diameter nitrocellulose filter that was placed on a Luria broth agar plate and incubated at 25°C overnight. The bacteria were resuspended in Davis minimal medium (17), and proper dilutions were plated on Davis minimal medium agar plates containing the appropriate antibiotics. The antibiotics concentrations used for *A. tumefaciens* were carbenicillin, 100 μg/ml; gentamicin, 50 μg/ml; and kanamycin, 50 μg/ml. For *E. coli*, ampicillin was used instead of carbenicillin, in the same concentration.

Preparation of RNA. Cell pellets were obtained from 50-ml cultures of *A. tumefaciens* C58 or from 25 ml of *E. coli* 71-18. Both cultures were growing

* Corresponding author. Phone: 972-3-6409379. Fax: 972-3-6429377. Electronic mail address: eliora@ccsg.tau.ac.il (Internet) or Ron@taunivm (Bitnet).

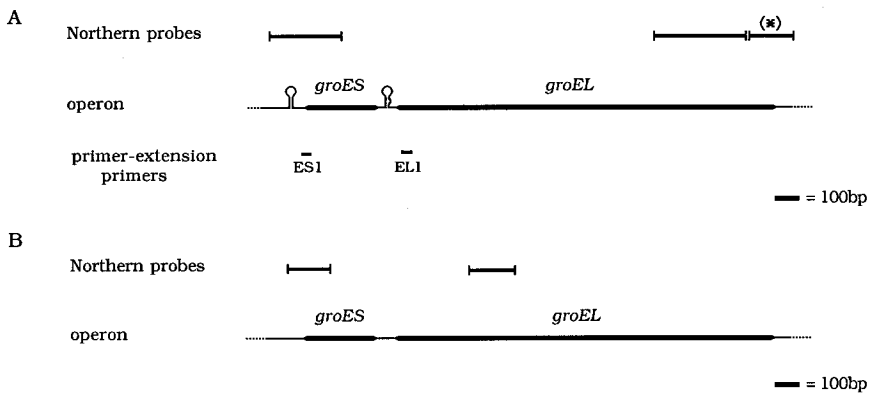

FIG. 1. Primers and probes used for the analysis of the *groESL* operon. (A) *A. tumefaciens groESL* operon. The lines above the operon indicate probes for Northern hybridization (*groES* probe, *groEL* probe, and *groEL** probe) (The asterisk above the 3′ end of the *groEL* gene marks the probe that was used for hybridization with the *groEL** gene; see Results for further explanations), and the lines under the operon indicate primer extension primers (ES1 and EL1). (B) *E. coli groESL* operon. The lines above the operon indicate probes for Northern hybridization (*coli-groES* and *coli-groEL*).

exponentially in Davis minimal medium. The treatment of the pellets, RNA blotting, and Northern (RNA) hybridization were done as previously described (29). Each lane in the Northern hybridizations contains 10 μg of RNA.

Primer extension analysis. Reverse transcriptase reactions were performed in reverse transcriptase buffer (50 mM Tris-Cl [pH 8.3], 8 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol) containing deoxynucleoside triphosphates (a 1 mM concentration [each] of dATP, dCTP, dTTP, and dGTP), RNasin (10 U), 5 pmol of labeled ([γ-^{32}P]ATP) primer, and 5 U of avian myeloblastosis virus reverse transcriptase (Molecular Biology Research, Inc., Milwaukee, Wis.). A total of 10 μg of the RNA preparation (preincubated for 10 min at 65°C) was used in a total reaction mixture volume of 6 μl. The reaction mixture was incubated for 5 min at 45°C and then incubated at 37°C for 30 min. After the addition of 4 μl of sequencing stop buffer, the samples were heated for 5 min at 90°C before being loaded on a standard sequencing gel. The reference nucleotide sequence was generated from the single-stranded M13 template, using the same primer.

Probes for Northern hybridization and primers for primer extension. The probes used for hybridization with the *A. tumefaciens groESL* operon were constructed from the plasmid pGS-AG3a (29). The *groEL* probe was a 418-bp *Pst*I-*Pst*I fragment (positions 1648 to 2065 of the sequence [29]). The *groES* probe was a 306-bp *Bst*EII-*Bss*HII fragment (positions 19 to 324 of the sequence [29]). The *groEL** probe was an ~200-bp fragment generated by a *Pst*I site (position 2065 of the sequence [29]) and a downstream *Ava*II site. The *groEL** probe can hybridize with the chromosomal *groEL* gene, as well as with the *groEL** gene on the plasmid (see Results for further explanations). The probes used for hybridization with the *E. coli groESL* operon were constructed from the plasmid pKT200 (4). The *coli-groEL* probe was a 181-bp *Asp*718-*Asp*718 fragment (positions 736 to 916 of the sequence [10]). The *coli-groES* probe was a 169-bp *Bst*EII-*Pst*I fragment (positions 58 to 226 of the sequence [10]). The oligonucleotides used for primers in the reactions and sequencing were the ES1 primer, 5′-TGCCTAATCCCTCGATC-3′ (positions 162 to 178 in the sequence), and the EL1 primer, 5′-CAGCATCTTTTCGCGCG-3′ (positions 586 to 602 in the sequence). The probes and primers for *A. tumefaciens* and *E. coli* are shown in Fig. 1.

DNA sequencing. The sequence was determined by the dideoxy chain termination method (27), using the Sequenase version II sequencing kit from United States Biochemicals.

RESULTS

Northern blot analysis of the *A. tumefaciens groESL* mRNA after heat shock. In a previous paper (29), Northern analysis of RNA derived from *A. tumefaciens* before and after heat shock demonstrated a large increase in the mRNA level of the *groEL* gene after heat shock. There were two major bands, 1.7 and 2.1 kb in size, that hybridized with the *groEL* probe. In order to further investigate the nature of these two bands we used two probes, one specific for the *groES* gene (*groES* probe) and one specific for the *groEL* gene (*groEL* probe), as shown in Fig. 1A. These two probes were hybridized to total RNA extracted from *A. tumefaciens* cells before and at different times after heat shock, as presented in Fig. 2A. The hybridization pattern was different with the two probes: hybridization with the *groEL* probe displayed the two bands, at 2.1 and 1.7 kb. The level of the 2.1-kb band increased for 10 min and then decreased until it almost disappeared after 30 min of heat shock, while the level of the 1.7-kb band increased more slowly (in comparison with that of the 2.1-kb band) and remained in large amounts after 20 and 30 min of heat shock. In contrast, the *groES* probe hybridized only with the 2.1-kb band and not with the 1.7-kb band. The hybridization pattern (activation and decrease) of the two probes with the 2.1-kb band was the same during the time checked, while the hybridization with the 1.7-kb band was restricted to the *groEL* probe.

Northern blot analysis of the *E. coli groESL* mRNA after heat shock. In order to determine if the hybridization pattern of the *groESL* operon of *A. tumefaciens* is general for bacterial *groESL* operons, the *E. coli groESL* operon was examined in the same way as described for *A. tumefaciens*. The *E. coli*

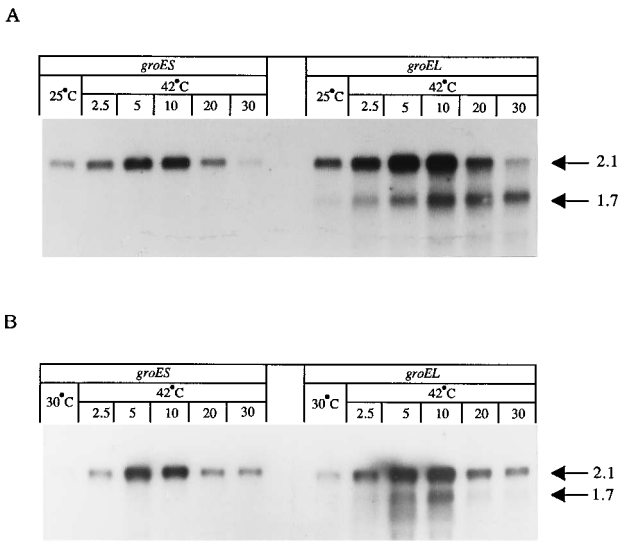

FIG. 2. Northern hybridization of the *groES* and the *groEL* mRNA. (A) Total RNA of *A. tumefaciens* was isolated before and at different times (2.5, 5, 10, 20, and 30 min) after heat shock from 25 to 42°C. The *groES* probe was the 306-bp *Bst*EII-*Bss*HII fragment, and the *groEL* probe was the 418-bp *Pst*I-*Pst*I fragment. (B) Total RNA of *E. coli* was isolated from cells grown at 30°C and treated as for panel A. The *coli-groES* probe was the 169-bp *Bst*EII-*Pst*I fragment, and the *coli-groEL* probe was the 181-bp *Asp*718-*Asp*718 fragment.

probes *coli-groES* and *coli-groEL* are shown in Fig. 1B. In the Northern analysis results presented in Fig. 2B, the two probes were hybridized to total RNA extracted from *E. coli* cells before and at different times after heat shock. The results indicate that in *E. coli*, hybridization with the two probes showed a major band of 2.1 kb that represents the polycistronic mRNA containing the *groES* and *groEL* genes. In the hybridization with the *coli-groEL* probe, there was a minor (in comparison with the 2.1-kb band) second band, in the size of 1.7 kb, that reflected in level the 2.1-kb band. This 1.7-kb band does not become the major mRNA of the operon after 20 or 30 min of heat shock, as was observed for the 1.7-kb band in *A. tumefaciens*. In the *E. coli* system, after 30 min of heat shock, there was a decrease in the level of the 2.1-kb polycistronic mRNA as well as of the 1.7-kb band. Although having a different kinetics of accumulation, the appearance of the 1.7-kb band in the *E. coli* operon may indicate that this phenomenon is conserved in *groE*SL operons.

Localization of the 5′ end of the 1.7-kb *groEL* transcript. In order to determine the 5′ end of the 1.7-kb *groEL* transcript, we used the EL1 primer located at the N-terminal end of the *groEL* gene for primer extension analysis and compared the results with those of the ES1 primer located at the N-terminal end of the *groES* gene (the primers are shown in Fig. 1A). Results of the primer extension analysis of these two primers with total RNA prepared from *A. tumefaciens* cells before and at different times after heat shock are presented in Fig. 3. The site of transcription initiation of the *groESL* operon (Fig. 3B) was the same as previously established using another primer (29), with heat shock induction reaching its maximum level 10 min after induction. The 5′ end of the *groEL* transcript (Fig. 3A) was located 27 bases after the *groES* stop codon and 49 bases before the *groEL* start codon, at base 503 in the sequence (29), and in the following sections we will regard it as the cleavage site. The RNA sequence between the *groES* and the *groEL* genes, the putative stem-loop structure located in this region, and the cleavage site are shown in Fig. 3C, and it can be seen that the *groEL* 1.7-kb fragment generated by the cleavage contains a stem-loop structure in its 5′ end. The *groES* fragment also contains a stem-loop structure at its 5′ end; this is the stem-loop structure located at the initiation site of the polycistronic transcript.

Analysis of the *A. tumefaciens groESL* mRNA after long periods at a high temperature. In order to investigate the heat shock response of the *groESL* operon after long periods at a high temperature, total RNA was extracted from *A. tumefaciens* cells and subjected to Northern analysis and primer extensions (Fig. 4). The Northern analysis (Fig. 4A) indicated that at 45 and 60 min after heat shock induction, the 2.1-kb band was absent and the 1.7-kb band was present at a low level, similar to the level of the 2.1-kb band before heat shock induction. In the primer extension with the ES1 primer (Fig. 4B), there was a product after 45 and 60 min at 42°C representing the start point of the *groESL* operon. The amount of this primer extension product appears very similar to that found in cells growing at 25°C (before heat shock induction) and represents the transcription level of the operon at high temperatures. The observation that the promoter located upstream of the *groES* gene was active at the same time when the 2.1-kb band could not be detected in the cell could be explained if the 2.1-kb polycistronic mRNA was formed but was cleaved to generate the 1.7-kb band containing the *groEL* gene.

When primer extension was performed with the EL1 primer (Fig. 4C), the amount of the cleavage product (representing the 1.7-kb band) increased until 45 min after the heat shock induction, and there was a slight decrease at 60 min after the

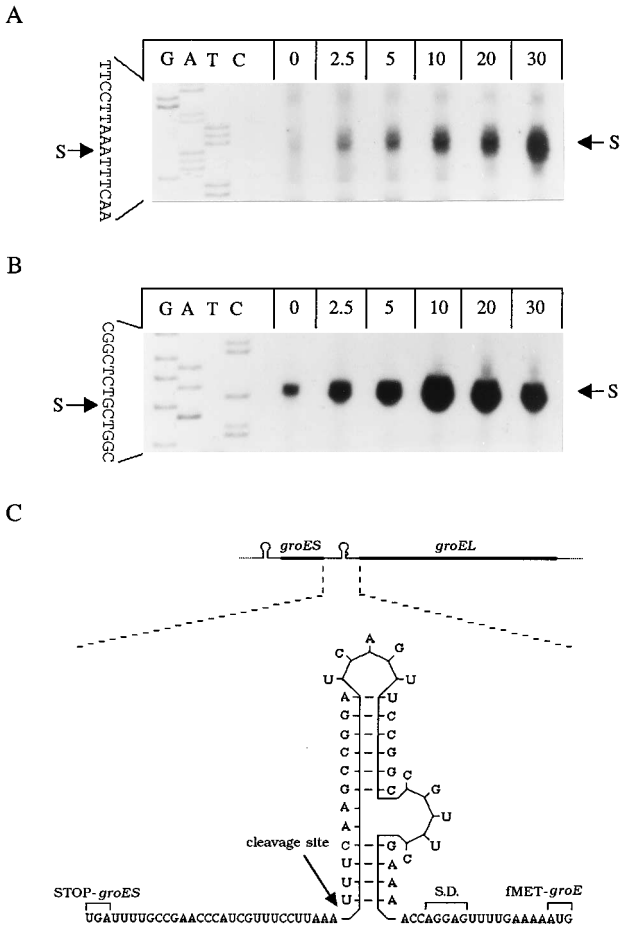

FIG. 3. Cleavage site between the *groES* and the *groEL* genes of *A. tumefaciens*. Total RNA (10 μg) from *A. tumefaciens* was hybridized to the EL1 primer (A) to determine the cleavage site or to the ES1 primer (B) to determine the 5′ end of the operon. The RNA was isolated before (0) and at different times (2.5, 5, 10, 20, and 30 min) after heat shock from 25 to 42°C. The primer extension products were analyzed on a sequencing gel. Lanes G, A, T, and C, products of the sequencing reaction obtained with the same primer. The sequence presented is of the sense strand. The arrow at S points to the base representing the 5′ end of the mRNA. (C) Putative stem-loop structure located in the region between the *groES* and the *groEL* genes of *A. tumefaciens*. The cleavage site that was determined by primer extension, the *groES* stop codon, the *groEL* start codon, and the putative *groEL* ribosomal binding site (S.D.) are marked.

induction. These results indicate that the 1.7-kb fragment is the major mRNA of the *groESL* operon at high temperatures, but even then the promoter located at the 5′ end of the operon is active. The decrease in the amount observed for the 1.7-kb band after 45 and 60 min in heat shock (Fig. 4A) was observed in the primer extension with the EL1 primer (Fig. 4C) only 60 min after heat shock induction. This apparent delay in the decrease was probably due to annealing of the primer to degradation products of the 1.7-kb band still present in the cell 45 min after the induction.

Distinction between mRNA cleavage and a second initiation site in the intergenic region of the *groESL* operon. All the results presented so far can be explained by mRNA cleavage, but in order to eliminate completely the alternative possibility of a second initiation site between the *groES* and the *groEL* genes, the system presented in Fig. 5 was constructed. This system is based on comparing two plasmids, one containing the promoter of the operon and the other without it. If there is a second promoter between the *groES* and the *groEL* genes, this

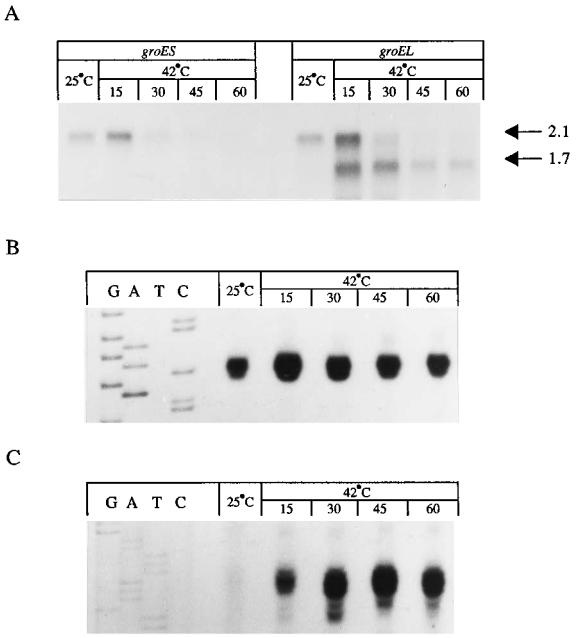

FIG. 4. Analysis of the *A. tumefaciens groESL* mRNA after long periods at a high temperature. Total RNA of *A. tumefaciens* was isolated before and at different times (15, 30, 45, and 60 min) after heat shock from 25 to 42°C. (A) Northern hybridization of the *groES* and the *groEL* mRNAs. (B) Primer extension analysis with the ES1 primer. The primer extension products were analyzed on a sequencing gel. Lanes G, A, T, and C, products of the sequencing reaction obtained by using the same primer. (C) The same as for panel B, but with the EL1 primer.

promoter should not be affected by the deletion of the promoter located at the 5′ end of the operon, as they are 410 bp apart. However, if there is a cleavage site between the *groES* and the *groEL* genes, the cleavage event would not take place if the polycistronic mRNA isn't synthesized from the plasmid.

In order to distinguish between the chromosomal *groESL* operon and the plasmid *groESL* operon, a 1.4-kb internal fragment was deleted from the plasmid *groEL* gene to generate the plasmid pGS-AG-22-S harboring the *groEL** gene (Fig. 5A, stage b). As a result, the *groESL** operon has an mRNA smaller than that of the chromosomal *groESL* operon and can be distinguished on a Northern blot. The plasmid pGS-AG-22-S was introduced into *A. tumefaciens*, and after it was confirmed that the 1.4-kb deletion had no effect on heat shock activation or on the cleavage event, a deletion of a 113-bp fragment that contains the promoter region and the stem-loop structure of the *groESL** operon was made, to generate the plasmid pGS-AG-22-S-D (Fig. 5A, stage c). By using plasmids pGS-SV-22-S and pGS-SV-22-S-D (Fig. 5B), it was then possible to distinguish between the existence of a second promoter and that of a cleavage site.

Total RNA was extracted before and at different times (5, 10, and 20 min) after heat shock induction, from *A. tumefaciens* cells containing plasmid pGS-SV-22-S or pGS-SV-22-S-D, and was hybridized with the *groEL** probe (Fig. 6). In the Northern hybridization with cells carrying the plasmid pGS-SV-22-S, the heat shock activation of the 0.7-kb band (the equivalent of the 2.1-kb band), as well as the appearance of the 0.3-kb band (the equivalent of the 1.7-kb band) generated by the cleavage event, was observed. Neither band was observed in cells carrying plasmid pGS-SV-22-S-D, indicating that both bands depended on the existence of the *groESL* operon promoter. The heat shock activation, as well as the cleavage product of the chromosomal *groESL* operon, was not affected by the presence of the plasmids.

In order to exclude the possibility that plasmid pGS-SV-22-S-D is unstable and therefore no hybridization could be detected with this plasmid, a part of the ampicillin resistance gene of plasmid pGS-SV1 was used as a control probe for Northern hybridization, and in addition viable counts were performed at the end of the experiment. The results obtained indicated that the plasmid pGS-SV-22-S-D was present in the cells during the experiment at essentially the same level as in the plasmid without the deletion (pGS-SV-22-S).

Effect of a translation inhibitor on the cleavage event. The heat shock response of the *groESL* operon of *A. tumefaciens* was studied with a translation inhibitor (tetracycline) (Fig. 7). When the translation inhibitor was added 7.5 min after the heat shock induction (Fig. 7B), two differences are seen in comparison with the results of Fig. 7A. (i) The presence of the 1.7-kb band was abolished; instead of having a maximum level 15 min after the heat shock induction, its amount decreased and it was almost absent 15 min after the induction. (ii) The polycistronic mRNA was present in large amounts for a longer period of time (maximum at 12.5 min in Fig. 7B, in contrast to 7.5 min in Fig. 7A) after the heat shock induction. There are several ways to explain the inhibition of cleavage of the polycistronic mRNA by the translation inhibitor, as will be pointed out in Discussion.

Analysis of the *A. tumefaciens groESL* mRNA after heat shock activation and return of the cells to 25°C. Total RNA was derived from *A. tumefaciens* cells that were heat shocked for 30 min and then returned to 25°C for 30 min. The Northern hybridization and primer extension analysis of RNAs extracted 15 and 30 min after the heat shock induction and 15 and 30 min after the cells were returned to 25°C (45 and 60 min, respectively, after the heat shock induction) are presented in Fig. 8. From a comparison of Fig. 8A and 4A, it is clear that 15 min after the cells were returned to 25°C, the polycistronic mRNA (the 2.1-kb band) had reappeared (Fig. 8A), while it was absent when the cells kept at 42°C (Fig. 4A). From the comparison of Fig. 8B and 4B, it can be seen that there was no difference in the pattern of the primer extension product generated with the ES1 primer, and there was no increase in the level of the product representing the 5′ end of the *groESL* operon after the cells were returned to 25°C. These results indicate that no activation of the *groESL* operon promoter was needed in order for the 2.1-kb band to reappear, and the reappearance of the polycistronic mRNA (2.1-kb band) probably occurred because the cleavage event stopped (or slowed down) at 25°C and the newly transcribed polycistronic mRNA was not cleaved. From comparing Fig. 8C and 4C, it can be seen that there was a decrease in the primer extension product with the EL1 primer 30 min after the cells were returned to 25°C. The delay between the appearance of the 2.1-kb band in the Northern hybridization (Fig. 8A), seen 15 min after the cells were returned to 25°C, and the decrease in the primer extension product with the EL1 primer (Fig. 8C), seen 30 min after the cells were returned to 25°C, probably reflects the annealing of the EL1 primer to degradation products of the 1.7-kb band that were still present 15 min after the cells were returned to 25°C. At any rate, the results of the Northern hybridizations and of the primer extensions indicate that the cleavage event stopped after the cells returned to 25°C.

DISCUSSION

The results presented in this paper provide evidence for the existence of site-specific cleavage in the *groESL* polycistronic mRNA of *A. tumefaciens* during heat shock. The cleavage site

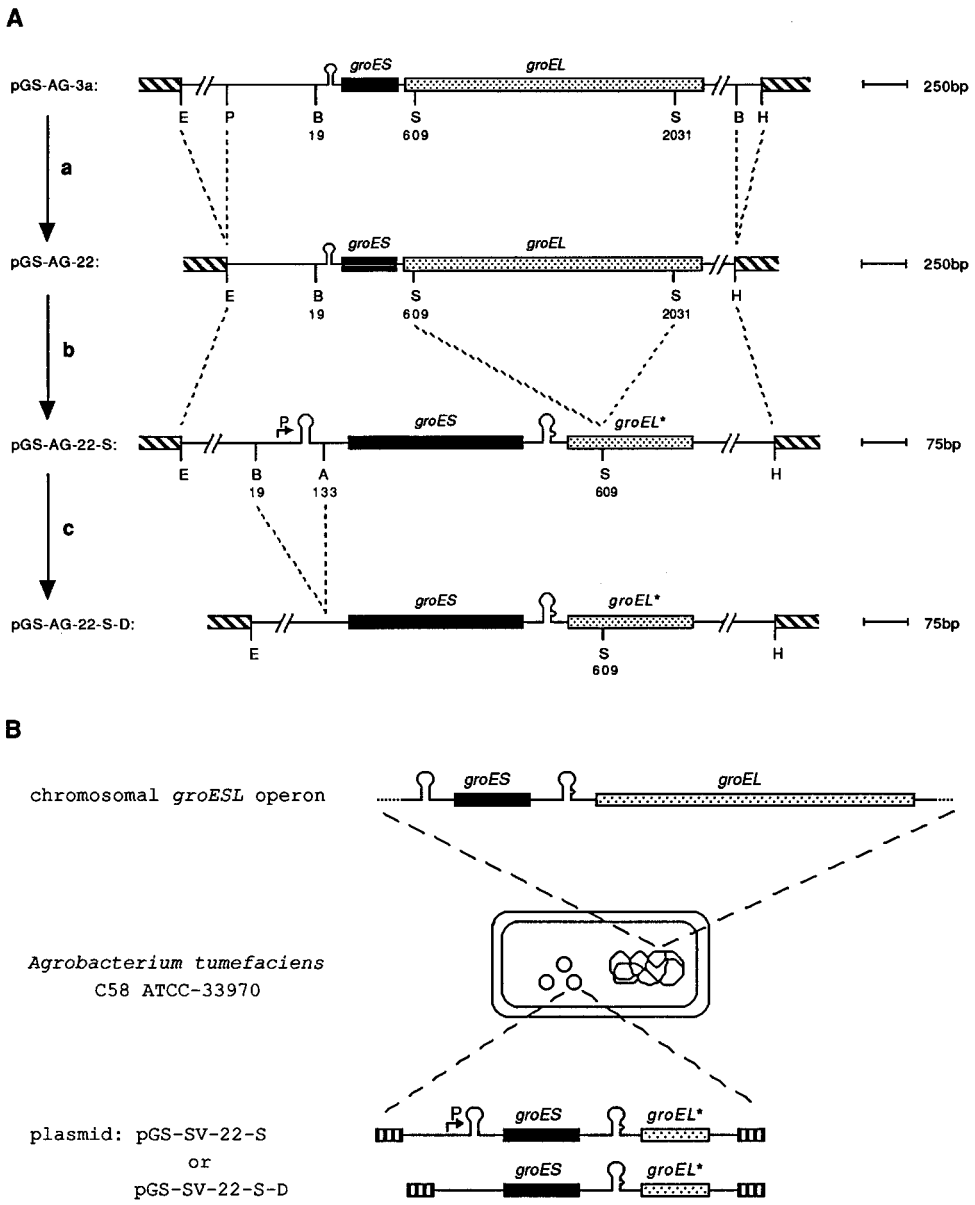

FIG. 5. System constructed in order to distinguish between a cleavage event and a second initiation between the *groES* and *groEL* genes. (A) Stages of plasmid manipulation. Stage a: plasmid pGS-AG-3a was digested with *Pvu*II and *Bst*EII (partial), and after the *Bst*EII site was filled in, the 3.6-kb fragment was cloned into the *Hin*dII site of pUC18, to generate pGS-AG-22. Stage b: the plasmid pGS-AG-22 was digested with *Sal*I, and the 1,422-bp internal fragment of the *groEL* gene was deleted by self-ligation, to generate pGS-AG-22-S. Stage c: plasmid pGS-AG-22-S was digested with *Bst*EII and *Ava*II (partial) and self-ligated after the two sites were filled in, to generate pGS-AG-22-S-D. The inserts of the plasmids pGS-AG-22-D and pGS-AG-22-D-S were cloned into pGS-SV1 by using the *Eco*RI and *Hin*dIII sites of pUC18, and the plasmids pGS-SV-22-S and pGS-SV-22-S-D were generated (stage not shown). (B) System in *A. tumefaciens* cells. The plasmid pGS-SV-22-S or pGS-SV-22-S-D was conjugated into *A. tumefaciens*, and the situation in the cells is schematically illustrated. ▨, pUC18; ■, *groES*; ▩, *groEL*; ▥, pGS-SV1. Restriction enzymes: A, *Ava*II; B, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; and S, *Sal*I. The letter P above the bent arrow indicates the promoter region and the transcription start site. *groEL**, truncated *groEL* gene that was generated after the deletion of the 1.4-kb *Sal*I fragment.

was located in the intergenic region between the *groES* and *groEL* genes and generates two monocistronic mRNAs. The *groEL* segment is stable and becomes the major mRNA of the operon after long periods at high temperatures, while the *groES* segment is unstable and probably subject to degradation.

The cleavage of the *groESL* polycistronic mRNA that occurs during heat shock appears to be a secondary process after the activation of the operon. Figures 2, 3, and 4 present Northern blots and primer extension analysis of the *groESL* mRNA of *A. tumefaciens* before (25°C) and at different times after heat shock induction (42°C). In the Northern blots (Fig. 2A and 4A), the 2.1-kb band, representing the polycistronic mRNA containing the *groES* and *groEL* genes, was the major mRNA of the *A. tumefaciens groESL* operon present in the cells before heat shock. This band was heat shock activated, reached its highest level 10 min after induction, declined 20 min after the heat shock induction, and was absent 45 and 60 min after the induction. The *groEL* cleavage product (the 1.7-kb band) was either absent or very weak before heat shock, and after heat shock activation it appeared more slowly than the 2.1-kb band,

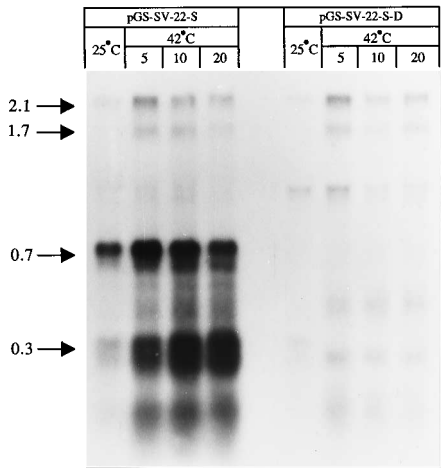

FIG. 6. Northern hybridization of RNA from *A. tumefaciens* cells containing the plasmids pGS-SV-22-S and pGS-SV-22-S-D. Total RNA was isolated from *A. tumefaciens* before and at different times (5, 10, and 20 min) after heat shock from 25 to 42°C. The hybridization was done with the ~200-bp *Pst*I-*Ava*II fragment *groEL** probe.

reaching its maximum level 15 min after the induction. The 1.7-kb band becomes the only mRNA representing the *groESL* operon that could be detected in the cells after a long time (45 and 60 min) at 42°C, and its level was similar to that of the 2.1-kb band before heat shock. These observations agree with the results of the primer extension with the ES1 primer, in which the amounts of the primer extension product before heat shock induction and 45 and 60 min after heat shock activation were also similar. Our assumption is that the lack of the cleavage product during vegetative growth, the delay between the heat shock activation and the cleavage event, and the reappearance of the polycistronic mRNA after the cells were returned to 25°C reflect a heat shock-dependent cleavage event that begins a few minutes after the heat shock induction and stops after the cells are returned to 25°C.

The cleavage event is expected to generate two products,

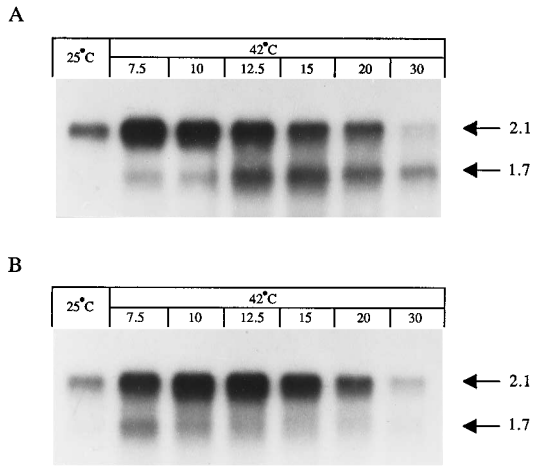

FIG. 7. Northern hybridization of the *groEL* mRNA. Total RNA of *A. tumefaciens* was isolated before and at different times (7.5, 10, 12.5, 15, 20, and 30 min) after heat shock from 25 to 42°C and hybridized to the *groEL* probe. (A) Without the addition of inhibitor. (B) The translation inhibitor tetracycline (12.5 μg/ml) was added 7.5 min after heat shock induction.

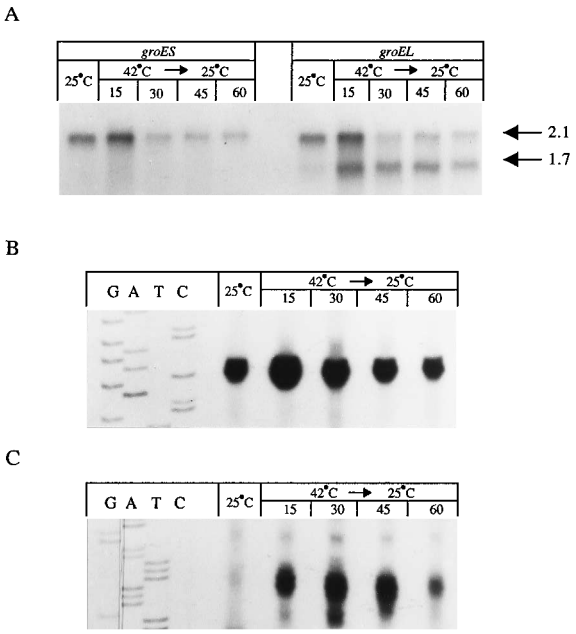

FIG. 8. Analysis of the *A. tumefaciens groESL* mRNA after long periods at a high temperature and return of the cells to 25°C. Total RNA of *A. tumefaciens* was isolated before and at different times (15, 30, 45, and 60 min) after heat shock from 25 to 42°C, and after 30 min at 42°C, the cells were returned to 25°C. The time points 45 and 60 min indicate 15 and 30 min, respectively, after the cells were returned to 25°C. (A) Northern hybridization of the *groES* and the *groEL* mRNA. (B) Primer extension analysis with the ES1 primer under the same conditions as for panel A. The primer extension products were analyzed on a sequencing gel. Lanes G, A, T, and C, products of the sequencing reaction obtained by using the same primer. (C) The same as for panel B, but with the EL1 primer.

one with the size of 1.7 kb (representing the *groEL* gene) and the second with a size of 0.4 kb (representing the *groES* gene), that we weren't able to detect. This fact suggests an alternative explanation for the appearance of the 1.7-kb band, namely, the existence of a second initiation site located between the *groES* and *groEL* genes rather than a cleavage of the polycistronic mRNA. However, the possibility of a second initiation site was ruled out, as the shorter mRNA (cleavage product) could not be observed after the 5′ *groESL* operon promoter was removed (Fig. 6). Another possibility that wasn't ruled out is a mechanism that assumes the existence of a downstream promoter whose function depends on the upstream promoter or its expression. This possibility appears unlikely, as the 5′ ends of the two mRNAs are more than 400 bp apart.

A similar mRNA processing event was demonstrated for the Pap pili of *E. coli* (1). This operon contains (in this order) the *papB*, *papA*, *papH*, *papC*, and *papD* genes, the promoter of the operon is located at the 5′ end of the *papB* gene, and there is a cleavage site between the *papB* and *papA* genes. The two segments resulting from the cleavage are (i) the 5′ end of the polycistronic mRNA that contains the *papB* gene, which is very unstable and degraded rapidly, and (ii) the 3′ end of the polycistronic mRNA that contains the *papAHCD* genes and which is further cleaved to generate the *papA* transcript, which is very stable and accumulates after the induction of the operon. The pattern of the mRNA cleavage at different times after induction is similar to that observed for the *groESL* operon of *A. tumefaciens*. In both cases, the 5′ segment is a short segment, unstable, and subjected to degradation and the 3′ segment is long and stable. There are additional genes in which differen-

tial stability of polycistronic mRNA was shown to play a role in the regulation of gene expression (3, 15, 23), but the *A. tumefaciens groESL* operon is the first one in which there is differential stability of mRNA during heat shock. As presented in the results, we examined the *E. coli groESL* operon and found out that this operon also contains two *groEL* transcripts: one contains the *groES* and *groEL* genes (2.1 kb), and the other contains only the *groEL* gene (1.7 kb). Although the *A. tumefaciens* operon has a kinetics of accumulation different from that of *E. coli*, the appearance of the 1.7-kb band in the *E. coli* operon may indicate that this phenomenon is conserved in *groE* operons.

The results presented in this paper demonstrate several features regarding the cleavage event. The cleavage hardly occurs at 25°C, as the major mRNA of the *groESL* operon detected in cells growing at 25°C was the polycistronic mRNA (Fig. 2A and 4). At 42°C there is an activation of the cleavage that stops when the cells are returned to 25°C (Fig. 8). These results can be explained by assuming that the cleavage is performed by a heat shock-activated endoribonuclease that presumably binds to the stem-loop structure. Alternatively, it could be an autocatalytic process generated by the stem-loop structure. If an endo-RNase is involved, it probably has a very short life span, even at high temperatures, as cleavage stopped after the addition of a translation inhibitor (Fig. 7B). Another possibility is that the tetracycline-induced ribosomal stalling interferes with the endonuclease cleavage. In the case of an autocatalytic process, the inhibition of the cleavage by tetracycline would probably be due to a conformational change in the mRNA stem-loop that is located 3 bases from the ribosomal binding site (Shine-Dalgarno sequence).

The role of this heat shock-dependent cleavage event as a part of the *A. tumefaciens* heat shock regulation is not known, but its involvement in the regulation of the *groESL* operon during heat shock may represent its role in other heat shock-dependent operons. Moreover, such a cleavage event could be important in the regulation of operons that are not heat shock activated but that have their mRNA cleaved under heat shock conditions, resulting in differential gene expression within a polycistronic operon.

As we have previously indicated, the DNA dyad symmetry element (stem-loop structure) located at the 5′ end of the *groESL* operon of *A. tumefaciens* is conserved in evolution and was found in the regulatory region of heat shock genes in a variety of phylogenetically distant bacteria: gram-positive bacteria (13, 19, 20, 25, 28, 29, 33, 35), cyanobacteria (6, 34), chlamydia (18), spirochetes (2), and several proteobacteria (9, 14, 26) including *A. tumefaciens* (29). The mechanism of transcriptional activation and the factor (or factors) operating are not known yet, but the involvement of the stem-loop structure in heat shock activation has already been established for *Lactococcus lactis* (33) and *Bacillus subtilis* (40). The additional, putative, stem-loop structure located between the *groES* and *groEL* genes (shown in Fig. 3C) may serve as a recognition site for an endonuclease that cleaves the polycistronic mRNA or for an autocatalytic process.

There exist at least two other ways in phylogenetically distant bacteria (24, 36) that could lead to a differential expression of the *groES* and *groEL* genes. In *Mycobacterium leprae* (a gram-positive bacterium) and in other mycobacteria (25), there are two *groEL* genes, and only one of them is arranged in an operon with a *groES* gene. In *M. leprae* both the *groESL* operon and the *groEL* gene contain the stem-loop structure, which may be involved in heat shock activation, at their 5′ ends. As this stem-loop structure is present in the gene and also in the operon, both of them are supposed to be activated under heat shock conditions and to give rise to a ratio other than 1:1 between the *groES* and *groEL* genes if no other regulation is involved. In *Leptospira interrogans* (a spirochete) (2), a second way for differential expression of the *groES* and *groEL* genes was found. In this case there is a second promoter, located in the middle of the *groES* gene, which transcribes the *groEL* gene. This promoter is in addition to the promoter of the operon, which is located upstream to the *groES* gene and near the conserved stem-loop structure presumably involved in heat shock activation. In this bacterium, like in *A. tumefaciens* (a proteobacterium), two transcripts are found after heat shock: one of the polycistronic mRNA and one of the *groEL* gene.

The cleavage event that was described for *A. tumefaciens* and the two other mechanisms described here can result in differential expression of the *groES* and *groEL* genes. All three mechanisms presumably give a ratio other than 1:1 between the *groEL* and the *groES* gene products and a larger amount of the *groEL* gene product. This observation is in agreement with the model in which the GroES and GroEL proteins catalyze their chaperonin reaction in a ratio of 1:2: the *groEL* as two multimers (7-mers) and the *groES* as one multimer (a 7-mer) (39).

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