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 subject 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*.

**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 alanA leu27897 Δ(lacZ)M15 gatI galK strA] (3) was used for transformations. *E. coli* 71-18 [F' lacIΔ(lacZ)M15 proABlac-proAB thi supE] (37) was used for RNA preparation and for M13 manipulations. *E. coli* SM-10 (thi thr leu supA) (31) was used for conjugations. Plasmid pKT200 (4) 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 *E. coli* probes, and plasmid pGS-AO3a (29) was used for construction of the *A. tumefaciens* probes. The plant cloning vector pPCV-702 (11) and the cloning vector pHS-1 (28) was used for construction of the 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...
RESULTS

Northern blot analysis of the A. tumefaciens groEL 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 groEL mRNA after heat shock. In order to determine if the hybridization pattern of the groEL operon of A. tumefaciens is general for bacterial groEL operons, the E. coli groEL operon was examined in the same way as described for A. tumefaciens. The E. coli probe displayed the two bands, at 2.1 and 1.7 kb.
probes \textit{coli}-groES and \textit{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 \textit{E. coli} cells before and at different times after heat shock. The results indicate that in \textit{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 \textit{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 \textit{A. tumefaciens}. In the \textit{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 \textit{E. coli} operon may indicate that this phenomenon is conserved in \textit{groESL} operons.

Localisation 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 \textit{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 \textit{groEL} 1.7-kb fragment generated by the cleavage contains a stem-loop structure in its 5' end. The \textit{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 \textit{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 \textit{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 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
Northern hybridization of the groES 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 ESI primer. The primer extension products were analyzed on a sequencing gel. Lanes 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 ESI 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
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.
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 amount 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, 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 pilus 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-
tional stability of polycistronic mRNA was shown to play a role in
the regulation of gene expression (3, 15, 23), but the A. tumefac-
iens 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. tumefaci-
iens 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 fea-
tures 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 auto-
catalytic 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 addi-
tion of a translation inhibitor (Fig. 7B). Another possibility is
that the tetracycline-induced ribosomal stalling interferences 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 bac-
teria (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 tran-
scriptional 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 Lacto-
coccus 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 dis-
tant bacteria (24, 36) that could lead to a differential expres-
sion 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 reg-
ulation 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 differ-
cential 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 (1 mer)
(39).

ACKNOWLEDGMENTS

This work was supported by the Israel Science Foundation. G.S. was
supported by the Clore Scholars program.

We thank J. Kuhin for providing plasmid pHG-165.

REFERENCES

mRNA with differential stability in the regulation of E. coli pilin gene
Adler. 1993. Molecular analysis of the hsp (gro) operon of Leptospira
1985. Differential expression of photosynthetic genes in R. capsulatus results
from segmental differences in stability within the polycistronic transcript.
two chaperon proteins of the cyanobacterium Synechocystis sp. PCC 6803. J.
heat shock gene products of Escherichia coli are essential for bacterial
tion, and functional expression in Escherichia coli chaperonin (groEL)
genes from the phototrophic sulfur bacterium Chromatium vinosum. J. Bacte-
riol. 175:1514–1523.
10. Henningsen, S. M., C. Woolford, S. M. van der Vies, K. Tilby, D. T. Dennis,
and bacterial proteins chaperon oligomeric protein assembly. Nature (Lon-
don) 333:330–337.
and J. Schell. 1989. High-frequency T-DNA-mediated gene tagging in
and GroE stress protein(s) and analysis of protein folding or macromolecular
shock response in Brucella abortus and isolation of the genes encoding
Zymomonas mobilis glf-czw-edd-glk operon is subjected to complex transcript