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The RpoH-Mediated Stress Response in *Neisseria gonorrhoeae* Is Regulated at the Level of Activity

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The general stress response in *Neisseria gonorrhoeae* was investigated. Transcriptional analyses of the genes encoding the molecular chaperones DnaK, DnaJ, and GrpE suggested that they are transcribed from σ^{32} (RpoH)-dependent promoters upon exposure to stress. This was confirmed by mutational analysis of the σ^{32} promoter of *dnaK*. The gene encoding the gonococcal RpoH sigma factor appears to be essential, as we could not isolate viable mutants. Deletion of an unusually long *rpoH* leader sequence resulted in elevated levels of transcription, suggesting that this region is involved in negative regulation of RpoH expression during normal growth. Transcriptional analyses and protein studies determined that regulation of the RpoH-mediated stress response is different from that observed in most other species, in which regulation occurs predominantly at the transcriptional and translational levels. We suggest that an increase in the activity of preformed RpoH is primarily responsible for induction of the stress response in *N. gonorrhoeae*.

When exposed to environmental stress, bacteria respond by rapidly increasing synthesis of a characteristic set of proteins that allows them to contend with the adverse environment, meanwhile down-regulating the expression of many housekeeping genes. Elevated temperatures have been widely used as a convenient means to study stress responses, and as a result, proteins induced upon increased temperature have been termed heat shock proteins (Hsps). There is strong evidence to suggest that the signal that induces the heat shock response is the accumulation of misfolded and denatured proteins that arise with stress (15).

Many of these stress-induced proteins are chaperones and proteases. Their biological role is to protect cells against the toxic effects generated by exposure to stress, but they also have an important role in protein function during normal growth conditions. The increase in production of these proteins following exposure to stress allows the bacteria to respond to the elevated level of misfolded proteins. The chaperones function to eliminate misfolded proteins in numerous ways, including (i) unfolding these proteins and subsequently promoting proper folding and (ii) targeting unfolded proteins for proteolysis (20). The best known of the chaperones are GroEL/GroES and members of the DnaK chaperone system, including DnaK, DnaJ, and GrpE. The DnaK chaperone system is well characterized in *Escherichia coli*, where it is the most abundant cytosolic chaperone system and cannot be replaced in vivo (30). These proteins are of particular interest in that in some species they are also involved in regulation of the stress response (14, 47). Induction of specific bacterial genes with stress is most often regulated at the transcriptional level (53), with the mechanisms controlling this induction varying greatly between species.

The alternative sigma factor RpoH is often used to regulate the stress response and has been identified in bacteria from different subdivisions of proteobacteria (36). The majority of species respond to stress by increasing the level of RpoH produced, using positive and/or negative regulatory processes. The positive regulation of *rpoH* expression in the alpha subdivision seems to occur primarily at the transcriptional and posttranslational levels. The *rpoH* genes from *Caulobacter crescentus* (51), *Rhodobacter capsulatus* (10), and *Agrobacterium tumefaciens* (33) are positively autoregulated from an RpoH-dependent promoter upon heat shock. In *C. crescentus*, down-regulation of the stress response is independent of DnaK and RpoH (9). In *A. tumefaciens*, DnaK-dependent regulation of RpoH has also been shown to occur at the level of activity (34). Some bacteria of this subdivision contain more than one *rpoH* gene, with each being regulated by different mechanisms (36).

In the majority of the organisms in the gamma subdivision of proteobacteria, regulation of *rpoH* occurs primarily by translational repression involving a thermosensitive secondary structure in the *rpoH* mRNA (55). Among the members of this group of bacteria studied, only *Haemophilus influenzae* (12) and *Buchnera aphidicola* (40) appear to lack this *rpoH* mRNA secondary structure. An additional regulatory process is the negative regulation of RpoH levels by the DnaK chaperone system. During nonstress conditions RpoH interacts with DnaK (14), and together with DnaJ and GrpE, RpoH is targeted to proteases for degradation (23). Upon exposure to heat shock DnaK is sequestered by misfolded proteins such that RpoH function is restored (14). Regulation of *rpoH* in most of these species can also occur, although to a minor extent, at the transcriptional level by way of different promoters which respond to various signals (11).

Other species negatively regulate heat shock genes at the transcriptional level by the controlling inverted repeat of chaperone expression (CIRCE)/HrcA repressor system (57). These

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include the gram-positive *Enterococcus faecium* (45) and *Streptococcus pyogenes* (50). The stress-responsive genes, such as *dnaK* and *groE*, are transcribed from conventional σ^{70} promoters, but their expression is modulated by GroE-dependent binding of the HrcA repressor protein to the CIRCE element (54). This system is also found in some gram-negative bacteria, including *Helicobacter pylori*, a member of the epsilon subdivision of the proteobacteria (46), and *Chlamydia* species (49). Only the *groE* operon of the alpha subdivision of the proteobacteria retains a CIRCE operator sequence (54). These operons are transcribed from a σ^{32} promoter, and the HrcA/CIRCE control system seems to act to repress *groE* transcription in nonstress conditions (39). An exception to this is *R. capsulatus*, which has a CIRCE element upstream of the *groE* operon but lacks the HrcA protein. It is suggested that the role of the CIRCE element in this species is to stabilize the *groE* mRNA (22).

Neisseria belongs to the beta subdivision of the proteobacteria. Homologues of the *rpoH*, *dnaK*, and *groE* genes have been identified in members of this subdivision. There have been limited investigations into the nature of the stress response in these species despite the evidence that molecular chaperones induce an immune response (24) and have a role in disease pathogenesis (37). Recent work on the heat shock response of *Neisseria meningitidis* using microarray technology suggested that the majority of genes are deregulated only at 45°C (16) rather than at 42°C as shown for *Neisseria gonorrhoeae* (48). Cloning and sequence analysis of the gonococcal *groES* and *groEL* homologues revealed that they were organized in a bicistronic operon, an arrangement similar to that found in most bacterial species (48). Transcription of these genes occurs from a σ^{70} promoter under nonstress conditions, and an elevated level of transcription occurs from an overlapping σ^{32} promoter following exposure to stress (48).

Here we report the transcriptional analysis of the genes encoding members of the gonococcal DnaK chaperone system and the RpoH sigma factor. We show that transcription of each of the genes encoding the chaperones is induced upon exposure of the gonococci to heat stress, that this transcription is mediated by RpoH, and that it is predominantly regulated at the level of RpoH activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *E. coli* strain used in all cloning experiments was DH5α [F^- *endA1 thi-1 hsdR17 supE44 relA1 ΔlacU169* (ϕ80 Δ*lacZ*M15)]. The *N. gonorrhoeae* strain used was MS11-A (41). JKD484 is a spontaneous rifampin-resistant mutant derivative of MS11-A which contains the conjugative plasmid p*tet*M25.2 (25). Gonococcal strains JKD487, JKD488, JKD489, JKD491, JKD492, and JKD493 were derived from JKD484. The growth conditions for *E. coli* and *N. gonorrhoeae* have been described previously (13).

Transformation and conjugation of *N. gonorrhoeae*. Transformation of *N. gonorrhoeae* was performed essentially as described previously (5). Erythromycin-resistant transformants from recombination between DNA from the Hermes-2 *E. coli* and *N. gonorrhoeae* shuttle plasmid and the p*tet*M25.2 conjugative plasmid (25) were selected on GC agar plates containing 7 μg of erythromycin/ml. Conjugations were performed by mixing 5×10^8 donor cells and 5×10^9 recipient cells on a small section of a GC agar plate which was incubated overnight at 37°C in a 5% CO_2 atmosphere. The growth was transferred into 500 μl of GC broth, and 100-μl aliquots were spread onto GC agar plates containing 7 μg of erythromycin and 10 μg of nalidixic acid/ml for selection of transconjugants.

TABLE 1. Oligonucleotide primers used in this study

Oligonucleotide	Sequence (5′→3′)	Reference or Source
3260	CACACTGGGACTGAGACATG	6
3261	CGGCAGTCTCATTAGAGTGC	6
4527	CATATTGACCCTAGCCGC	This study
5490	CGCCATTCCCATCATGCG	This study
5492	CAAGTGCCGATTTATGCG	This study
5493	TTGGATGGCGGGTAATGC	This study
5494	TCGGTAGCTGCTCTTGCC	This study
5495	TTTCCTACTGTCTCGACG	This study
5608	GTACCCTATTTCCAAACG	This study
5609	CGGCTTTGAACATGGACG	This study
5610	TGCCAGAGGTCGAAACCG	This study
5611	CGGCATACGGGTTGACCG	This study
6034	TCATCGAGTCTTCACACG	This study
6035	ACAAGAGTTGGTTGTACC	This study
7070	CAGGATGAGTTGTTTGGC	This study
7071	ACCGCCGATACGCAGTTTCAGCC	This study
7078	CGGCGGGCTGTTTCCCGCTACAGCATGGC	This study
7079	GCCATGCTGTAGCGGGAAACAGCCCGCCG	This study
7080	CGATGGGCTGTAAATCTGGCGGGCGGCGGG	This study
7081	CCCGCCGCCCGCCAGATTTACAGCCCATCG	This study
7082	TGTAAACCTGATAGCTCAATTCG	This study

Recombinant DNA techniques and RNA analysis. The techniques used were performed as described previously (13). Oligonucleotide primers used are listed in Table 1. Plasmids used are listed in Table 2. Total RNA was prepared from exponentially growing cultures of *E. coli* and *N. gonorrhoeae* as described previously (13). The methods used for RNA dot blot and primer extension analysis have been described previously (13). Probes used were 16S rRNA, an 0.83-kb PCR product amplified from *N. gonorrhoeae* MS11-A using oligonucleotide primers 3260 and 3261 (to confirm equivalent amounts of RNA); *dnaK*, a 1.37-kb HindIII/ClaI fragment from pJKD1926; *dnaJ*, a 0.53-kb DraI/ClaI fragment from pJKD2107; *grpE*, a 0.95-kb PCR product amplified from pJKD2108 using oligonucleotide primers 5610 and 5611; and *rpoH*, a 0.59-kb HincII/StuI fragment from pJKD2101.

Determination of CAT levels. Cell extracts required for enzyme assays were prepared from exponentially growing cultures, the protein concentration was determined, and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (13).

Western blotting. Western blotting was performed as described previously (27). The dilution of the polyclonal antibody raised against *E. coli* RpoH antiserum was 1/4,000. The first antibody was detected using peroxidase-conjugated anti-rabbit immunoglobulin at a 1/3,000 dilution.

RESULTS

Identification of the *dnak*, *dnaJ*, and *grpE* genes of *N. gonorrhoeae*. A BLAST analysis (1) of the *N. gonorrhoeae* strain FA1090 (GenBank accession number AE004969) genome database revealed regions where the derived amino acid sequence displayed significant similarity with that of the *E. coli* DnaK/Hsp70 (3), DnaJ/Hsp40 (4), and GrpE (28) proteins (72.4, 59.1, and 34.3% amino acid sequence identity, respectively [data not shown]). Each of these predicted gonococcal proteins has the domains and amino acid residues shown to be important for its function. Each gene appears to form an independent transcriptional unit, unlike the equivalent genes in other species (43). Each gene was amplified or cloned from *N. gonorrhoeae* MS11-A (Table 2).

Transcription of the *dnak*, *dnaJ*, and *grpE* genes of *N. gonorrhoeae* is induced upon exposure to heat shock. RNA dot blot hybridization was employed to determine whether transcription of the gonococcal *dnaK*, *dnaJ*, and *grpE* genes was induced when cells were exposed to heat shock. Probes for each of the genes are described in Materials and Methods.

TABLE 2. Plasmids used in this study

Plasmid	Description	Reference or source
Hermes-2	*E. coli*/*N. gonorrhoeae* shuttle vector	25
pKK232-8	Promoter selection vector	7
pSU2718	Chloramphenicol-resistant cloning vector	29
pUC18	Ampicillin-resistant cloning vector	52
pJKD1595	Hermes-2 containing a promoterless *cat* gene	5
pJKD1926	Clone from a *N. gonorrhoeae* MS11-A Sau3A1 library in pUC18 (*dnaK*)	17
pJKD2101	2.37-kb PCR product amplified from *N. gonorrhoeae* MS11-A genomic DNA with oligonucleotide primers 5490 and 5492, filled in, and inserted into HincII-digested pSU2718 (*rpoH*)	This study
pJKD2107	1.5-kb PCR product amplified from *N. gonorrhoeae* MS11-A genomic DNA with primers 5608 and 5609, filled in, and inserted into HincII-digested pUC18 (*dnaJ*)	This study
pJKD2108	0.95-kb PCR product amplified from *N. gonorrhoeae* MS11-A genomic DNA with primers 5610 and 5611, filled in, and inserted into HincII-digested pSU2718 (*grpE*)	This study
pJKD2122	1.1-kb cassette containing *recA* promoter::*cat* transcriptional fusion inserted into HincII pJKD2101; same orientation as *rpoH*	This study
pJKD2124	1.1-kb cassette containing *recA* promoter::*cat* transcriptional fusion inserted into HincII pJKD2101; opposite orientation to *rpoH*	This study
pJKD2238	0.33-kb PCR product amplified from *N. gonorrhoeae* MS11-A genomic DNA with oligonucleotide primers 6034 and 6035 (Fig. 2A), filled in, and inserted into HincII-digested pUC18 (wild-type *dnaK* upstream region)	This study
pJKD2239	PCR fusion product amplified with oligonucleotide primers 6034 and 6035 using the PCR products amplified from pJKD2238 with oligonucleotide primer combinations 6034–7080 and 6035–7081 (Fig. 2A) as templates; 0.33-kb product was filled in and inserted into HincII-digested pUC18 (mutated *dnaK* −10 region)	This study
pJKD2240	PCR fusion product amplified with oligonucleotide primers 6034 and 6035 using the PCR products amplified from pJKD2238 with oligonucleotide primer combinations 6034–7078 and 6035–7079 (Fig. 2A) as templates, the 0.33-kb product was filled in and inserted into HincII-digested pUC18 (mutated *dnaK* −35 region)	This study
pJKD2266	0.33-kb BamHI-HindIII fragment of pJKD2239 inserted into pKK232-8	This study
pJKD2267	0.33-kb BamHI-HindIII fragment of pJKD2240 inserted into pKK232-8	This study
pJKD2268	0.48-kb PCR product amplified from *N. gonorrhoeae* MS11-A genomic DNA with oligonucleotide primers 4527 and 7070 (Fig. 4), filled in, and inserted into HincII-digested pUC18 (wild-type *rpoH* upstream region)	This study
pJKD2270	PCR fusion product amplified with oligonucleotide primers 4527 and 7070 using the PCR products amplified from pJKD2101 with oligonucleotide primer combinations 4527-7082 and 7070-7071 (Fig. 4) as templates; the 0.36-kb product was filled in and inserted into HincII-digested pSU2718 (deleted *rpoH* upstream region)	This study
pJKD2273	0.33-kb BamHI-HindIII fragment of pJKD2238 inserted into pKK232-8	This study
pJKD2282	Fragment from pJKD2273 containing wild-type *dnaK* promoter::*cat* transcriptional fusion in Hermes-2	This study
pJKD2283	Fragment from pJKD2266 containing mutated *dnaK* −10 promoter::*cat* transcriptional fusion in Hermes-2	This study
pJKD2284	Fragment from pJKD2267 containing mutated *dnaK* −35 promoter::*cat* transcriptional fusion in Hermes-2	This study
pJKD2319	0.48-kb BamHI-HindIII fragment of pJKD2268 inserted into pKK232-8	This study
pJKD2320	0.36-kb BamHI-HindIII fragment of pJKD2270 inserted into pKK232-8	This study
pJKD2325	Fragment from pJKD2319 containing wild-type *rpoH* promoter::*cat* transcriptional fusion in Hermes-2	This study
pJKD2326	Fragment from pJKD2320 containing deleted *rpoH* promoter::*cat* transcriptional fusion in Hermes-2	This study

Total RNA was extracted from cultures of *N. gonorrhoeae* MS11-A following their exposure to the higher temperature of 42°C for various times. A substantial increase in the level of *dnaK*-, *dnaJ*-, and *grpE*-specific transcripts was observed following exposure to heat shock at 42°C for 10 min (Fig. 1A), indicating that transcription of these genes is induced upon exposure to stress. The amount of transcripts continued to increase slightly with longer exposure to a 42°C environment. The detection of transcripts at 37°C indicates that the proposed σ^{32}-dependent promoters for *dnaK*, *dnaJ*, and *grpE* are functional under nonstress conditions.

To confirm induction of transcription of these genes, and to locate the promoters responsible for their transcription, the transcriptional start points (TSPs) were mapped by primer extension using the same RNA preparations. Oligonucleotide primers 6035 (Fig. 2A; Table 1), 5494 (Fig. 2B; Table 1), and 5495 (Fig. 2C; Table 1) were used to identify the TSPs of *dnaK*, *dnaJ*, and *grpE*, respectively, and were also used in sequencing reactions. Primer extension products were obtained for all the genes at 37°C; however, the signals were greatly intensified with a shift to 42°C (Fig. 1B). In each case, the primer extension product obtained increased in intensity with continued exposure to heat shock. The TSP for *dnaK* under physiological and stress conditions was mapped to an A residue situated 61 bp upstream of the putative initiation codon (Fig. 1B and 2A). The TSP for *dnaJ* under stress and nonstress conditions mapped to a G residue and increased with a thermal upshift (Fig. 1B and 2B). Transcription of *grpE* was initiated from an A residue 42 bp upstream of the putative start codon (Fig. 1B and 2C). The sequence upstream of each stress-induced TSP displayed similarity to the −10 and −35 regions of σ^{32}-dependent promoters (Fig. 2) (8).

***dnaK* is transcribed from a σ^{32} promoter.** To verify that the putative σ^{32} promoters were responsible for the increased transcription observed under stress conditions, site-directed mutagenesis of the promoter upstream of *dnaK* was per-

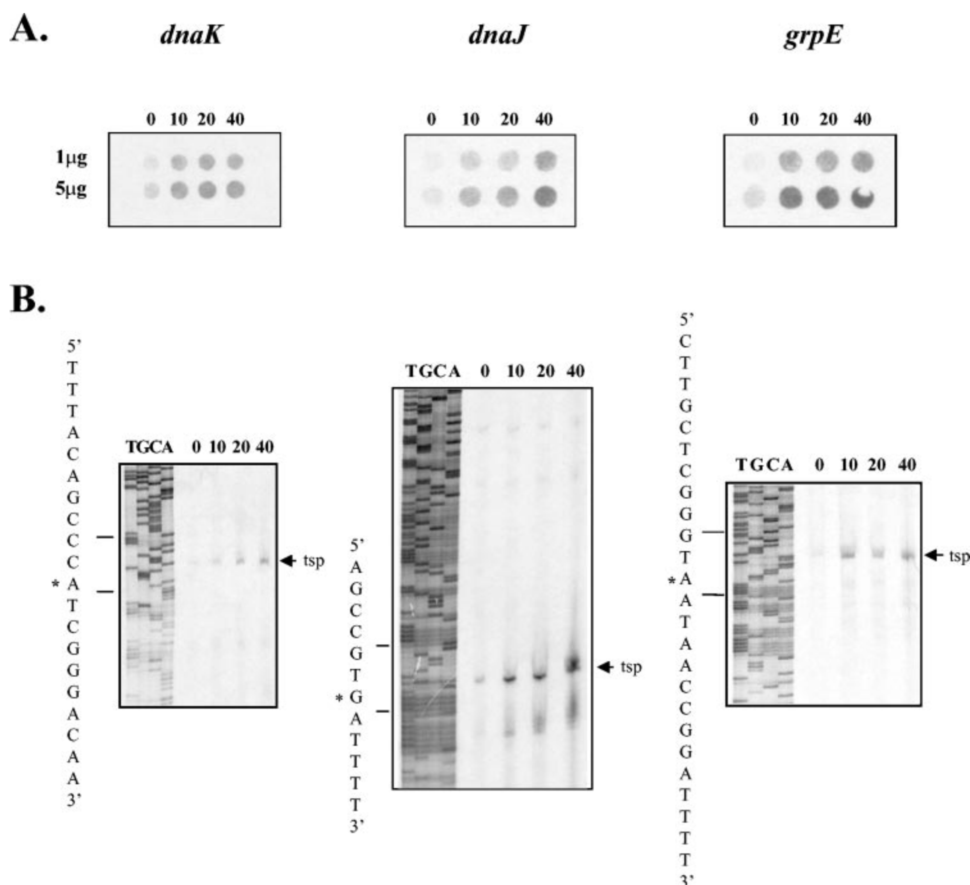

FIG. 1. Induction of the gonococcal *dnaK*, *dnaJ*, and *grpE* genes upon exposure to heat shock. (A) RNA dot blot hybridizations of RNA extracted from cells exposed to heat shock at 42°C for the number of minutes indicated above each panel. The amount of RNA (μg) transferred to the membranes is indicated to the left. Filters were probed with the gonococcal *dnaK*, *dnaJ*, and *grpE* genes as indicated. (B) Primer extension analysis of the promoter regions of these genes in *N. gonorrhoeae* MS11-A. Total RNA (50 μg per lane) was extracted from cells that had been heat shocked at 42°C for the number of minutes indicated above each lane. Primer extension products obtained are indicated by arrows. Sequencing ladders adjacent to the primer extension reactions are marked TGCA. The TSPs are indicated by the asterisks on the sequences at the left of each panel and shown in Fig. 2.

formed. The nucleotide changes made to bases within the −10 and −35 regions (Fig. 2) were designed to render RpoH incapable of recognizing the promoter. The wild-type, mutated −10, and mutated −35 promoter regions were fused to a promoterless *cat* gene and introduced into *N. gonorrhoeae* JKD484 using plasmids pJKD2282, pJKD2283, and pJKD2284 (Table 2), generating strains JKD491, JKD492, and JKD493, respectively. As a negative control, a promoterless *cat* gene was also introduced into this background using plasmid pJKD1595 (Table 2) to give strain JKD487.

Exponentially growing cultures of the gonococcal strains containing the promoter::*cat* transcriptional fusions were subjected to heat shock at 42°C for 20 min. Cell extracts of the cultures were prepared, and CAT levels were determined (Fig. 3). Basal levels of CAT were obtained for the negative control strain JKD487. As expected, much higher levels of CAT were obtained for strain JKD491, which contains the wild-type *dnaK* promoter region and establishes the activity of the σ^{32}-dependent promoter in this background. The CAT levels obtained for strains JKD492 and JKD493, which contain the mutated −10 and −35 boxes, respectively, were greatly reduced, approximately 17- and 80-fold, respectively. These results indicate that the putative σ^{32}-dependent promoter is responsible for transcription of the *dnaK* gene and the bases mutated in the −35 region have a more pronounced effect on sigma binding than those mutated in the −10 region (Fig. 2A).

Identification and nucleotide sequence analysis of the *rpoH* gene from *N. gonorrhoeae*. A BLAST search (1) of the *N. gonorrhoeae* strain FA1090 (GenBank accession number AE004969) genome database with the *E. coli* RpoH amino acid sequence (26) indicated the presence of an *rpoH* homologue in *N. gonorrhoeae* (50% amino acid similarity [data not shown]). Based on flanking FA1090 sequences, oligonucleotide primer pair 5490–5492 (Table 1) was designed to amplify *rpoH* and flanking regions from *N. gonorrhoeae* MS11-A genomic DNA by PCR. Flanking regions were included to enable subsequent construction of an *rpoH* mutant (see below). Analysis of the gonococcal RpoH sequence indicates that proposed functional domains and residues have been conserved. These sequences include regions required for promoter recognition and binding to RNA polymerase (32, 35). *rpoH* was cloned from *N. gonorrhoeae* MS11-A (Table 2).

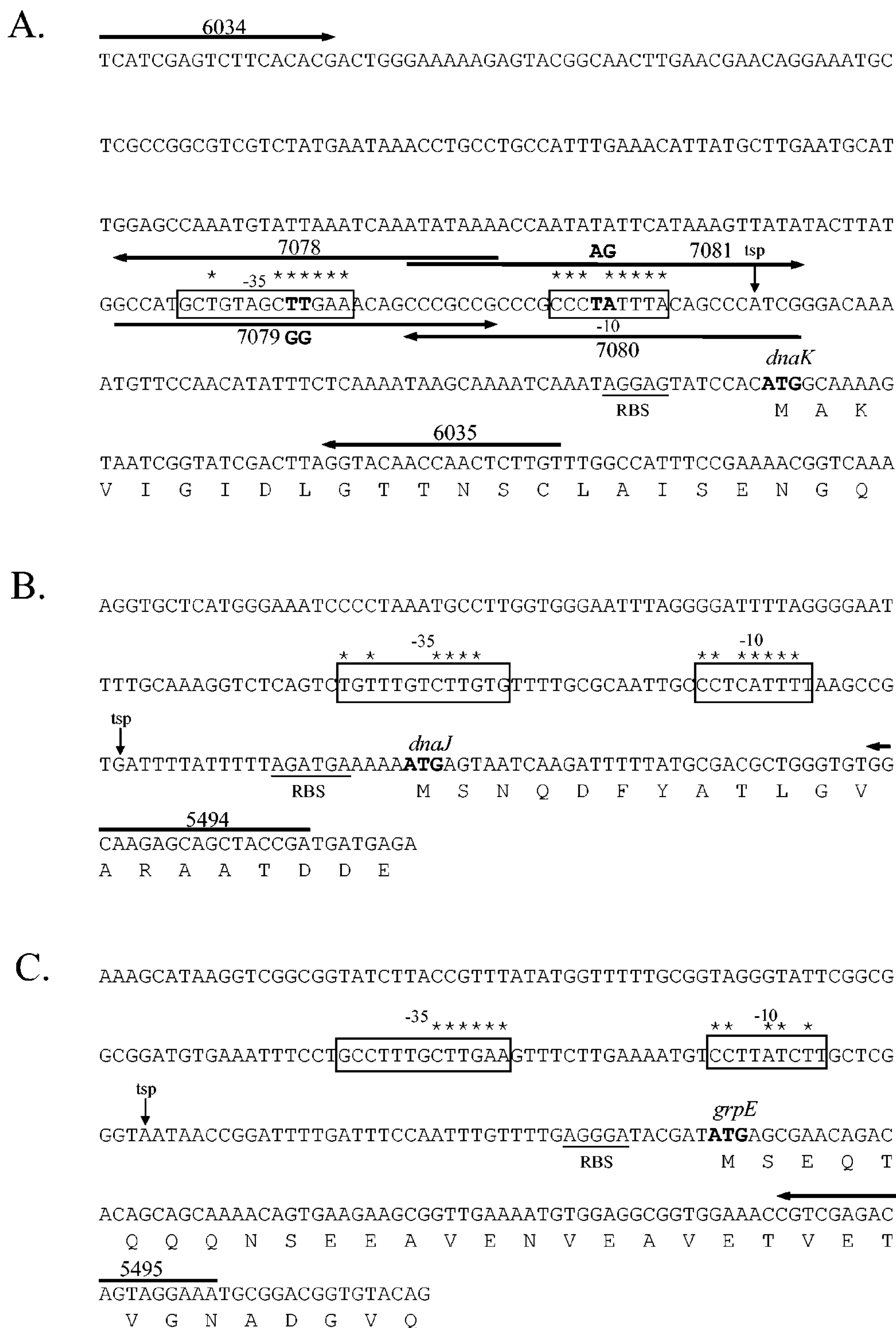

FIG. 2. Nucleotide sequences of the promoter regions of the *dnaK* (A), *dnaJ* (B), and *grpE* (C) genes from *N. gonorrhoeae*. The initiation codon for each gene is shown in boldface. The deduced amino acid sequences of the proteins are shown beneath the corresponding nucleotide sequences. Putative ribosome binding sites (RBS) are underlined. Oligonucleotide primers are indicated by numbered arrows. The σ^{32} promoter sequences are boxed (−35 and −10), and asterisks indicate nucleotides identical to those in the consensus sequence (8). In panel A, boldface letters in the −35 and −10 boxes indicate those nucleotides changed in site-directed mutagenesis experiments. The introduced changes are indicated above or below the boxes. Downward-pointing arrows represent TSPs determined by primer extension analysis (Fig. 1).

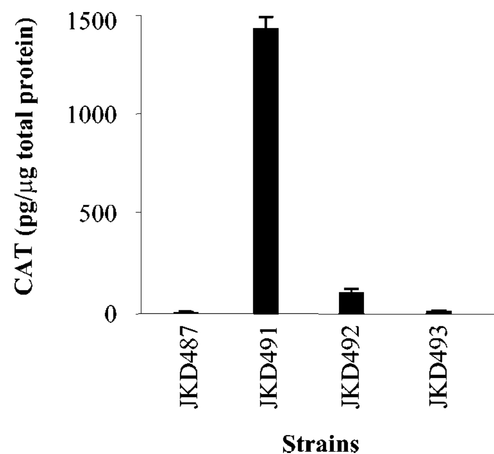

FIG. 3. Transcriptional analysis of the gonococcal *dnaK* upstream region in *N. gonorrhoeae* strains JKD491, JKD492, and JKD493, which contain promoter::*cat* transcriptional fusions integrated into the conjugative plasmid p*tet*M25.2. These strains carry the wild-type *dnaK* promoter region, the mutated −10 region, and the mutated −35 region, respectively. Strain JKD487 was the negative control containing a promoterless *cat* gene. Cell extracts were prepared from strains exposed to heat shock at 42°C for 20 min. The CAT levels shown are the means of results of four separate assays. The error bars represent standard deviations.

The *rpoH* gene from *N. gonorrhoeae* appears to be essential for growth. An attempt was made to mutate the *rpoH* gene from *N. gonorrhoeae* by insertional activation with an antibiotic resistance cassette. A 1.1-kb cassette containing the gonococcal *recA* promoter fused to a promoterless *cat* gene (5) was cloned into a HincII site located 70 bp downstream of the putative translation start site in the *rpoH* gene in pJKD2101 (Fig. 4). The antibiotic resistance cassette was cloned in both orientations, giving plasmids pJKD2122 and pJKD2124 (Table 2). Each plasmid was transformed into *N. gonorrhoeae* MS11-A in an attempt to detect integration into the gonococcal chromosome by allelic exchange at the *rpoH* locus. As an *E. coli rpoH* mutant was temperature sensitive (56), plates were incubated at 30 and 37°C to determine whether this phenotype applied to the gonococcal mutant. No chloramphenicol-resistant transformants were obtained for cultures incubated at 30°C. Only a few chloramphenicol-resistant transformants were recovered from those cultures incubated at 37°C. Southern hybridization suggested that these transformants resulted from either random integration into sites other than the *rpoH* gene or a single crossover event, resulting in both an inactivated and an intact copy of the *rpoH* gene. These results strongly suggest that the *rpoH* gene of *N. gonorrhoeae* may be essential for growth at 30 and 37°C.

The *rpoH* gene from *N. gonorrhoeae* is transcribed from a σ^{70} promoter under steady-state and stress conditions. RNA dot blot hybridization was used to determine the transcriptional levels of *rpoH* after heat shock. Total RNA was extracted from an exponentially growing culture of *N. gonorrhoeae* MS11-A following exposure to heat shock at 42°C. The *rpoH*-specific mRNA increased upon heat shock, but only after 20 min, and became much more intense after 40 min of exposure (Fig. 5A), indicating that transcription of *rpoH* is induced by stress. The delayed increase in the level of *rpoH* mRNA

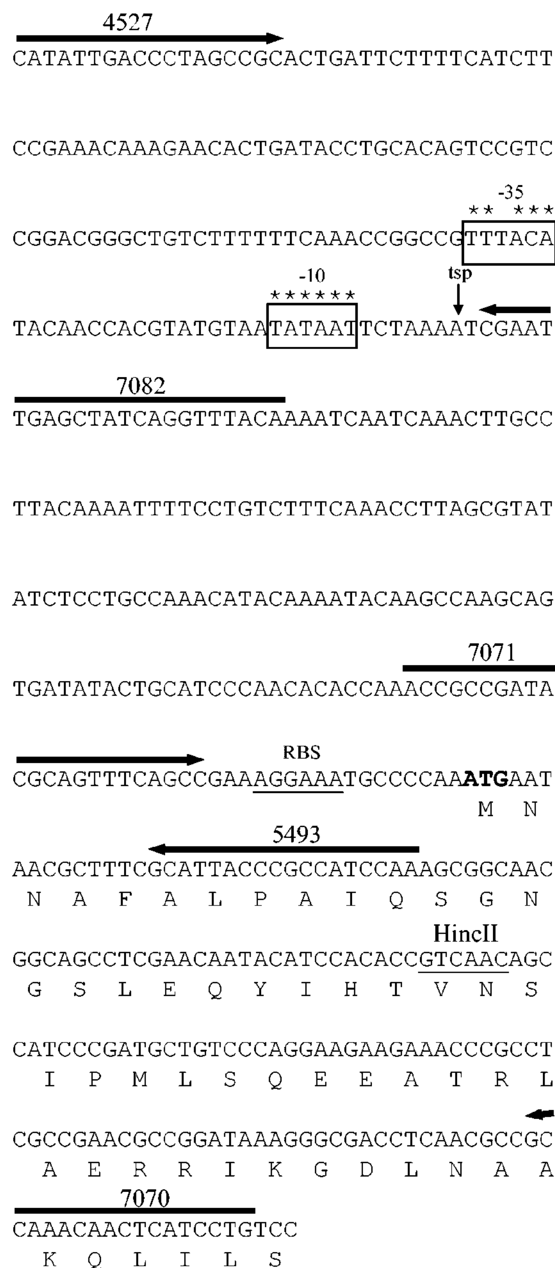

FIG. 4. Nucleotide sequence of the promoter region of the *rpoH* gene from *N. gonorrhoeae*. The ATG initiation codon is shown in boldface. The deduced amino acid sequence of the gene is shown beneath the corresponding nucleotide sequences. The putative ribosome binding site (RBS) is underlined. Oligonucleotide primers are indicated by the numbered arrows. The σ^{70} promoter sequences (−35 and −10) are boxed, and asterisks indicate nucleotides identical to those in the consensus sequence (18). The TSP is indicated by the downward-pointing arrow. The HincII site used in attempting to construct the *rpoH* mutant is indicated.

compared to *dnaK*, *dnaJ*, and *grpE* mRNA (Fig. 1) suggests that an increase in *rpoH* transcription is not responsible for the increased transcription of these genes.

To map the *rpoH* promoter, primer extension analysis was performed using the same RNA preparations. The oligonucleotide primer 5493 was used for the primer extension and se-

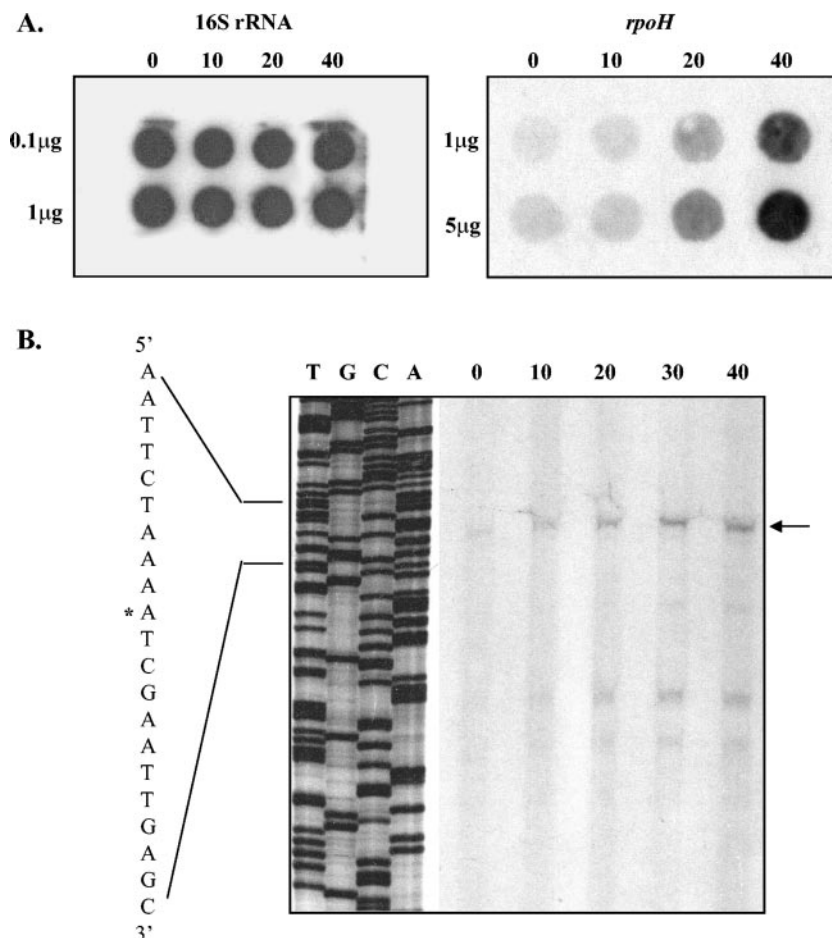

FIG. 5. Induction of the gonococcal *rpoH* gene upon exposure to heat shock. (A) RNA dot blot hybridizations of RNA extracted from cells exposed to heat shock at 42°C for the number of minutes indicated above each well. The amount of RNA (μg) transferred to the membranes is indicated to the left of each panel. Filters were probed with the gonococcal 16S rRNA and *rpoH* genes as indicated. (B) Primer extension analysis of the *rpoH* upstream region of *N. gonorrhoeae* MS11-A. Total RNA (50 μg per lane) was extracted from cells heat shocked at 42°C for the number of minutes indicated above each lane. Primer extension products obtained using oligonucleotide primer 5493 (Fig. 4) are indicated by the arrow. The sequencing ladder adjacent to the primer extension reaction lanes is marked TGCA. The TSP is indicated by the asterisk on the sequence at the left and is also marked on Fig. 4.

quencing reactions (Fig. 4). A weak product was obtained for *rpoH* at 37°C, and this signal intensified following the temperature shift (Fig. 5B). The increase in transcription was slight at 10 min and more prominent at 20 min, consistent with the RNA dot blot hybridization results (Fig. 5A). The TSP mapped to an A residue located 181 bp upstream of the putative start codon (Fig. 4). The sequence upstream of the TSP displayed strong similarity to the −10 and −35 regions of σ^{70}-dependent promoters (18). Several shorter products (Fig. 5B) appear to be artifactual, as they are not associated with consensus promoter sequences. Therefore, *N. gonorrhoeae* appears to use a single σ^{70}-dependent promoter for transcription in the absence or presence of stress conditions.

RpoH synthesis is induced by heat shock. Expression of the gonococcal RpoH protein was investigated to establish whether the rate of synthesis increases with the observed increase in transcription. Exponentially growing cells of *N. gonorrhoeae* MS11-A were heat shocked at 42°C and subsequently harvested. Cell extracts were prepared, and equivalent amounts of protein were separated on a sodium dodecyl sulfate-polyacrylamide gel. Western blot analysis was performed with a polyclonal antibody raised against *E. coli* RpoH (Fig. 6). Fortuitously, the antibody cross-reacted with several proteins in the gonococcal extracts, demonstrating an equivalent amount of protein in each sample. These proteins were not recognized by normal rabbit serum. A protein of approximately 32 kDa that was recognized by the antibody was substantially induced following incubation at 42°C. This induction seemed to occur at around 15 min and increased with prolonged exposure to heat shock. It coincided with the gradual increase in *rpoH* transcription as determined by RNA dot blot analysis and primer extension (Fig. 5). Transcription of *dnaK*, *dnaJ*, and *grpE* is induced as early as 10 min after a thermal upshift. The delayed increase in RpoH protein levels supports the suggestion that neither an increase in transcription nor an increase in translation of *rpoH* is responsible for induction of the stress genes.

RpoH synthesis does not seem to be subject to translational regulation. A search for elements involved in expression of *rpoH* in other species was performed. In the majority of gamma

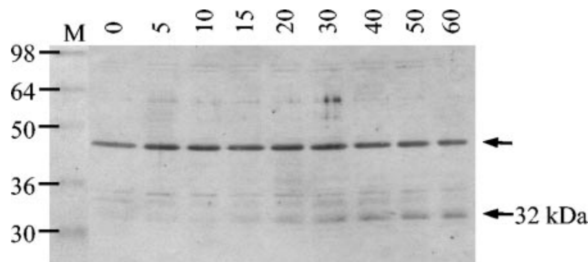

FIG. 6. Western blot analysis of *N. gonorrhoeae* MS11-A exposed to heat shock at 42°C for the number of minutes indicated above each lane and whole cell extracts subsequently prepared. Equal volumes of extract from each sample were fractionated on a 12% polyacrylamide gel electrophoresis gel. The membrane was incubated with the polyclonal antibody raised against *E. coli* RpoH. Prestained standards (in kDa) served as the markers (M). Proteins recognized by the antiserum are indicated by arrows.

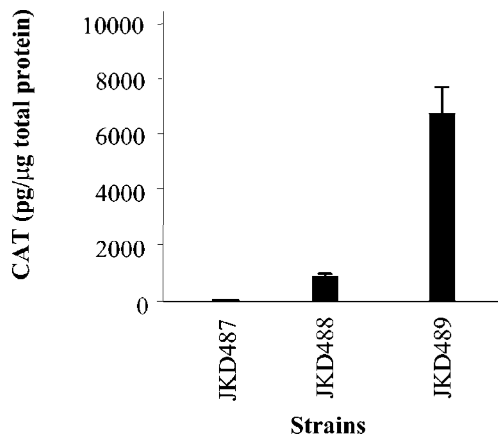

FIG. 7. Transcriptional analysis of the gonococcal *rpoH* upstream region in *N. gonorrhoeae* strains JKD488 and JKD489, which contain promoter::*cat* transcriptional fusions integrated into the conjugative plasmid p*tet*M25.2. These strains carry the wild-type *rpoH* promoter region and that with the deleted leader sequence, respectively. Strain JKD487, containing a promoterless *cat* gene, was the negative control. Cell extracts were prepared from strains exposed to heat shock at 42°C for 20 min. The CAT levels shown are the means of results from four separate assays. The error bars represent standard deviations.

proteobacteria, *rpoH* regulation seems to occur primarily at the translational level. The 5′ end of the mRNA contains a sequence that forms an internal secondary structure involving the ribosome binding site, initiation codon, and downstream 20 nucleotides, termed the downstream box, which represses *rpoH* translation at physiological temperatures due to the inaccessibility of the ribosome binding site (55). Following a temperature increase, the structure partially melts, permitting an enhanced level of translation due to the complementarity of the downstream box with 16S rRNA (31). Sequence analysis indicates that the gonococcal *rpoH* transcript lacks the ability to form such a structure, in part due to the absence of the downstream box (data not shown). This was confirmed via a prediction of the secondary structure of the gonococcal *rpoH* mRNA (data not shown) using the MULFOLD program (21) and implies that regulation of *rpoH* in the gonococcus is unlike the regulation in most gamma proteobacteria.

The leader sequence may regulate transcription of the gonococcal *rpoH* gene. The discovery that the *rpoH* gene appears to be regulated in part at the transcriptional level led to closer examination of the upstream region and a search for potential regulatory elements. An unusual finding was the presence of the relatively long leader sequence of 172 bp. To determine the transcriptional significance, if any, of the leader sequence, the sequence was deleted, and transcriptional activity from the *rpoH* promoter region in *N. gonorrhoeae* was evaluated. The wild-type and deleted promoter regions were fused to a promoterless *cat* gene and introduced into *N. gonorrhoeae* JKD484 using plasmids pJKD2325 and pJKD2326 (Table 2), generating strains JKD488 and JKD489, respectively. As a negative control, a promoterless *cat* gene was also introduced into this background by using plasmid pJKD1595 (Table 2) to give strain JKD487.

Exponentially growing cultures of the gonococcal strains containing the promoter::*cat* transcriptional fusions were subjected to heat shock at 42°C for 20 min, since *rpoH* transcription increases substantially by this time (Fig. 5). Cell extracts of the cultures were prepared, and CAT levels were determined (Fig. 7). Basal levels of CAT were obtained for the negative control strain JKD487. Deletion of the leader sequence in strain JKD489 resulted in an approximate sevenfold increase in CAT compared to that in JKD488 containing the wild-type *rpoH* upstream region, suggesting an important role for the leader sequence in the transcription of *rpoH*. Analyses of *E. coli* indicated that the same TSP is used in the deleted construct and that transcript levels from this construct are higher than those in the nondeleted construct (data not shown).

DISCUSSION

The stress responses of a number of species have been studied, and expression of the chaperones DnaK, DnaJ, and GrpE has generally been found to be regulated at the transcriptional level upon exposure to heat shock (54). We investigated the heat shock response in *N. gonorrhoeae* and characterized the genes encoding the molecular chaperones DnaK, DnaJ, and GrpE from *N. gonorrhoeae* FA1090 and MS11-A. Analysis of flanking regions indicated that in the gonococci, these genes were not arranged in an operon, an organization that is common in many species (43). It is possible that this is because different levels of *dnaK* and *dnaJ* expression are required in *N. gonorrhoeae*. However, this end result has been achieved with *E. coli* because of the infrequent translation initiation of *dnaJ* mRNA (4, 44). To confirm induction of these genes upon heat shock, RNA dot blot hybridization and primer extension analyses were employed (Fig. 1). All three genes were significantly induced upon exposure to heat stress. A similar result had previously been obtained for the gonococcal *groE* operon (48).

There appear to be two primary groups of transcriptional regulatory strategies employed by bacteria to regulate the *dnaK* chaperone system. The majority of gram-positive bacteria and several gram-negative bacteria utilize a negative control mechanism in which stress exposure displaces a repressor protein bound to a CIRCE element located near the promoter. This element is highly conserved among these species but absent from *N. gonorrhoeae*. Several species of gram-negative bacteria have adopted a positive mode of regulation of the

dnaK operon where the alternative sigma factor, RpoH, directs transcription under stress (12, 32, 38). Tauschek et al. (48) demonstrated that transcription of the gonococcal *groE* operon during stress is initiated from a σ^{32} promoter.

Primer extension analysis of *dnaK*, *dnaJ*, and *grpE* revealed that transcription under nonstress and heat shock conditions emanated from promoters with similarity to the -10 and -35 regions of σ^{32} promoters (Fig. 2). Alignment of the consensus sequences for heat shock promoters of *E. coli* (8) and alpha purple proteobacteria (42) with those identified in this study and that from the gonococcal *groE* operon (48) revealed that the gonococcal σ^{32} promoters shared similarity to both of the consensus sequences. In the -10 box of the gonococcal genes there is some variation among the stretch of C residues found in the *E. coli* consensus. Based upon the above alignment, site-directed mutagenesis was performed on the potential *dnaK* promoter to assess its activity in *N. gonorrhoeae* (Fig. 3). This confirmed that the σ^{32} promoter identified by primer extension analysis is in fact responsible for *dnaK* transcription.

We were unable to create a gonococcal *rpoH* mutant by insertional inactivation, suggesting that this gene may be essential for viability as is the case in *E. coli* (56). This finding was not surprising since a basal level of the proteins encoded by the heat shock genes it transcribes would be required for normal growth and the maintenance of cellular functions.

To determine the mechanisms responsible for regulation of the gonococcal *rpoH* gene, a search for the regulatory elements operative in other organisms was performed. The lack of a σ^{32} consensus sequence in the *rpoH* upstream region suggested that this gene is not autoregulated (Fig. 4). The positive regulatory element, the downstream box, was lacking from the gonococcal transcript, and the 5′ region did not form the characteristic secondary structure (data not shown) responsible for thermal regulation in *E. coli* and several other gamma subdivision proteobacteria (31, 32, 55). RNA dot blot hybridization and primer extension analyses were performed (Fig. 5). As for *dnaK*, *dnaJ*, and *grpE*, transcription of *rpoH* increased substantially following temperature stress. The primer extension experiments showed that this increase was evident at 20 min. Transcription emanated from a σ^{70} promoter (Fig. 4), and no other promoters were identified under the conditions tested. This is in contrast to the situation in many other species, in which transcription occurs from multiple promoters (11). The level of RpoH protein was investigated following heat shock and appeared to increase at around 15 min (Fig. 6). The concomitant increase in transcription and translation suggests that *rpoH* from *N. gonorrhoeae* is controlled, at least partially, at the level of transcription following heat shock. However, the levels of *dnaK*, *dnaJ*, and *grpE* increased prior to the increase observed for *rpoH*, suggesting that activation of preformed RpoH was sufficient to induce the heat shock response. Such an energy-efficient mode of regulation would be particularly advantageous to *N. gonorrhoeae* since it could respond rapidly to stress stimuli in vivo. Therefore, the increased amount of RpoH obtained by transcription late in the heat shock response is probably a subsidiary mechanism for sustaining the elevated expression of Hsps. The immediate increase in heat shock protein synthesis prior to an increase in the RpoH level has also been observed for *A. tumefaciens* (34).

In *E. coli*, the DnaK chaperone system modulates the cellular level of RpoH by regulating its synthesis, stability, and activity (14, 47). The control of activity and stability is exerted by preferential binding of the DnaK system to RpoH under normal conditions, rendering the DnaK system sensitive to proteolysis and sequestering it away from core RNA polymerase (19). This form of activity and stability control of RpoH may be occurring in *N. gonorrhoeae*. With stress, the DnaK chaperone system could preferentially bind to misfolded or denatured proteins so that RpoH was free to bind to core RNA polymerase and induce transcription of the heat shock genes.

To further elucidate the regulatory mechanism responsible for the transcriptional regulation of *rpoH* from *N. gonorrhoeae*, the uncharacteristically long leader sequence was deleted, and transcriptional activity in *N. gonorrhoeae* was assessed. As determined by measuring CAT protein levels (Fig. 7), deletion of the leader sequence led to a significantly higher level of transcription. A similar result was found for *E. coli*. This may be a result of the different secondary structures adopted by each of the individual transcripts which may effect transcription or translation. The secondary structure of the intact *rpoH* promoter region would be more extensive and complex than the deleted one and may therefore decrease mRNA stability or increase the frequency of premature transcription termination (2). Alternatively, these results could suggest the binding of a repressor to the *rpoH* leader sequence. This is to be investigated.

Overall, the data presented here suggest that the gonococcal stress response is regulated predominantly at the level of activity immediately following a thermal upshift. An increase in *rpoH* transcription and a subsequent increase in RpoH levels are then observed following ongoing exposure to heat shock. The stability of RpoH, the mechanism involved in regulation of *rpoH* transcription, and the role of the leader sequence have yet to be determined.

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