Microbial pathogenesis in cystic fibrosis: co-ordinate regulation of heat-shock response and conversion to mucoidy in Pseudomonas aeruginosa

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Summary
Conversion of Pseudomonas aeruginosa to the mucoid phenotype plays a major role in the pathogenesis of respiratory infections in cystic fibrosis (CF). One mechanism responsible for mucoidy is based on mutations that inactivate the anti-σ factor, MucA, which normally inhibits the alternative sigma factor, AlgU. The loss of MucA allows AlgU to freely direct transcription of the genes responsible for the production of the exopolysaccharide alginate resulting in mucoid colony morphology. In Escherichia coli, a close homologue of AlgU, σE, directs transcription of several genes under conditions of extreme heat shock. Here we examined whether AlgU, besides its role in controlling alginate production, affects the heat-shock response in P. aeruginosa. The P. aeruginosa rpoH gene encoding a homologue of the major heat-shock sigma factor, σ32, was found to be transcribed by AlgU containing RNA polymerase from one of its promoters (P3) identified in this study. Transcription of rpoH from P3 was elevated upon exposure to extreme heat shock in an algU-dependent manner. Importantly, the AlgU-dependent promoter of rpoH was found to be activated in mucoid mucA mutants. In keeping with this observation, introduction of a wild-type mucA gene abrogated AlgU-dependent rpoH transcription in mucoid P. aeruginosa laboratory isolates and CF isolates. These results suggest that conversion to mucoidy and the heat-shock response are co-ordinately regulated in P. aeruginosa. The simultaneous activation of both systems in mucA mutants, selected in the lungs of CF patients, may have significance for the inflammatory processes characteristic of the establishment of chronic infection and ensuing clinical deterioration in CF.

Introduction
Chronic respiratory infections and associated inflammation are the leading cause of high mortality and morbidity in patients with cystic fibrosis (CF) (Boat, 1989; Govan and Deretic, 1996). The overproduction of the exopolysaccharide alginate, which results in mucoid colony morphology, is a characteristic pathogenic determinant expressed by Pseudomonas aeruginosa in CF (Govan and Deretic, 1996). The muc locus, a major site of genetic alterations responsible for the conversion to the mucoid phenotype, has initially been described by Fyfe and Govan (1980). The recent molecular characterization of the genes within the muc locus at 67.5 min of the P. aeruginosa map resulted in identification of the algU mucABCD gene cluster as the major regulatory site which encodes both positive and negative factors controlling mucoidy (Boucher et al., 1996; Martin et al., 1993a; Martin et al., 1993b; Martin et al., 1993c; Schurr et al., 1994). Similar findings have been reported by others, resulting in alternative designations of algU as Pa σE or algT (DeVries and Ohman, 1994; Hershberger et al., 1995; Wozniak and Ohman, 1994) and mucB as algN (Goldberg et al., 1993). The initial analysis of algU showed that its predicted gene product was homologous to the Bacillus stationary phase and sporulation sigma factor SpoOH (Martin et al., 1993a). In a subsequent study, Martin et al. (1994) showed that the −35 and −10 regions of promoters transcribed by AlgU shared considerable similarity with the known promoter consensus sequence for σE, the second heat-shock sigma factor identified at the biochemical level in Escherichia coli (Erickson and Gross, 1989). These and subsequent sequence analyses permitted the identification of E. coli and Salmonella typhimurium rpoE genes based on striking homologies of their gene products with AlgU (Hiratsu et al., 1995; Martin et al., 1994; Raina et al., 1995; Rouvière et al., 1995). These observations and additional analyses have uncovered the existence of a broader family of novel alternative sigma factors termed ECF (Lonetto et al., 1994) or σE-like factors (Deretic et al., 1994).

The initial experimental evidence supporting the hypothesis that algU encodes the P. aeruginosa equivalent of σE was presented when E. coli rpoE was shown to complement an algU mutation and to induce mucoidy in P. aeruginosa (Yu et al., 1995). Biochemical evidence that
algU encodes a sigma factor has been reported recently (Hershberger et al., 1995; Schurr et al., 1995b). AlgU activity is controlled at a post-translational level by the products of the downstream genes (mucABCD) that encode its negative regulators. Recently, it has been demonstrated that MucA probably functions as an anti-sigma factor (Schurr et al., 1996; Xie et al., 1996). Mutations in mucA and other muc genes located in the 67.5 min region are responsible for the conversion to mucoidy in CF isolates and laboratory strains (Martinet al., 1993c). As a result of mucA inactivation, AlgU is relieved from the inhibition by its cognate anti-σ factor and is available to direct transcription of the subordinate promoters. AlgU directs transcription of its own gene (Schurr et al., 1995a) and that of algR, a response regulator controlling critical alginate genes (Martin et al., 1994; Wozniak and Ohman, 1994). AlgU and AlgR co-operate to activate transcription of the algD gene (Martin et al., 1994; Schurr et al., 1993) encoding GDP mannose dehydrogenase (Deretic et al., 1987; Roychoudhury et al., 1989), which catalyses the first committed step in alginate synthesis. Because of this cascade of regulatory interactions, the net result of a loss of mucA is alginate overproduction and mucoid colony phenotype.

The equivalence of E. coli σE and P. aeruginosa AlgU suggests that there may be broader physiological overlaps in the function of these sigma factors (Yu et al., 1995). The sigma factor σ32 that controls the heat-shock response in E. coli is encoded by the rpoH gene (Grossman et al., 1984). E. coli σ32 controls expression of genes encoding heat-shock proteins such as GroEL, DnaJ, DnaK, and Lon (Yura et al., 1993). Interestingly, one of the E. coli rpoH promoters (P3) is controlled by σE under conditions of extreme heat shock (Erickson and Gross, 1989; Wang and Kaguni, 1989). In order to address the question of whether AlgU, in addition to regulating mucoidy in P. aeruginosa, affects the heat-shock response in this organism, the promoter region of the recently characterized P. aeruginosa rpoH gene (Naczynski et al., 1995) and the effects of algU and mucA mutations on rpoH transcription were analysed. We report that AlgU is responsible for transcriptional initiation of one of the rpoH promoters (Fig. 1). We also show that mucA mutations in P. aeruginosa not only induce alginate production and mucoidy in this organism but also activate rpoH transcription, which may have downstream effects on the entire heat-shock response. As chronic inflammation is a well-recognized sequela of P. aeruginosa infections in CF, it is possible that the expected increased expression in mucA mutants of highly conserved antigens such as heat-shock proteins may further complicate immune processes and contribute to the pathogenesis in CF.

Results

S1 nuclease protection mapping of the rpoH mRNA 5' ends

In order to investigate whether rpoH is transcribed by AlgU (Pa-σE) in P. aeruginosa and to map the rpoH promoters in this organism, the region immediately upstream of the rpoH gene was cloned and examined by S1 nuclease protection analysis. Several bands of protection corresponding to rpoH mRNA 5’ ends were observed (Fig. 2). One 5’ mRNA end was located 25 bp upstream of the rpoH initiation codon (Figs 1 and 2). One 5’ mRNA end was located 25 bp upstream of the rpoH initiation codon (Figs 1 and 2). A second band of protection corresponded to the 5’ mRNA end at the position 41 bp upstream of the rpoH translational start (Fig. 2A, lane 1). When P. aeruginosa was grown on minimal media, an additional 5’ mRNA end was observed. This band of protection was located 36 bp upstream of the rpoH initiation codon (Figs 1 and 2). A second band of protection corresponded to the 5’ mRNA end at the position 41 bp upstream of the rpoH translational start (Fig. 2A, lane 1). When P. aeruginosa was grown on minimal media, an additional 5’ mRNA end was observed. This band of protection was located 36 bp upstream of the initiation codon (Fig. 2B), was not visible in RNA extracted from cells grown in rich medium (Fig. 2A), and was more prominent when RNA was extracted from PAO6852 (algUTcR) (Fig. 2B, lanes 3 and 4). These results suggest...
that the P. aeruginosa rpoH gene is transcribed from at least three promoters (P1, P2, and P3) corresponding to the mRNA start sites mapped in this study at 25 bp, 36 bp, and 41 bp upstream of the initiation codon (Fig. 1). Of the mapped mRNA 5′ ends, P1 and P3 were present under most conditions, while P2 was detectable only on minimal media. Interestingly, the P2 signal was enhanced in algU null mutants (Fig. 2A, lanes 3 and 4) suggesting a possible interference of initiation from the P2 and P3 promoter or masking of the signal.

**Induction of rpoH P3 by extreme heat shock and its dependence on algU**

A strong consensus σ70 (AlgU) promoter sequence, previously noted upstream of the rpoH coding sequence (Benvenisti et al., 1995; Deretic et al., 1994; Naczynski et al., 1995), was located at the canonical −35 and −10 positions in relation to the P3 band. In order to determine if rpoH P3 was AlgU-dependent, RNA from P. aeruginosa PAO6852 (algU::TcR) was subjected to S1 nuclease protection analysis. The P3 band was absent in these samples (Fig. 2A, lanes 3 and 4) while it was present as expected (Martin et al., 1994; Schurr et al., 1995a) in samples with RNA from algU+ cells (Fig. 2A, lanes 1 and 2). These results indicate that the rpoH P3 signal is dependent on the presence of a functional algU gene.

The AlgU promoters of algU (P1 and P3) and algR (Pp) are induced during exposure to extreme heat shock (Martin et al., 1994; Schurr et al., 1995a). In order to examine whether the P3 promoter of rpoH can be induced by extreme heat shock, P. aeruginosa PAO1 RNA was isolated from cells that were exposed to a temperature shift from 37°C to 50°C. This treatment resulted in a dramatic increase in rpoH P3 transcription (Fig. 2A, lane 2) as compared to cells grown at 37°C (Fig. 2A, lane 1). The observed activation of rpoH transcription from P3 under conditions of extreme heat shock (Fig. 2A, lane 2) and the absence of the corresponding transcripts in algU mutants (Fig. 2A, lanes 3 and 4) are consistent with the interpretation that rpoH P3 is an AlgU-dependent promoter.

**In vitro transcriptional analysis of AlgU and σ70 promoters of P. aeruginosa rpoH**

Purified AlgU has been used in vitro to demonstrate its activity as a sigma factor on one of the promoters of algU (P1) (Schurr et al., 1995b). In order to examine whether AlgU directs transcription of the rpoH P3 promoter, purified AlgU was preincubated with core RNA polymerase and added to the standard in vitro transcriptional run-off assay (Schurr et al., 1995b) with rpoH as a template. The predicted distance from P3 initiation to the end of the truncated rpoH is 77 nucleotides and a transcript of this size was observed upon addition of AlgU to the mixture (Fig. 3A, lanes 2 and 3). This transcript had the expected size and was absent when the same template was incubated with RNA polymerase core alone (Fig. 3A, lane 1). These findings support the conclusion that the rpoH P3 promoter is transcribed by the AlgU (Pa σ70) RNA polymerase holoenzyme.

In a set of complementary experiments, in vitro transcription of P1 and P2 was investigated. Examination of the −10 (TAcAcT) and −35 (TGTcag) regions of the strong rpoH P1 promoter suggested the possibility that this promoter may be transcribed by the σ70 holoenzyme. In E. coli, it is known that σ70 directs transcription of several rpoH promoters (Erickson et al., 1987; Fujita et al., 1987; Nagai et al., 1990). In order to examine the possibility that σ70 directs transcription of the P1 promoter of P. aeruginosa rpoH, in vitro transcriptional run-off analyses were performed either with core RNA polymerase reconstituted with exogenously added σ70 or with σ70 saturated
Fig. 3. AlgU directs transcription of the $rpoH$ P$_3$ promoter and $\sigma^{70}$ directly transcribes from $rpoH$ P$_1$ and P$_2$ promoters.

A. *In vitro* transcription from the $rpoH$ P$_3$ promoter. Lanes: 1, core RNA polymerase; 2 and 3, core RNA polymerase with AlgU. Open triangle, $rpoH$ P$_3$ transcript.

B. $\sigma^{70}$-directed *in vitro* transcription from the $\lambda P_R$ promoter and the $rpoH$ P$_1$ and P$_2$ promoters. Lanes: 1, RNA polymerase holoenzyme and the $\lambda P_R$ promoter; 2, core RNA polymerase with $\sigma^{70}$ and the $\lambda P_R$ promoter; 3, RNA polymerase holoenzyme and the $rpoH$ promoter; 4, core RNA polymerase with $\sigma^{70}$ and the $rpoH$ promoter.

C. Schematic representation of $rpoH$ promoter positions. Open box, $rpoH$ P$_3$ transcript; striped box, $rpoH$ P$_1$ and P$_2$ promoters; filled triangle, $\lambda P_R$ transcript; striped triangles, $rpoH$ P$_1$ and P$_2$ transcripts.

**Discussion**

In this study, we have mapped the 5’ mRNA ends of the *P. aeruginosa* $rpoH$ gene and found that there are three promoters located at positions $-25$ (P$_1$), $-36$ (P$_2$) and $-41$ (P$_3$) relative to the start codon. The conclusion that *P. aeruginosa* $rpoH$ contains an AlgU-dependent promoter (P$_3$) is supported by the following experimental work:
Fig. 4. Induction of AlgU-dependent transcription of algU and rpoH in mucA mutants.
A. S1 nuclease protection analysis. Lanes: 1, PAO381 (mucA<sup>+</sup>mucB<sup>+</sup>); 2, PAO578I (mucA<sup>22</sup>); 3, PAO6857 (mucB::Tc<sup>R</sup>) using an algU probe generated with oligonucleotide UU-1; 4, 5 and 6, same as lanes 1, 2 and 3 using an algU probe generated with oligonucleotide UR-23; 7, 8 and 9, same as lanes 1, 2 and 3 using an rpoH probe generated with oligonucleotide HR-2.
B and C. Schematic representation of probes, oligonucleotides and restriction sites used in S1 nuclease protection analyses. Negative numbers show relative positions of promoters with respect to the initiation codons of algU and rpoH. Circles represent AlgU-dependent promoters; filled or patterned boxes and circles indicate other promoters; FP, band of full protection. All algU promoters (P<sub>1</sub>–P<sub>5</sub>) shown here have been previously described (Schurr et al., 1995a).
observations and additional considerations: (i) P₃ transcription is absent in algU null mutants of P. aeruginosa; (ii) P₃ transcription is initiated in vitro by AlgU-RNA polymerase holoenzyme; (iii) P₃ is induced under conditions of extreme heat shock; (iv) P₃ expression is increased in mucA mutant strains; and (v) the −35 (GAACTT) and −10 (TCAgA) regions of P₃ conform with the AlgU (σF) consensus promoter sequence. These findings are also consistent with the interpretation that conversion to mucoidy and transcriptional activation of the heat-shock response in P. aeruginosa are co-ordinated and probably occur simultaneously in mucA mutants. This is evidenced by: (i) increased rpoH P₃ transcription in mucA mutant strains; and (ii) suppression of rpoH P₃ transcription by plasmid-borne mucA⁺ introduced into laboratory and CF mucA mutants. MucA has been demonstrated to negatively regulate AlgU (Fig. 6A) and recent findings support the hypothesis that it does so by binding to AlgU (Martin et al., 1993c; Schurr et al., 1994; 1996; Xie et al., 1996). Since MucA, as shown here, also controls expression of rpoH it will be of interest to examine, in future studies, the extent of the effects of mucA mutations on the expression of rpoH-dependent genes. Such analyses are expected to reveal additional details and uncover the full extent of overlaps between the conversion to mucoidy and heat-shock response in P. aeruginosa. It is possible that selection of mucA mutants in CF reflects not only the advantages due to the alginate coating but may provide additional protection to the pathogen because of the co-induction of other stress-response systems.

Two of the three mapped rpoH promoters (P₁ and P₂) are initiated by σ⁷₀ as shown in this study. A previous
report (Naczynski et al., 1995) examined transcription of P. aeruginosa rpoH in the heterologous host E. coli. One of the mRNA 5’ ends observed in our study (P1 at −25°) matches with the initiation start sites reported by Naczynski et al. (1995). However, the P3 transcriptional start site, mapped in our study in five strains to the same position (−41), did not match any of the other bands reported by Naczynski et al. (1995) for plasmid-borne P. aeruginosa rpoH expressed in E. coli. A third band corresponding to full probe protection (Fig. 4A, lanes 7–9; FP) was also observed in our studies, suggesting the existence of at least one additional transcription initiation site. Such a transcript has not been reported by Naczynski et al. (1995). These and additional discrepancies are best explained by potential expression differences of the P. aeruginosa rpoH gene in E. coli and P. aeruginosa. The transcription from P2 observed in this study was detectable only on minimal media, which is reminiscent of medium-dependence for expression of one of the E. coli rpoH promoters (Nagai et al., 1990). It is also worth noting that it was difficult to detect P2 activity in vivo unless AlgU-dependent transcription from P3 was absent. At present, we do not know whether the physical proximity of the canonical −10 and −35 regions for the P2 and P3 promoters or some other factors may be the basis for this apparent interference. However, as the two promoters are active under different physiological conditions, the occupancy of P2 and P3 may be mutually exclusive. Interestingly, the intensity of P1 and P2 transcripts in vitro (Fig. 3B) was in favour of P2 in contrast to the relative intensity in vivo (Fig. 2B). However, the intensity of signals obtained in vitro may also reflect transcript size. It is also important to note that P3 transcription was stronger than P2 transcription under all conditions when both promoters were active.

Several studies have implicated stress proteins from different bacteria in the induction of autoimmune disease (Lamb et al., 1989; Oldstone, 1987; Young, 1990). Heat-shock proteins of many pathogenic bacteria (e.g. Salmonella spp., E. coli, Chlamydia trachomatis, Mycobacterium spp., and Brucella spp.) are recognized by the immune system (Cameron et al., 1994; Cerrone et al., 1991; Elzer et al., 1994; Johnson et al., 1991; Roop et al., 1994; Tatum et al., 1994; van Eden et al., 1988) and have been implicated in bacterial virulence or in immunopathology associated with the sequelae of bacterial infections (Lamb et al., 1989; van Eden et al., 1988; Young, 1990). One of the recently studied proteins that belongs in this category is HtrA (DegP) (Lipinska et al., 1990; Strauch and Beckwith, 1988). HtrA is important for virulence of Salmonella (Johnson et al., 1991) and appears to play a role in the virulence of Brucella abortus (Elzer et al., 1996) and Yersinia enterocolitica (Li et al., 1996). In P. aeruginosa, this factor has two homologues, algW and mucD (Boucher et al., 1996). The mucD gene is believed to be under the control of AlgU. These relationships and the increased sensitivity to heat killing of algU mutants (Yu et al., 1995) have provided the basis for suggestions that the control of mucoidy and the heat-shock response may be intimately associated in P. aeruginosa. Such views are now corroborated in this study and extended to include the major heat-shock sigma-factor gene rpoH. Several general properties of the heat-shock response in P. aeruginosa have been investigated (Allan et al., 1988). It has been reported that 17 proteins with apparent molecular masses ranging from 15.7–103.4 kDa are induced in response to temperature shifts from 37°C to 42°C. It has also been shown that two of these proteins (61 kDa and 76 kDa) are immunologically similar to E. coli GroEL and DnaK, respectively (Allan et al., 1988). These studies have also confirmed that GroEL is one of the predominant proteins responding to heat shock in P. aeruginosa. GroEL (Hsp 60) is a highly immunodominant molecule and is frequently recognized by antibodies in bacterial infections (Cerrone et al., 1991; van Eden et al., 1988). It is worth mentioning that a vigorous immune response to GroEL has been noted as a result of bacterial infections in CF (Jensen et al., 1993).

There are several lines of evidence indicating that an exuberant but apparently ineffective activation of the host immunological response contributes to the extensive tissue damage observed in chronic infections by P. aeruginosa in CF lungs (Boat et al., 1989). Furthermore, autoimmune pathology and rheumatoid abnormalities such as episodic or chronic erosive arthropathy, hypertrophic pulmonary osteoarthropathy, rheumatoid arthritis and diabetes mellitus have been associated with the chronic disease in CF (Coffey et al., 1989). While these processes are not understood at present, questions associated with these phenomena may be central to the issues of inflammation and associated morbidity and mortality in CF. It has been proposed that the highly conserved stress antigens of P. aeruginosa such as heat-shock proteins may play an important role in the immunopathology seen in CF (Jensen et al., 1995; Jensen et al., 1993). Thus it is possible that the putative induction of the heat-shock response, as a collateral effect of conversion to mucoidy (Fig. 6B) or as a part of selection for strains with upregulated stress systems during chronic colonization of the lung, may contribute to such processes in CF. Further in vitro and in vivo investigations of the rpoH-dependent regulon, which with a few exceptions (Fujita et al., 1993; Jensen et al., 1995) remains to be characterized in P. aeruginosa, and antigens whose production may be induced in mucA mutants may reveal important targets or modulators of the immune response in the context of the hypersensitivity reactions and inflammation leading to various manifestations of immune pathology in CF.
**Experimental procedures**

**Bacterial strains, plasmids and growth conditions**

PAO1 is the standard genetic *P. aeruginosa* strain (Holloway, 1955). PAO6852 is an AlgU·Tc derivative of PAO1 described previously (Martin et al., 1994). PAO6857 is a mucB·Tc derivative of PAO1 described previously (Schurr et al., 1996). CF23 and CF1 are mucC CF patient isolates that have been previously described (Martin et al., 1993c). pTac-mucA is a pVDCmac derivative carrying mucA under the control of the tac promoter as previously described (Martin et al., 1993c).

**pRK2013** is the plasmid that contains the tra functions used in triparental conjugations (Figurski and Helinski, 1979). pETU-1610 is the AlgU expression clone that produced the purified AlgU as previously described (Schurr et al., 1995b). *P. aeruginosa* was grown in Pseudomonas isolation agar (PIA) supplemented with 300 µg of carbenicillin or 300 µg of tetracycline when required. *E. coli* was grown in Luria–Bertani (LB) medium supplemented with 25 µg ml⁻¹ kanamycin, 30 µg ml⁻¹ chloramphenicol and 40 µg ml⁻¹ ampicillin as needed. Polymerase chain reaction (PCR) fragments were subcloned into the Invitrogen pCRII vector when cloned. *E. coli* BL21(DE3) pLysS (Studier and Moffat, 1986) was used for the overproduction and purification of AlgU.

**RNA isolation and S1 nuclease protection assay**

RNA was isolated as previously described (Schurr et al., 1995a). Uniformly labelled single-stranded probes were generated from M13 derivatives carrying the appropriate promoter region (algU or rpoH). The AlgU probe has been described previously (Martin et al., 1994; Schurr et al., 1995a). The *P. aeruginosa* rpoH gene was cloned as a 967 bp PCR fragment using oligonucleotides HF-1 (5′-AGGCGGATCAGGACC-3′, -109 to -94 relative to the start codon) and HR-1 (5′-CGATCGGCCGAGAATC-3′, +941 to +958 relative to the ATG) and cloned into pCRII and termed pCR rpoH. The AlgU promoter region was subcloned from pGAlgU into M13mp18 as an XmnI/EcoRI 385bp fragment. The oligonucleotide HR-2 (5′-CGAGTGCAGGCTTTCA-3′, +50 to +69 relative to the start codon) was used to generate a 188-nucleotide uniformly labelled α-³²P probe as previously described (Schurr et al., 1995a). This probe contained 10 bp from the pCRII vector. The probe was hybridized to 50 µg of RNA and treated with S1 nuclease as previously described (Martin et al., 1994).

In vitro transcriptional run-off assay

The *in vitro* run-off assay using AlgU with the P₁ promoter of algU has been described previously (Schurr et al., 1995b). His₁₀-AlgU was renatured by dilution (1:10) into renaturation buffer (total volume 30 µl) consisting of 100 mM Tris-HCl (pH7.5), 33% glycerol, 10 mM KCl, 10 mM MgCl₂, 0.3 mM ATP, 0.33 mg ml⁻¹ GroEL and 0.2 mg ml⁻¹ GroES (Epicentre Technologies) and incubated for 20 min at 25°C. A standard sigma-factor/core RNA polymerase preincubation reaction (6 µl) consisted of 3 µl with 0.6 pmol core RNA polymerase (Epicentre Technologies) freshly diluted in 10 mM Tris-HCl (pH8.0), 10 mM KCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.4 mg ml⁻¹ bovine serum albumin, 0.1% Triton X-100 and 0.72 pmol His₁₀-AlgU in 3 µl of renaturation buffer. After 10 min on ice, RNA template (0.1 pmol) was added to the RNA polymerase/sigma-factor mixture and incubated in transcription buffer (40 mM Tris-HCl, pH7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT) for 10 min at 37°C. Transcription was carried out in a final volume of 20 µl and was initiated by addition of ATP, CTP, and GTP (200 µM each), 20 µM [α-³²P]-UTP (8 Ci mmol⁻¹) to the DNA template/core RNA polymerase/sigma-factor mix and samples incubated for 15 min at 37°C. The reaction was stopped and products separated on a sequencing gel. The rpoH P₁–4 47 bp DNA template used in the *in vitro* run-off assay was generated by PCR using the oligonucleotides HF-1 and HR-3 (5′-GGAAACAAGGATCAGGAGG-3′, +19 to +38 relative to the rpoH start codon).

**AlgU purification**

AlgU was purified from *E. coli* BL21(DE3) pLysS (Studier and Moffat, 1986) using the expression vector pETU-1610 as previously described (Schurr et al., 1995b). *E. coli* BL21(DE3) pLysS cells containing pETU-1610 were grown in LB supplemented with 30 µg of chloramphenicol and 40 µg of ampicillin to an OD₅₉₀ of 0.4. AlgU production was induced by the addition of 1 mM IPTG and incubated for 1 h. The cells were collected and lysed in metal chelate affinity chromatography buffer containing 8 M urea and 80 mM imidazole. The cell-free extract was chromatographed on an Ni-NTA agarose column washed with 100 mM imidazole. AlgU was eluted from the column by the addition of 200 mM imidazole. Fractions containing AlgU were pooled and AlgU was renatured prior to use as described above.

**Acknowledgements**

This study was supported by Grant AI31139 from the National Institute of Allergy and Infectious Diseases. M.J.S. was supported by Grant SCHURR95IO from the Cystic Fibrosis Foundation.

**References**


