Influence of salt on the transcription of the gas-vesicle genes of *Haloferax mediterranei* and identification of the endogenous transcriptional activator gene

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The transcription of the 14 *gvp* genes of the gas-vesicle-encoding mc-vac region was investigated, using RNA from 25% and 15% (w/v) salt cultures of the moderately halophilic archaeon *Haloferax mediterranei*. Transcription occurred only from two promoters, located in front of the mc-*gvpA* and mc-*gvpD* genes. In both cultures transcripts spanning the entire mc-*gvpDEFGHIJKLM* transcription unit were formed only during the exponential growth phase. Amounts of these transcripts were larger in the 25% salt culture, in which the 2.0 kb mc-*gvpD* mRNA and large amounts of 1.3 kb and 0.45 kb partial mc-*gvpD* transcripts were also synthesized during the stationary phase. The levels of the mc-*gvpD* transcripts and of the 324 nt mc-*gvpA* mRNA increased in parallel during the stationary phase of the 25% salt culture. Only under these conditions were mRNAs spanning the entire mc-*gvpACNO* transcription unit observed, and gas-vesicles were formed. Investigation of the influence of the mc-*gvpDE* genes on both mc-vac promoters in transformants revealed that by themselves they were nearly inactive. The addition of mc-*gvpE*, however, resulted in a high level of constitutively produced mc-*gvpA* and mc-*gvpD* mRNA, indicating a transcriptional activator function for the mc-*gvpE* product.

Keywords: *Haloferax mediterranei*, gas-vesicle genes, archaea, gene regulation, transcriptional activator

INTRODUCTION

The moderately halophilic archaeon *Haloferax mediterranei*, isolated from salterns near Alicante, Spain (Rodriguez-Valera *et al.*, 1983), synthesizes gas-vesicles during the stationary phase when grown in media containing 17–30% (w/v) salt. Cells grown in 15% salt medium are gas-vesicle-free (Englert *et al.*, 1990). Gas-vesicles are also formed by *Halobacterium salinarum* and various bacteria, for example cyanobacteria (for a review, see Walsby, 1994). Gases dissolved in the cytoplasm diffuse freely through the ribbed gas-vesicle wall, which consists of a single hydrophobic protein, named GvpA.

In the case of halophilic archaea, 14 genes are involved in gas-vesicle synthesis, which are clustered in a genomic region, such as the p-vac and the c-vac region of *Hb. salinarum* (located on a plasmid and on the chromosome,

respectively) and the mc-vac region of *Hf. mediterranei* (Horne *et al.*, 1991; Jones *et al.*, 1991; Englert *et al.*, 1992a). In these regions, the *gvp* genes are arranged as two units: *gvpACNO*, and upstream and oppositely oriented, *gvpDEFGHIJKLM*. The boundaries of the mc-vac region have been defined by transformation experiments using the Vac[−] species *Haloferax volcanii* as recipient: the lack of mc-*gvpO* or mc-*gvpM* leads to Vac[−] transformants (Englert *et al.*, 1992b). Similar results have also been obtained with the p-vac region of *Hb. salinarum* PHH1 (Offner *et al.*, 1996). The involvement of only ten *gvp* genes in the homologous p-vac region of plasmid pNRC100 was reported by DasSarma *et al.* (1994); however, some of the results obtained by insertional mutations conflict with data obtained in our laboratory (see Offner *et al.*, 1996). In the cyanobacterium *Calothrix* sp. two *gvpA* homologues (*gvpA1* and *gvpA2*) and *gvpC* form one transcription unit (Damerval *et al.*, 1987), whereas in *Anabaena flos-aquae*

multiple copies of the *gvpA* gene were detected, and four additional genes similar to the other halobacterial *gvp* genes *gvpN*, *gvpJ*, *gvpK* and *gvpF/L* are located further downstream of this unit (see Walsby, 1994; Hayes & Powell, 1995). The exact number of genes involved in gas-vesicle formation of cyanobacteria has not yet, however, been determined.

In all cases, the *gvpA* and *gvpC* genes encode the only gas-vesicle structural proteins known to date: the major 8 kDa hydrophobic protein GvpA and the more hydrophilic 42 kDa GvpC, which appears to be located on the gas-vesicle surface. GvpC possibly stabilizes the overall structure, as shown for gas-vesicles of cyanobacteria (Hayes *et al.*, 1992). Halobacterial GvpC proteins are detectable in gas-vesicle preparations, but a stabilizing function has not yet been determined (Halladay *et al.*, 1993; Englert & Pfeifer, 1993). The functions of the other *gvp* genes and their products still need to be elucidated, but there is strong evidence that mc-*gvpD* is involved in the repression of gas-vesicle synthesis: *Hf. volcanii* transformants containing the mc-vac region with a deletion within mc-*gvpD* (ΔD) overproduce gas-vesicles, and the addition of the mc-*gvpD* gene on a second vector construct reduces their numbers back to wild-type levels (Englert *et al.*, 1992b; Pfeifer *et al.*, 1994). Additional support for such a function comes from the investigation of the p-vac region of *Hb. salinarum*: here, artificially large amounts of the 5'-terminal part of the p-*gvpD* mRNA result in the lack of the p-*gvpF-M* mRNA and a reduction in the number of gas-vesicles (Offner & Pfeifer, 1995).

Transcriptional analysis of the mc-vac region indicates the strong 324 nt mc-*gvpA* mRNA, which appears together with minor mRNAs spanning mc-*gvpAC*, mc-*gvpACN* and mc-*gvpACNO* in the early stationary phase, 40 h prior to the detection of gas-vesicles (Englert & Pfeifer, 1993). The mc-*gvpD-M* genes appear to be organized in two separate transcription units: a 4.8 kb mRNA spanning mc-*gvpF-M* occurs predominantly during the exponential growth phase, whereas 1.9 and 1.1 kb mc-*gvpD* transcripts appear during the stationary phase (Englert *et al.*, 1992a). Two divergent promoters are located in front of mc-*gvpA* and mc-*gvpD*; however, a third promoter within mc-*gvpE*, as found in the p-vac region of *Hb. salinarum*, could not be detected (Englert *et al.*, 1992a; Englert, 1992; Offner & Pfeifer, 1995). Salt-dependent gas-vesicle formation in *Hf. mediterranei* has only been investigated in the case of the mc-*gvpA* mRNA: its level increases sevenfold in a culture grown in 25% salt medium (producing gas-vesicles), as compared to a 15% salt culture, in which cells are gas-vesicle-free (Englert *et al.*, 1990).

In the present report, we determined all transcripts derived from the mc-vac region in *Hf. mediterranei*, using RNA from cells grown in both 25% and 15% salt media. In addition, the regulation of the two mc-vac promoters was analysed with respect to the influence of the mc-*gvpDE* genes. Halobacterial transformants carrying subfragments of the mc-vac region were then constructed, and transcripts investigated by Northern analyses.

METHODS

Growth of *Hf. mediterranei* and *Hf. volcanii*. *Hf. mediterranei* R4 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ 1411^T) and *Hf. volcanii* WFD11 was from W. F. Doolittle. *Hf. mediterranei* growth medium contained either 4 M NaCl (25% salt medium) or 2.3 M NaCl (15% salt medium), 60 mM MgSO₄, 20 mM KCl, 50 mM Tris/HCl pH 7.5 and 1.5% (w/v) peptone (Oxoid). *Hf. volcanii* growth medium contained either 3.4 M NaCl (25% salt medium) or 1.9 M NaCl (15% salt medium), 150 mM MgSO₄, 40 mM KCl, 50 mM Tris/HCl pH 7.5 and 1.5% peptone. Liquid cultures were grown at 37°C and aerated on a gyratory shaker at 250 r.p.m. Colonies were cloned on growth media solidified with 1.8% (w/v) agar at 42°C.

Transformation of *Hf. volcanii* WFD11. *Hf. volcanii* was transformed with DNA constructs containing the mc-vac region or part of it. All constructs contained the halobacterial vector plasmid pWL102 (Lam & Doolittle, 1989). The 10177 bp *Pst*I–*Bgl*II (BP) construct (Englert *et al.*, 1992b) carried the entire mc-vac region; the 9259 bp ΔD construct (Englert *et al.*, 1992b) was a variant of the BP construct, carrying a 918 bp *Xho*I deletion within the mc-*gvpD* gene; the A construct contained the 623 bp *Eco*RI–*Mlu*I mc-*gvp(C)A(D)* fragment (containing the mc-*gvpA* gene and parts of mc-*gvpC* and mc-*gvpD* cloned into pWL102; the A + D construct contained the 2586 bp *Eco*RI–*Dra*I mc-*gvp(C)AD(E)* fragment cloned into pWL102; the A + D + E construct contained the 3310 bp *Eco*RI–*Cla*I mc-*gvp(C)ADE(F)* fragment cloned into pWL102; the A + ΔD + E construct was a variant of the A + D + E construct, carrying a 918 bp *Xho*I deletion within the mc-*gvpD* gene. Prior to transformation of *Hf. volcanii*, each construct was passaged through the *E. coli* dam strain GM1674 (Palmer & Marinus, 1994) to avoid a halobacterial restriction barrier (Holmes *et al.*, 1991). Transformation was done as described by Pfeifer & Ghahraman (1993) using 15% salt medium (in which transformants are most easily obtained), and transformants were selected on agar plates containing 6 µg mevinolin ml⁻¹. The presence of the desired construct in each transformant was verified by Southern analyses. Transformants were subsequently grown and analysed in 25% salt medium, in order to ensure the expression of the mc-vac region.

Isolation of total RNA from cultures of *Hf. mediterranei* and *Hf. volcanii* and transcript analyses. Total RNA was isolated from *Hf. mediterranei* grown in 25% or 15% salt medium by lysis in guanidine thiocyanate buffer and ultracentrifugation through a caesium chloride cushion, according to Ausubel *et al.* (1988). RNA from *Hf. volcanii* transformants was isolated using the RNeasy kit from Qiagen, followed by DNase I digestion. Transformant RNA was isolated from 50 ml exponential-growth-phase cultures (OD₆₀₀ 0.1–0.2), or from 5 ml stationary-phase cultures (OD₆₀₀ ≥ 2).

Northern analyses involved electrophoresis of RNA on denaturing, formaldehyde-containing 1.2% (w/v) agarose gels, followed by transfer to nylon membranes (Ausubel *et al.*, 1988): 10 µg of each RNA sample was used in the case of *Hf. mediterranei*, but only 5 µg RNA from *Hf. volcanii* transformants because the pWL102 vector constructs are present in multiple copies in each cell. Strand-specific RNA probes were synthesized using the following fragments cloned in pBluescript as templates: the 849 bp *Eag*I–*Nru*I *gvpN/O* probe, the 367 bp *Eco*RI–*Xcm*I *gvpA* probe, the 304 bp *Mlu*I–*Xho*I 5' *gvpD* probe, the 610 bp *Sal*I–*Sac*I probe from the middle of mc-*gvpD* (Englert *et al.*, 1992a), the 580 bp *Nru*I–*Kpn*I *gvpD/E* probe, the 414 bp

EagI–*ClaI* *gvpF* probe and the 483 bp *SaI*–*BstEII* *gvpL* probe. The [α - ^{32} P]UTP-labelled RNA probes were synthesized using the T3/T7 polymerase system produced by Stratagene. Northern hybridization was generally carried out as described by Ausubel *et al.* (1988), except that the hybridization solution contained 10% (w/v) dextran sulfate (Sigma) and 0.5 μ g *E. coli* 16S and 23S rRNA ml $^{-1}$ (Boehringer Mannheim), in order to minimize nonspecific binding of the probe.

The 5' and 3' ends of transcripts were determined by S1 analyses according to Nasmyth (1983) and Murray (1986). For the determination of the 5' end of the 0.45 kb RNA from *mc-gvpD*, the 5' end-labelled oligonucleotide 5'-TGTCCTGATCGACGCGGG-3' (position 4511–4528 of the *mc-vac* sequence) was used as the primer for probe synthesis. Two different *mc-gvpD* restriction fragments were used for the investigation of the 3' end of the 0.45 kb RNA from *mc-gvpD*: a 726 bp *Sau3AI*–*BstXI* fragment and an 887 bp *MluI*–*BstXI* fragment were radioactively labelled at the 5' overhanging end (generated by *Sau3AI* or *MluI*), by the fill-in reaction using the Klenow DNA polymerase and [α - ^{32} P]dATP (Ausubel *et al.*, 1988).

Analyses of DNA sequence data. The palindromic region in the 5'-terminal part of *mc-gvpD* was detected and analysed for a possible secondary structure of the mRNA, using the BESTFIT and MFOLD programs from the UWGCG package.

RESULTS

Growth of *Hf. mediterranei* in media containing 25% or 15% salt and isolation of total RNA

Cells of *Hf. mediterranei* grown in media containing 25% or 15% salt were examined for the formation of gas-vesicles by phase-contrast microscopy. Samples for the isolation of RNA were taken from both cultures throughout growth: sample 1 was from the mid-exponential growth phase, samples 2 and 3 were from the late exponential growth phase, and samples 4 and 5 were from the stationary phase (Fig. 1). In the 25% salt culture, light-refractile bodies (i.e. gas-vesicles) were first observed at the beginning of the stationary phase (see Fig. 1, sample 4) and up to three such inclusions were observed inside most cells 9 h later. No gas-vesicles were formed during growth in the 15% salt medium (Rodriguez-Valera *et al.*, 1983; Englert *et al.*, 1990; and this paper). At time point 5, the OD $_{600}$ of the 25% salt culture decreased, and many cell fragments were observed. This was probably due to problems with the particular batch of the carbon source used for the medium. Therefore, samples 1–4 of both cultures were used for RNA extraction and for the investigation of *mc-vac* transcripts by Northern and S1 analyses.

Transcription of the *mc-gvpACNO* genes

From each sample of the 25% or 15% salt culture, 10 μ g RNA was separated electrophoretically on denaturing agarose gels and hybridized to strand-specific RNA probes synthesized from either of the two DNA strands of the *mc-gvpA* gene (Fig. 2) or the *mc-gvpNO* genes. No antisense transcripts were observed with the respective probes (data not shown).

Transcripts formed in the 25% salt culture. Northern analysis with the *gvpA* probe detected a 0.32 kb transcript in all four samples taken throughout growth (see Fig. 2).

Fig. 1. Growth of *Hf. mediterranei* in 25% (■) and 15% (○) salt cultures. Gas vesicles were only formed in the 25% salt culture and the arrow indicates their appearance in sample 4 at the beginning of the stationary phase. Samples were taken for the isolation of RNA at the time points indicated by numbers 1–5. Samples 1 (OD $_{600}$ ~ 0.5), 2 (OD $_{600}$ ~ 1.5) and 3 (OD $_{600}$ ~ 2) were from the exponential growth phase, samples 4 (OD $_{600}$ 3–4) and 5 were from the stationary phase.

The 324 nt *mc-gvpA* mRNA (Englert *et al.*, 1990) appeared in the mid-exponential growth phase, increased markedly during the following 17 h (Fig. 2, samples 1 and 2) and remained at a constant level during late exponential and early stationary phase (Fig. 2, samples 3 and 4). The *gvpA* probe also detected minor transcripts of 0.8 kb (also encompassing *mc-gvpA*), 1.8 kb and 2.4 kb (both encompassing *mc-gvpAC*) and 3.0 kb (encompassing *mc-gvpACNO*; Englert & Pfeifer, 1993). The 0.8 kb and 2.4 kb RNAs were formed in addition to shorter mRNAs spanning the same genes; they were considered to be by-products of transcription and probably to have no specific function. The relative amounts of the *mc-gvpACNO* transcripts decreased in the order 1.8 kb > 2.4 kb > 3.0 kb. They appeared together with the *mc-gvpA* mRNA (Fig. 2, sample 2), but their levels had already decreased at the beginning of stationary phase (Fig. 2, samples 3 and 4). This was verified by the hybridization pattern obtained with the *gvpNO* probe (data not shown).

Transcripts formed in the 15% salt culture. The 0.32 kb *mc-gvpA* mRNA was also observed in the 15% salt culture (Fig. 2). It appeared only in the late exponential growth phase, but its level markedly increased at the beginning of the stationary phase, remaining nearly constant during the following 33 h (Fig. 2, and data not shown). The 1.8 kb and 2.4 kb *mc-gvpAC* and the 3.0 kb *mc-gvpACNO* mRNAs were only detected in small amounts in early stationary phase (Fig. 2, *gvpA* probe). Similarly weak signals were also obtained using the *gvpNO* probe (data not shown).

Fig. 2. Northern analyses of the transcription of the *mc-vac* region in *Hf. mediterranei* grown in 25% (b–d) or 15% (e–g) salt cultures. (a) The *mc-vac* region, with the *mc-gvpACNO* genes and the oppositely oriented *mc-gvpDEFGHIJKLM* genes, is shown at the top. The arrows represent the two *mc-vac* promoters. The probes used in the Northern analyses (*gvpA*, 5′*gvpD* and *gvpL*) are indicated as black bars below the respective genes. (b–g) Northern analyses: (b, e) *gvpL* probe, (c, f) 5′*gvpD* probe, (d, g) *gvpA* probe. Lanes 1–4 contain RNA samples (10 µg) taken at the time points 1–4, respectively, indicated in Fig. 1. The size of the hybridizing mRNAs is indicated in kb.

Comparison of these data revealed the following differences in the transcription of the *mc-gvpACNO* genes of *Hf. mediterranei* grown in media containing 25% or 15% salt: the 0.32 kb *mc-gvpA* mRNA increased markedly during the late exponential growth phase of the 25% salt culture (Fig. 2, samples 1 and 2), whereas a comparable increase was delayed in the 15% salt culture for 22 h, until the stationary phase (Fig. 2, samples 3 and 4). In the 25% salt culture only, *mc-gvpA* mRNA synthesis was accompanied by the formation of substantial amounts of the 1.8 kb, 2.4 kb and 3.0 kb *mc-gvpACNO* mRNAs during early stationary phase, and gas-vesicles were produced.

Transcription of the *mc-gvpDEFGHIJKLM* genes

For the Northern analyses of transcripts of *mc-gvpDEFGHIJKLM*, strand-specific RNA probes were derived from the 5′-terminal part of the *mc-gvpD* gene (see

Fig. 2, 5′*gvpD* probe), from the middle of *mc-gvpD*, from the 3′ end of *mc-gvpD* and part of *mc-gvpE*, from *mc-gvpF* and from *mc-gvpL* (*gvpL* probe). No antisense RNAs were detected with any of these probes (data not shown). We present here only the analyses with the 5′*gvpD* probe and the *gvpL* probe, since they indicate all transcripts found in this region.

Transcripts formed in the 25% salt culture. The main signals observed with the 5′*gvpD* probe corresponded to transcripts of 2.0 kb, 1.3 kb and 0.45 kb (Fig. 2). They appeared only weakly in the RNA of sample 1, but hybridized strongly to RNA from samples collected during late exponential and stationary phase. The amount of the 2.0 kb *mc-gvpD* mRNA was lower at the beginning of the stationary phase (sample 4). The 1.3 kb and 0.45 kb RNAs, covering only parts of *mc-gvpD*, appeared in large amounts together with the *mc-gvpA* mRNA (see above).

Fig. 3. Schematic representation of the transcription of the *mc-vac* region in *Hf. mediterranei*. A genetic map of the *mc-vac* region is shown with both promoters represented as arrows. The bars depict the probes used for the investigation of *mc-vac* transcripts in *Hf. mediterranei*: *gvpNO*, *gvpA*, 5'*gvpD*, a probe from the middle of *mc-gvpD* also used in previous experiments (Englert *et al.*, 1992a), *gvpDE*, *gvpF* and *gvpL*. Arrows below the map indicate the direction and relative strength of transcripts as observed in Northern analyses. Transcript lengths are given in kb. The transcripts are grouped according to their appearance during exponential and stationary phases of the culture.

Weak transcripts of 3.0 kb and up to 7 kb were observed mainly during the exponential growth phase (samples 1 and 2). The 3.0 kb transcript was long enough to span the *mc-gvpDEF* genes, and the 7 kb mRNA spanned the entire *mc-gvpDEFGHIJKLM* gene cluster. The *gvpL* probe detected only the 7 kb transcript during the exponential growth phase (Fig. 2). The detection of the 7 kb transcript with the probes from both ends of *mc-gvpD-M* showed that this region forms a single transcription unit, and no additional promoter is located in *mc-gvpE* as reported by Englert *et al.* (1992a). This was also indicated by the results obtained with three other probes from the *mc-gvpDEF* region (data not shown). The transcripts detected in Northern analyses with all the probes are summarized in Fig. 3.

Transcripts formed in the 15% salt culture. Similar Northern analyses using the 5'*gvpD* probe revealed small amounts of the 2.0 kb and 1.3 kb transcripts during the exponential growth phase; in the stationary phase the amount of the 2.0 kb transcript decreased (Fig. 2). The 0.45 kb RNA was detected only in very low amounts in the stationary phase (Fig. 2). As seen before, faint signals corresponding to transcripts of up to 7 kb appeared during the exponential growth phase, which were also detected with the *gvpL* probe (Fig. 2, samples 1 and 2).

Summarizing these results, the transcripts derived from the *mc-gvpDEFGHIJKLM* gene cluster were similar in both cultures, but were formed in larger amounts in the 25% salt culture. A major difference was seen with the 0.45 kb RNA derived from the 5' terminus of *mc-gvpD* that was mainly formed in the 25% salt culture. Apparently, the activity of the *mc-gvpD* promoter markedly increased during the late exponential growth phase of the 25% salt culture, leading to the 2.0 kb *mc-gvpD* mRNA and to partial transcripts of *mc-gvpD*, but not to transcripts spanning the entire *mc-gvpD-M* gene cluster. This abortion of transcription could be due to RNA degra-

dation, processing or early transcriptional termination, and prevents the expression of the genes located downstream of *mc-gvpD*, despite increased *mc-gvpD*-promoter activity. To determine a possible transcriptional terminator for the most abundant 0.45 kb RNA, the 5' and 3' ends of this transcript were investigated.

Determination of the 5' and 3' ends of the 0.45 kb *mc-gvpD* 5'-transcript by S1 analysis

S1 analysis of the 5' terminus of the 0.45 kb RNA revealed the previously determined start site of *mc-gvpD* (Englert *et al.*, 1992a; data not shown). The DNA sequence near the putative 3' end of the 0.45 kb transcript indicated an interesting feature: 513 bp downstream of the transcriptional start site of *mc-gvpD* there was a 159 bp region containing two overlapping palindromic sequences (Fig. 4) that could represent a transcriptional terminator or processing signal.

For the determination of the 3' terminus, RNA from the late exponential growth phase of the 25% and 15% salt cultures was hybridized to an *mc-gvpD* fragment derived from the 5'-terminal region including the palindromic sequence. This fragment was radioactively labelled at the hybridizing 3' end. The unprotected 5'-terminal part of the DNA probe was then degraded with S1 nuclease, and the length of the protected DNA strand determined on a sequencing gel. Multiple lengths of protected fragments were detected that occurred only in the S1 analysis with RNA from the 25% salt culture, indicating multiple transcript 3' termini in a region between 210 nt and 481 nt downstream of the *mc-gvpD* start site (data not shown, Fig. 4). The major stop sites occurred at positions +303, +364, +375 and +398 relative to the *mc-gvpD* start site, and at least 115 nt upstream of the palindromic sequence. The detection of more than one stop site was unexpected, but was verified by the use of a second DNA probe that revealed the same distribution of protected fragments.

Fig. 4. Palindromic region in the 5'-terminal part of *mc-gvpD*. (a) DNA sequence of this region (EMBL accession number X64701), numbered according to the distance from the transcriptional start site (+1) of *mc-gvpD*. The ATG start codon is indicated by bold letters. The *Mlu*I and *Sau*3A1 restriction sites indicate the radioactively labelled 3' ends of the DNA fragments used for the determination of the 3' terminus of the 0.45 kb *mc-gvpD* RNA. Vertical arrows indicate the major stop sites observed (positions +303, +364, +375 and +398). Horizontal arrows indicate the location of palindromes (positions 4742–4900 of the *mc-vac* sequence, or +513 to +671 relative to the *mc-gvpD* transcriptional start site). (b) A possible secondary structure formed by this palindromic region, modified after a structure detected with the MFOLD program from the UWGCG package.

Fig. 5. Fragments of the *mc-vac* region used for the transformation of *Hf. volcanii*. The *gvpA* and the 5'*gvpD* probes used in Northern analyses are indicated by black bars below the respective *mc-vac* genes. Selected restriction sites defining the *mc-vac* subfragments are indicated. The *mc-vac* fragments used for transformation experiments are indicated by bars. The *Xho*I-deletion in the ΔD and $A + \Delta D + E$ construct (see text) is represented as a dotted line.

Fig. 6. Northern analyses of *Hf. volcanii* transformants. (a, b) Results obtained using the *gvpA* probe, (c, d) those obtained using the 5'*gvpD* probe (see Fig. 5 for location). RNA was from the *Hf. volcanii* transformants indicated at the top (see also Fig. 5). Five micrograms of RNA was applied from exponential (lanes e) and stationary (lanes s) phase cultures. The length of hybridizing transcripts is indicated in kbp.

Thus, the 0.45 kb RNA has no defined 3' terminus but rather represents a range of RNAs of different lengths.

Influence of mc-*gvpDE* on the activities of the mc-*gvpA* and mc-*gvpD* promoter

Transcript analysis revealed that the mc-vac region of *Hf. mediterranei* is transcribed from two divergent promoters, located in front of mc-*gvpA* and mc-*gvpD*. In order to investigate the endogenous regulation of the mc-vac region and to identify possible regulator genes, the activities of the two mc-vac promoters were assessed in *Hf. volcanii* transformants. The mc-vac subfragments used are shown in Fig. 5 and contained the promoter region plus different numbers of adjacent genes: the A construct contained the mc-*gvpA* gene and both promoters, the A+D construct contained mc-*gvpA* plus mc-*gvpD*, and the A+D+E construct contained mc-*gvpA* plus mc-*gvpDE*. The A+ΔD+E construct was a variant of the A+D+E construct that incurred a 918 bp deletion within mc-*gvpD*. This deletion causes strong gas-vesicle overproduction in transformants carrying the entire mc-vac region (ΔD transformant; Englert *et al.*, 1992b). As controls, *Hf. volcanii* transformants carrying the entire mc-vac region (BP-construct; Englert *et al.*, 1992b) or the ΔD construct were investigated. Each of these fragments was ligated to the same vector plasmid. Transformants were grown in 25 % salt medium, and total RNA was extracted

from exponential and stationary phase cultures and analysed for mc-*gvpA* and mc-*gvpD* transcripts by Northern analyses, using the *gvpA* and the 5'*gvpD* probe (Fig. 6).

Northern analyses of the BP wild-type, and the gas-vesicle-overproducing ΔD transformant indicated that transcription from both promoters occurred mainly in the stationary phase in BP, but was constitutive in ΔD (Englert *et al.*, 1992b; and Fig. 6, BP and ΔD). Furthermore, the analysis of the ΔD transformant with the *gvpA* probe indicated a larger amount of mc-*gvpACNO* mRNAs than in the BP transformant. The ΔD transformant also contained larger amounts of the ≤ 5.2 kb mc-*gvpΔD-M* mRNAs (Fig. 6, 5'*gvpD* probe). The ~2 kb transcript detected with the 5'*gvpD* probe corresponded to the mc-*gvpΔDEF* mRNA in the ΔD transformant. Thus, the mc-*gvpEF* genes were transcribed in ΔD throughout growth, and in larger amounts than in BP.

Similar analyses were performed with the transformants carrying subfragments of the mc-vac region. The A and A+D transformants contained only traces of the 0.32 kb mc-*gvpA* mRNA in exponential and stationary phases (Fig. 6, *gvpA* probe). No mc-*gvpD* transcripts could be detected in the RNA of the A transformant, since these lacked the DNA sequences complementary to the 5'*gvpD* probe. However, low amounts of the 2.0 kb mc-*gvpD* mRNA and shorter transcripts were detected in the A+D transformant throughout growth (Fig. 6, 5'*gvpD* probe).

These results demonstrate that the *mc-gvpA* and the *mc-gvpD* promoters are only weakly active by themselves. Addition of *mc-gvpE* in the A + D + E transformant, however, led to the formation of large amounts of the 0.32 kb *mc-gvpA* mRNA throughout growth, as also seen in the A + ΔD + E transformant. In the case of the *mc-gvpD* promoter, high amounts of *mc-gvpD* transcripts were formed in the A + D + E and A + ΔD + E transformant as well. Both contained transcripts spanning *mc-gvpDE* in the exponential and stationary phases: as weak 2.9 kb *mc-gvpDE* mRNA in A + D + E, and as 2 kb *mc-gvp Δ DE* mRNA in A + ΔD + E (also seen in ΔD , see above). These results demonstrate that *mc-gvpE* encodes the transcriptional activator of the *mc-gvpA* and probably also of the *mc-gvpD* promoter (thus activating its own transcription), and that an intact *mc-gvpD* gene has no influence on this *mc-gvpE*-mediated activation in the A + D + E transformant.

DISCUSSION

Gas-vesicle production in the moderately halophilic archaeon *Hf. mediterranei* depends on growth in media containing at least 17% salt and occurs in the stationary phase. This salt-dependent gas-vesicle formation has been shown to involve transcriptional regulation: the transcription of *mc-gvpA*, encoding the major gas-vesicle structural protein, correlates closely with the formation of gas-vesicles (Englert *et al.*, 1990). In the present analysis, transcription of the *mc-vac* region was investigated during growth in 25% versus 15% salt medium. We did not differentiate between the effects of osmolarity and oxygen supply (salt decreases the solubility of O₂ in water), because the latter would have required the use of more sophisticated equipments than presently available.

The 14 *mc-gvp* genes are transcribed as two units (*mc-gvpACNO* and *mc-gvpD-M*) from two divergent promoters located in front of *mc-gvpA* and *mc-gvpD* (Englert *et al.*, 1992a; and this report): in the present analysis the *mc-gvpD-M* genes were shown to represent a single transcription unit because long RNAs spanning this entire gene cluster were detected. No antisense RNAs could be detected in the *mc-vac* region.

The two promoters of the *mc-vac* region were both active in cells grown in 25% or 15% salt media (although at different levels), despite the different Vac phenotypes observed with *Hf. mediterranei*. Major differences were that in the 25% salt culture (i) very large amounts of transcripts starting at the *mc-gvpD* promoter appeared in late exponential and stationary phases, comprising only *mc-gvpD* or parts of it, including a 0.45 kb transcript, and (ii) the earlier appearance of *mc-gvpA* mRNA led to the formation of *mc-gvpACNO* mRNAs, and finally to gas-vesicle synthesis. Thus, the *mc-vac* transcripts formed during gas-vesicle production included *mc-gvpA* and *mc-gvpACNO* mRNAs and (partial) *mc-gvpD* transcripts, which all appeared in high amounts during late exponential growth phase. The function of the latter transcripts during gas-vesicle synthesis is not clear. Interestingly, these transcripts stopped upstream of *mc-gvpE*, encoding

the transcriptional activator gene of the *mc-vac* region. Attempts to identify the 3' end of the most abundant 0.45 kb RNA yielded no information about the mechanism of its generation, because different 3' termini were detected. An extensive palindromic region was observed downstream of these multiple 3' termini, but no relation with respect to transcriptional termination or processing could be defined.

The transcript pattern of the *mc-vac* region differs markedly from the transcription and expression of the plasmid-borne *p-vac* region of *Hb. salinarum* PHH1, which is expressed from four promoters located in front of *p-gvpA*, *p-gvpD*, *p-gvpF* and *p-gvpO*. The *p-vac* region promotes gas-vesicle formation throughout the growth cycle: the *p-gvpA* mRNA occurs in all stages of growth together with smaller amounts of longer transcripts spanning *p-gvpACNO* (Horne *et al.*, 1991; Englert *et al.*, 1992a; Offner & Pfeifer, 1995). In addition, *p-gvpO* is also transcribed from its own promoter, underlining the importance of this gene for gas-vesicle synthesis. (Offner *et al.*, 1996). The *p-gvpD-M* cluster is transcribed as two units: the *p-gvpDE* mRNA occurs in the stationary phase, whereas the *p-gvpFGHIJKLM* mRNA appears only in the exponential growth phase, starting from an additional promoter located in *p-gvpE* (Offner & Pfeifer, 1995). It is interesting to note that the termination or processing observed behind and within *mc-gvpD* in the stationary phase in the *mc-vac* region results in the same exponential-growth-phase-dependent *gvpF-M* mRNA formation as in the *p-vac* region. In the constitutively expressed *p-vac* region, the *gvpDE* genes are not required for gas-vesicle synthesis (Offner & Pfeifer, 1995). The *p-gvpA* promoter is active by itself, thus the *p-gvpE* gene product might not be necessary. Therefore, an additional promoter located within *p-gvpE* could drive the expression of the *p-gvpF-M* gene cluster.

We also investigated the endogenous regulation of both *mc-vac* promoters in response to products of the adjacent *mc-gvpDE* genes. Earlier investigations indicated very low expression of the *mc-gvpA* gene when present by itself in *Hf. volcanii*, whereas the gene was expressed at a higher levels in transformants containing larger *mc-vac* sub-fragments (Englert *et al.*, 1992b). In addition, these transformation experiments suggested a repressor function for the *mc-gvpD* gene and its product, since a *mc-vac* region containing an in-frame deletion within *mc-gvpD* (ΔD) resulted in a gas-vesicle overproducing transformant. This effect could be reduced to wild-type levels by the addition of *mc-gvpD* (Englert *et al.*, 1992b; Pfeifer *et al.*, 1994).

The constructs used in the present studies contained the *mc-gvpA* gene by itself, or *mc-gvpA* together with *mc-gvpD*, *mc-gvpDE* or *mc-gvp Δ DE*. In the absence of *mc-gvpE*, both *mc-vac* promoters were nearly inactive, whereas the presence of the *mc-gvpE* gene (A + DE and A + Δ DE transformants) led to large amounts of *mc-gvpA* and *mc-gvpD* mRNAs. This indicates that *mc-GvpE* is a transcriptional activator for the *mc-gvpA* promoter, and probably also for the *mc-gvpD* promoter. A stability effect on the *mc-gvpDE* mRNA seems unlikely, since low

amounts of the mc-*gvpD* mRNA were already observed in the A + D transformant. In contrast to the growth-phase-dependent expression of mc-*gvpA* in transformants containing the entire mc-vac region (Englert *et al.*, 1992b; and this report), in the A + D + E and the A + Δ D + E transformants the transcripts from both promoters were found during all stages of growth. This implies that there must be another mc-vac gene product inhibiting the GvpE-mediated activation of the mc-*gvpA* promoter during exponential growth. Such a function as previously assigned to the mc-*gvpD* gene product (see above); however, the results presented here demonstrate that mc-GvpD cannot mediate this effect by itself. Experiments to detect the additional factor inhibiting the activation of mc-*gvpA* in early growth stages are currently in progress; and the function of this protein possibly depends on the presence of GvpD. In this respect it is interesting that the GvpD protein contains a conserved motif (P-loop; Saraste *et al.*, 1990) characteristic of the nucleotide-binding site for ATP/GTP-binding proteins.

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