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The *pilE* Gene of *Neisseria gonorrhoeae* MS11 Is Transcribed from a σ^{70} Promoter during Growth In Vitro

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Type 4 pili are essential for virulence in *Neisseria gonorrhoeae*. The gonococcal pilin subunit is encoded by *pilE*, upstream of which three putative promoter sequences (P1, P2, and P3) have been identified. P1 and P2 are σ^{70}-like promoters and are functional when a *PpilE*::*cat* transcriptional fusion is expressed in *Escherichia coli* DH5α. P3 is σ^{54} dependent and overlaps the P1 sequence. Site-directed mutagenesis of the *pilE* promoters followed by transcriptional analysis in *E. coli* indicated that in the absence of an appropriate activator protein, binding of RNA polymerase-σ^{54} to P3 inhibits transcription from P1 on the order of 30-fold. Transcription from P3 was undetectable in *E. coli*. However, PilR-dependent, P3-associated expression was detected in *Pseudomonas aeruginosa* PAK containing a *PpilE*::*cat* fusion, with P3 the only intact promoter. A similar analysis was performed on gonococcal reporter strains containing wild-type and mutated *PpilE*::*cat* cassettes recombined into the chromosome. In such piliated gonococcal recombinants cultured in vitro, P1 was responsible for *cat* expression and almost certainly for transcription of *pilE*. Transcription from P2 and P3 was not detectable under these conditions. Inhibition of transcription from P1 by σ^{54} binding to P3 was not apparent in *N. gonorrhoeae* MS11-A, suggesting that σ^{54} was either absent or unable to bind to P3 in these cells.

The type 4 pili produced by virulent isolates of *Neisseria gonorrhoeae* are thought to mediate specific adhesion to human mucosal cells, initiating the infectious process (13). The *pilE* gene, encoding the pilin subunit in *N. gonorrhoeae* MS11, has been cloned and sequenced, and a transcription start point (tsp) has been mapped 144 bp upstream of the ATG start codon (26). This tsp was located appropriately 6 bp downstream of a consensus −10 box of a σ^{70} promoter, although no sequence resembling a −35 box was identified.

Following the cloning and sequence analysis of type 4 pilin genes from organisms such as *Pseudomonas aeruginosa* (30) and the identification of a σ^{54} promoter as being responsible for expression of the pilin-encoding gene (*pilA*) in that organism (18), the promoter region of the gonococcal *pilE* gene was reexamined. It was recognized that a consensus binding sequence for σ^{54} (GG-N_{10}-GC) overlaps the previously identified σ^{70} promoter, with a predicted tsp within 3 bp of that mapped by Meyer et al. (26). By extrapolating from the results obtained from studies on *pilA* expression in *P. aeruginosa*, it was later reported that the putative σ^{54} promoter upstream of the gonococcal *pilE* gene was responsible for the expression of that gene (15, 40).

Subsequent studies were aimed at determining which of these two promoters was responsible for expression of the cloned gonococcal *pilE* gene in *Escherichia coli* (10). We reported that in an *rpoN* mutant, *pilE* was overexpressed to a level that was lethal to the *E. coli* cells. This was confirmed following the construction of a fusion between the *pilE* promoter and a promoterless *cat* gene (*PpilE*::*cat*), which allowed the level of transcription from the *pilE* promoter in various genetic backgrounds to be quantified. It has been reported (34) that the RNA polymerase (RNAP)-σ^{54} holoenzyme is able to spontaneously bind to and form a closed complex with its target sequence. However, in the absence of protein-protein contact between RNAP and an activator protein bound to an upstream activator site (UAS), the enzyme cannot initiate transcription through formation of an open complex. We proposed that in the absence of the appropriate activator protein, the RNAP-σ^{54} holoenzyme acts as a transcriptional repressor of *pilE* by inhibiting the binding of RNAP-σ^{70} to the overlapping promoter and subsequent transcription initiation (10).

Evidence that σ^{54}-dependent transcription of *pilE* can occur in *E. coli* was obtained following the introduction of a cloned *nifA* gene into an *E. coli* strain containing a *pilE*::*lacZ* transcriptional fusion. The increased level of β-galactosidase activity observed in this system was dependent on a functional *rpoN* gene (4).

Here we report the identification of a second putative σ^{70} promoter upstream of *pilE* with activity in *E. coli*. The tsp associated with this promoter is 37 bp downstream of the previously mapped tsp for *pilE* (26). Consequently, we have designated the *pilE* promoter sequences identified so far as P1 (upstream σ^{70}), P2 (downstream σ^{70}), and P3 (overlapping P1, σ^{54} dependent).

Site-directed mutagenesis of these promoter sequences and subsequent transcriptional analysis of *PpilE*::*cat* fusion constructs in *E. coli*, *P. aeruginosa*, and *N. gonorrhoeae* revealed major differences in promoter activity in each of these backgrounds. These observations have implications for the understanding of the regulation of *pilE* expression in *N. gonorrhoeae*.

(A preliminary report of these findings was presented at the Ninth International Pathogenic Neisseria Conference, Winchester, England, September 1994.)

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strain used in all cloning experiments and transcriptional analyses was DH5α [F⁻ *endA1 thi-1 hsdR17 supE44 relA1* Δ*lacU169* (ϕ80 *lacZ*ΔM15)]. The *rpoN* mutant strain YMC18 was derived from YMC10 (35). The *N. gonorrhoeae* strain used was MS11-A (38). Gonococcal *PpilE*::*cat* reporter strains JKD351, JKD352, JKD353, and JKD354 were derived from MS11-A. The wild-type *P. aeruginosa* strain used was PAK (David Bradley, Memorial University of Newfoundland); the *pilR* mutant PAK R94 has

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been described previously (14). Plasmid pJKD860 containing the wild-type promoter *PpilE*::*cat* fusion has been described previously (10). Plasmid pJKD862 was constructed by subcloning the *PpilE*::*cat* fusion from pJKD860 on a *Hin*dIII-*Nhe*I fragment into pGEM3-z (Promega) digested with *Hin*dIII and *Xba*I. The plasmids containing mutated promoter *PpilE*::*cat* cassettes were derived from pJKD862 as described under Recombinant DNA techniques. For transcriptional analysis of the *pilE* promoters in *E. coli*, *Bam*HI fragments containing the wild-type or mutated promoter *PpilE*::*cat* fusions were subcloned into the *Bam*HI site of pBR322 (2) in the opposite orientation from the *tet* gene. The reporter plasmids thus generated were pJKD1477 ($P1^+$ $P2^+$ $P3^+$), pJKD1253 ($P1^+$ $P2^+$ $P3^-$), pJKD1251 ($P1^+$ $P2^-$ $P3^+$), pJKD1531 ($P1^-$ $P2^+$ $P3^+$), pJKD1471 ($P1^+$ $P2^-$ $P3^-$), pJKD1490 ($P1^-$ $P2^+$ $P3^-$), pJKD1489 ($P1^-$ $P2^-$ $P3^+$), and pJKD1488 (promoterless *cat*). Plasmid DNA was transformed into *E. coli* DH5α. The *P. aeruginosa* plasmid pJKD731 was constructed by subcloning the *PpilE*::*cat* *Bam*HI cassette ($P1^-$ $P2^-$ $P3^+$) from pJKD1489 into pAH121 (17) digested with *Bam*HI.

Media and culture conditions. *E. coli* and *P. aeruginosa* strains were routinely grown at 37°C in Luria-Bertani (LB) medium (36). *E. coli* plasmids were maintained with ampicillin (50 μg ml^{-1}), whereas *P. aeruginosa* plasmids were maintained with carbenicillin (500 μg ml^{-1}). Preparation of competent cells and transformation of *E. coli* were performed by the method of Hanahan (12). Transformation of *P. aeruginosa* was performed as previously described (8). Gonococcal strains were routinely cultured on GC agar base (Oxoid) or in GC broth supplemented with 1% (vol/vol) DMIV, a modified form of IsoVitaleX (37). When appropriate, GC plates were supplemented with chloramphenicol (10 μg ml^{-1}). Plate cultures were incubated in a 5% CO_2 atmosphere, whereas GC broth was supplemented with 1% (vol/vol) $NaHCO_3$ as a CO_2 source. Gonococcal transformations were performed as previously described (1). Chloramphenicol-resistant (Cm^r) transformants were selected on GC plates containing chloramphenicol (10, 8, and 0 to 15 μg ml^{-1} gradient). Transformant colonies were detected following 24 h of incubation.

Recombinant DNA techniques. All DNA manipulations were performed by standard procedures (36). DNA sequencing was performed with the PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems), and DNA was analyzed on an Applied Biosystems model 373A DNA sequencing system. Oligonucleotide primers were synthesized on an Applied Biosystems 381A oligonucleotide synthesizer and eluted from columns according to the manufacturer's instructions.

The oligonucleotide primers used in site-directed mutagenesis of the *pilE* promoters were 278 (5′-AACTGCGTGCTAGCAAGCAAGA-3′) and the complement 231; 346 (5′-CCGATGGATCGATACATTGC-3′) and the complement 347; and 383 (5′-GATTCGTGATTTTATG-3′) and the complement 384. The strategy used was essentially as described previously (16). Overlapping DNA fragments were amplified by PCR with an FTS-1 Thermal Sequencer (Corbett Research), using 30 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C, followed by one cycle of 1 min at 94°C, 1 min at 45°C, and 5 min at 72°C. Low-melting-temperature agarose gel slices containing the appropriate DNA fragments were used as the template in PCR fusion reactions, which involved 30 cycles of 1 min at 91°C, 1 min at 37°C, and 2 min at 72°C, followed by 1 min at 91°C, 1 min at 37°C, and 5 min at 71°C. The PCR fusion products containing the mutated *pilE* promoter regions were digested with *Ava*I and *Sau*I and ligated with pJKD862 that had been similarly digested. The inserts contained in the resulting clones were sequenced on both strands to verify the introduced nucleotide changes and to check that no PCR errors had been incorporated.

PCR amplification of a 2.9-kb DNA fragment from the gonococcal *iga* gene was performed with MS11-A chromosomal DNA as the template and oligonucleotide primers 968 (5′-ATAGCCCGACGTCTACGACG-3′) and 970 (5′-CTATGAACCCCATAAAGCTTG-3′). Thirty cycles of 1 min at 92°C, 1 min at 55°C, and 1 min at 72°C were followed by one cycle of 1 min at 92°C, 1 min at 55°C, and 5 min at 72°C. To make ends flush for blunt-end cloning, the resulting product was treated according to the Double Geneclean protocol (Bresatec) and cloned into the *Sma*I site of pUC18 (28).

Primer extension analysis. Total RNA was extracted from exponential-phase cultures of *E. coli*, *P. aeruginosa*, or *N. gonorrhoeae* by the single-step method (6) with Trisolv (Biotecx Laboratories Inc.). The extraction procedure was essentially that described by the manufacturer except that following homogenization and prior to the addition of chloroform, the homogenate was heated at 65°C for 10 min and then cooled to room temperature. This modification resulted in an increased yield of high-molecular-weight RNA species. The oligonucleotide primers 935 and 1768, complementary to the *pilE* and *PpilE*::*cat* sequences, respectively, were 5′-end labelled with 30 μCi of [γ-^{32}P]ATP (at 3,000 Ci $mmol^{-1}$) and T4 polynucleotide kinase, and primer extension reactions were performed as described in the instructions for use of the avian myeloblastosis virus reverse transcriptase primer extension system (Promega). Fifty micrograms of total RNA was used in each reaction, and half of the precipitated products were subjected to electrophoresis in an 8% polyacrylamide gel (8 M urea), next to a sequencing ladder generated from plasmid pJKD862 with primer 1768. Gels were then subjected to autoradiography.

Determination of CAT levels in total cell extracts. Cell extracts of *E. coli* strains for the determination of chloramphenicol acetyltransferase (CAT) levels were prepared from exponential-phase cultures by passaging through a French pressure cell (Aminco, Silver Spring, Md.). Gonococcal cell extracts were prepared from exponential-phase cultures by repeated freezing and thawing. Protein concentration was determined by the method of Lowry et al. (24), with bovine serum albumin as the standard. CAT levels were determined with the CAT enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim) according to the manufacturer's instructions. The A_{405} of samples was read with a Bio-Rad microplate reader (model 450).

RESULTS AND DISCUSSION

Mapping of *pilE* transcription start points in *N. gonorrhoeae* and *E. coli*. We prepared total RNA from exponential-phase cultures of the gonococcal strain MS11-A and *E. coli* DH5α containing pJKD1477. Figure 1A shows the results of primer extension analysis of 25 μg of total RNA from these strains. The binding sites of primers 935 and 1768 on the *pilE* and *PpilE*::*cat* sequences, respectively, are shown in Fig. 1B. The main primer extension product (P1a) obtained for MS11-A, 6 bp downstream of the TATAAT hexamer (Fig. 1A), was consistent with the tsp mapped previously (26). An equivalent primer extension product was obtained for DH5α(pJKD1477), but at a lesser intensity. In both cases, a second band (P1b), representative of a tsp 3 nucleotides (nt) further downstream, was observed. P1b was a less abundant product in the case of MS11-A, whereas for DH5α(pJKD1477), the two bands were of equal intensity.

Neither P1a nor P1b was the main primer extension product obtained for DH5α(pJKD1477). In this case, an intense band (P2a), 37 nt smaller than P1a, was observed, with a less intense band (P2b), 2 nt larger than P2a. Figure 1B shows that these two products represent a tsp 6 to 8 bp downstream of a sequence, TTAAAT, which is preceded, 17 bp upstream, by the hexamer TTGTCG. Both of these sequences have 4 of 6 bp identical with the consensus −10 and −35 boxes of a σ^{70} promoter. Minor bands at positions equivalent to P2a and P2b were observed for MS11-A. However, they appeared to represent part of a ladder of products extending a further 8 nt upstream, which may have formed as a result of reverse transcriptase pausing, often associated with A-U-rich regions.

Site-directed mutagenesis of putative *pilE* promoters and measurement of their activities in *E. coli*. It was possible from the primer extension results (Fig. 1A) that P1a and P1b represented the tsps associated with the overlapping promoters P1 and P3. To determine whether this was the case, and also to verify the promoter activity of P2, site-directed mutagenesis of the *pilE* promoter sequences was performed, introducing the nucleotide changes shown in Fig. 2. These changes were expected to prevent the recognition of the individual promoters by the appropriate σ factors. Total RNA was prepared from mid-exponential-phase DH5α cells containing each of the seven *PpilE*::*cat* reporters, and primer extensions were performed with radiolabelled 1768 to prime the reactions. The results shown in Fig. 3A clearly indicated that the products designated P1a and P1b (Fig. 1A) were both associated with the σ^{70} promoter, P1. Changing the sequence of the −10 box from TATAAT to GCTAGC resulted in the loss of both of these products (Fig. 3A, lanes 1, 2, and 3). In addition, an increased abundance of P1a and P1b was observed when the P3 −24 box was changed from TCGGC to TGATC (Fig. 3A, lanes 4 and 6). The same intense bands corresponding to P1a and P1b were observed when a primer extension was performed on RNA prepared from an *E. coli rpoN* mutant (35) containing the wild-type *PpilE*::*cat* reporter pJKD1477 (data not shown). These results are consistent with our previous hypothesis (10) that transcription from P1 is repressed by the binding of σ^{54} to the overlapping P3 promoter sequence.

Figure 3A (lanes 1 and 5) shows that when the putative −10 box of P2 was changed from TTAAAT to ATCGAT, both the

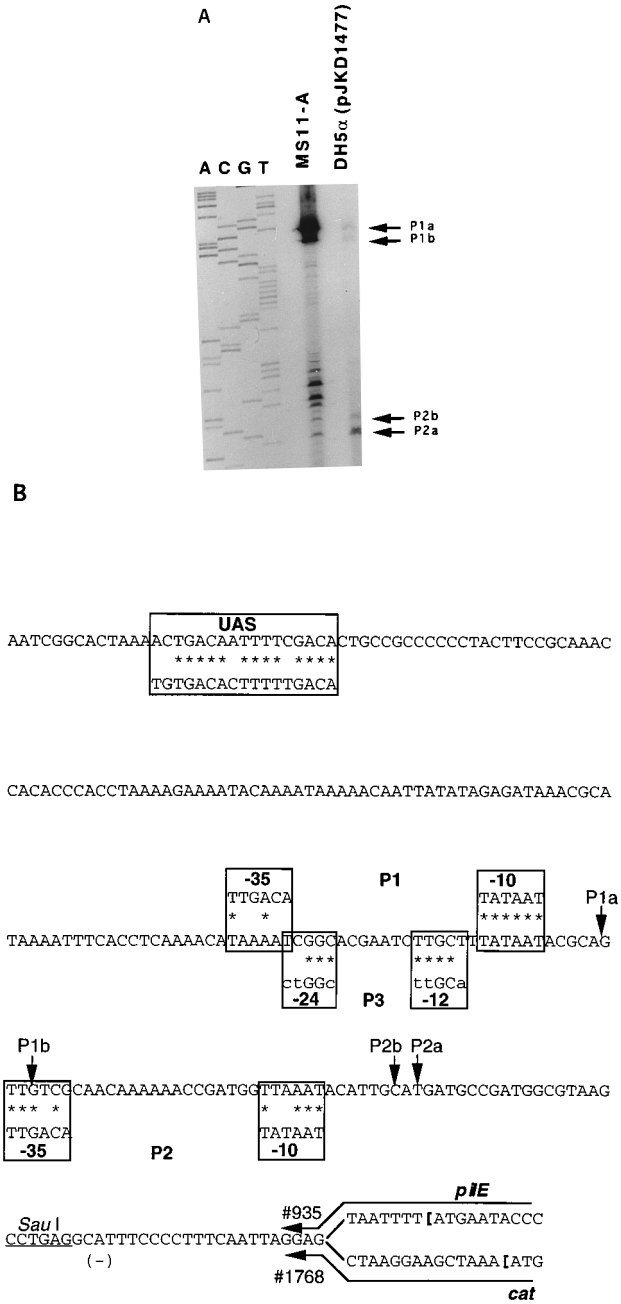

FIG. 1. Identification of *pilE* promoter activity in *E. coli* and *N. gonorrhoeae*. (A) Primer extension analysis of RNA prepared from gonococcal strain MS11-A and *E. coli* DH5α(pJKD1477). The reactions were primed with radiolabelled oligonucleotides 935 and 1768, respectively. A sequencing ladder (ACGT), complementary to the coding strand, was generated from pJKD862 (*PpilE*::*cat*) with primer 1768. Reaction mixes were electrophoresed through an 8% polyacrylamide sequencing gel. (B) Nucleotide sequence of the *pilE* promoter region of MS11-A, showing the primer binding site for 935, and the *PpilE*::*cat* fusion region, showing the binding site for primer 1768. The putative −10 and −35 hexamers of P1 and P2 are boxed along with the consensus sequences for a σ^{70} promoter. Likewise, the −24 and −12 boxes of P3 are compared with the σ^{54} consensus sequences. The boxed region labelled UAS is compared with the putative UAS upstream of the *P. aeruginosa pilA* gene (31). In all cases, asterisks indicate identical nucleotides. Tsps identified by primer extension (P1a, P1b, P2a, and P2b) are indicated. A minus sign in parentheses beneath a nucleotide indicates that it is absent in the cloned *pilE* gene (10).

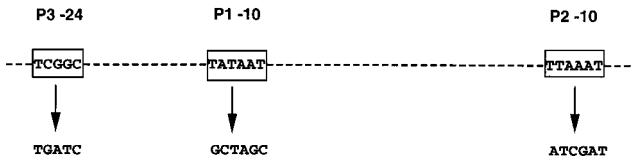

FIG. 2. Site-directed mutagenesis of the gonococcal *pilE* promoters. The nucleotide changes were introduced into the −10 box of P1 (with complementary primers 278 and 231), the −10 box of P2 (primers 346 and 347), and the −24 box of P3 (primers 383 and 384).

P2a and P2b bands disappeared from the primer extension products associated with plasmids pJKD1489 ($P1^-$ $P2^-$ $P3^+$) and pJKD1251 ($P1^+$ $P2^-$ $P3^+$). However, in the case of pJKD1471 ($P1^+$ $P2^-$ $P3^-$), with derepressed P1 activity, a product of the same size as P2a was observed, along with several larger products (Fig. 3A, lane 4). A likely explanation for these extra bands, as in the case of those observed for MS11-A (Fig. 1A), is that they are artifacts caused by reverse transcriptase pausing. Clearly, they are not associated with other promoters, as they are not observed in the absence of the P1 products.

A quantitive measure of the level of transcription from each promoter individually and in combination with the others was obtained by performing CAT ELISAs on total cell extracts prepared from the same *E. coli* strains harvested in exponential phase. The CAT levels detected in these extracts, shown in Fig. 3B, were consistent with the primer extension results. The difference in CAT levels between DH5α(pJKD1471) with P1 as the only intact promoter (7 ng of protein μg^{-1}) and DH5α(pJKD1251) with both P1 and P3 intact (0.23 ng of protein μg^{-1}) indicated that RNAP-σ^{54} binding to P3 inhibited P1 transcription on the order of 30-fold.

No transcriptional activity associated with P3 (or any other sequence other than P1 and P2) was detected on the basis of CAT levels. The most likely explanation for this observation was the lack of an appropriate activator protein in *E. coli*. However, the possibility remained that in the process of mutating the P1 −10 box, the nucleotide changes introduced immediately downstream of the P3 −12 box, although not altering the consensus sequence per se, may have resulted in reduced binding of σ^{54} to this promoter.

Detection of transcription from the σ^{54} promoter P3 in *P. aeruginosa*. In a previous study (10), we identified a sequence (UAS) 140 bp upstream of the predicted tsp for P3 (see Fig. 1B), which has 13 of 17 bp identical to a sequence upstream of the *pilA* promoter region in *P. aeruginosa* thought to be involved in the transcriptional activation of that gene (31). The activator protein associated with enhanced transcription of *pilA* (from a σ^{54} promoter) is the product of the *pilR* gene (14, 19).

To determine whether transcription from P3 due to activation by the PilR protein could be detected in *P. aeruginosa*, the *PpilE*::*cat* cassette was subcloned on a *Bam*HI fragment from pJKD1489 ($P1^-$ $P2^-$ $P3^+$) into the *P. aeruginosa* vector pAH121 (17) to yield pJKD731. *P. aeruginosa* PAK and PAK R94 (a *pilR* mutant) were transformed with pJKD731, total RNA was prepared, and primer extensions were performed with oligonucleotide 1768 to prime the reactions. A P3-associated product, in the form of a doublet, was obtained from RNA prepared from PAK(pJKD731) but not from PAK R94(pJKD731), as shown in Fig. 4. The size of the larger, more abundant product was consistent with a tsp 2 bp downstream of the predicted tsp for P3 and was 1 nt larger than the P1-

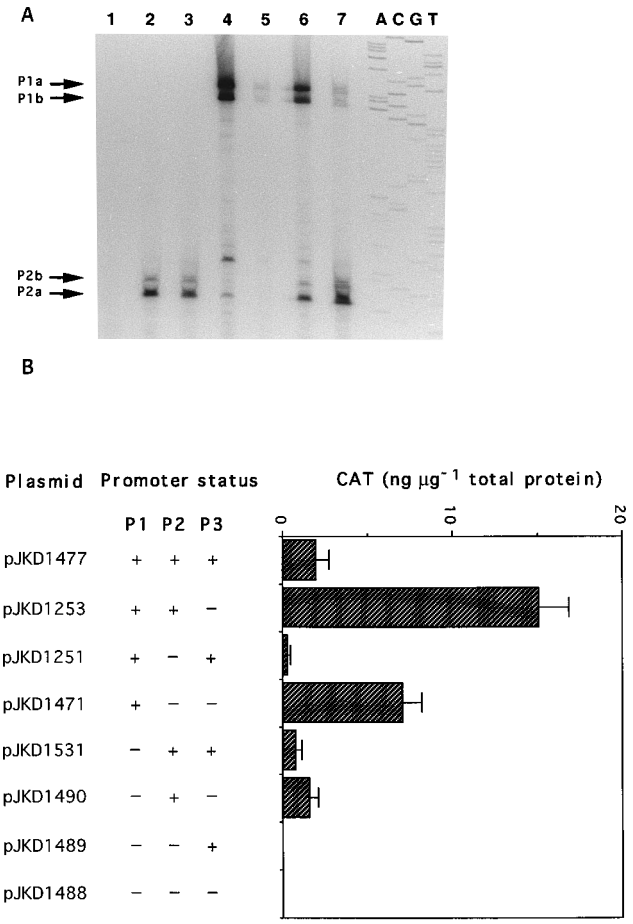

FIG. 3. Transcriptional analysis of wild-type and mutated *PpilE*::*cat* fusions in *E. coli*. (A) Primer extension reactions performed on RNA from DH5α cells containing the *PpilE*::*cat* reporter plasmids. Reactions were primed with radiolabelled oligonucleotide 1768, the same primer used to generate the sequencing ladder (from pJKD862 DNA), which is complementary to the coding strand (ACGT). Reaction mixes were electrophoresed through an 8% polyacrylamide sequencing gel. The products associated with each plasmid (for promoter status, see Fig. 3B) are as follows: lane 1, pJKD1489; lane 2, pJKD1490; lane 3, pJKD1531; lane 4, pJKD1471; lane 5, pJKD1251; lane 6, pJKD1253; and lane 7, pJKD1477. (B) CAT levels present in total cell extracts prepared from DH5α cells containing the same reporter plasmids. The results shown are averages for four separate experiments. Error bars represent one standard deviation.

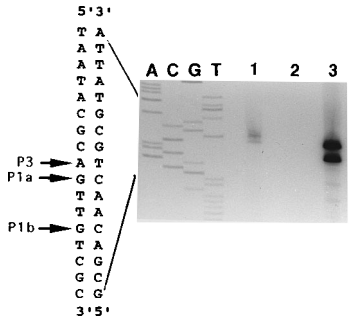

FIG. 4. Demonstration of transcription from the σ^{54} *pilE* promoter (P3) in *P. aeruginosa*. Primer extensions were performed on RNA prepared from strains PAK and PAK R94 (a *pilR* mutant), each containing the *PpilE*::*cat* reporter plasmid pJKD731 ($P1^-$ $P2^-$ $P3^+$). The products of these reactions are shown in lanes 1 and 2, respectively. A similar reaction performed on RNA from *E. coli* DH5α containing pJKD1471 ($P1^+$ $P2^-$ $P3^-$), shown in lane 3, was included for comparison. All extension reactions were primed with radiolabelled oligonucleotide 1768, the same primer used to generate the sequencing ladder, from pJKD862 DNA, which is the complement of the coding strand. Reaction mixes were electrophoresed through an 8% polyacrylamide sequencing gel. The tsps labelled P1a and P1b on the corresponding sequence are associated with promoter P1. The position of the tsp associated with the P3 promoter is labelled P3.

associated product P1a obtained with RNA from *E. coli* DH5α(pJKD1471).

These results led to the conclusions that (i) the P3 σ^{54} promoter was functional in *P. aeruginosa*, despite the nucleotide changes introduced immediately downstream, and (ii) the *P. aeruginosa pilR* gene product was capable of activating the gonococcal σ^{54} *pilE* promoter. The PilR binding site, within the previously identified UAS, upstream of *pilA*, has recently been characterized (21). Studies are under way in our laboratory to determine whether the sequence upstream of *pilE*, displaying similarity to the *pilA* UAS, is in fact responsible for PilR activation of P3.

Construction of gonococcal reporter strains containing *PpilE*::*cat* cassettes within the *iga* gene. We then determined which of the promoters identified as playing a role in transcription of *pilE* in either *E. coli* or *P. aeruginosa* was active in gonococcal strain MS11-A. The comparison of primer extension products obtained with gonococcal and *E. coli* RNA (Fig. 1A) suggested that in contrast to the situation demonstrated for the cloned gene in *E. coli*, P1, and perhaps P3, was responsible for the majority of *pilE* transcription in MS11-A.

Transcription from P2 was apparently insignificant when gonococci were grown under these conditions. Before a detailed analysis of promoter activity in MS11-A could be performed, it was necessary to construct a series of vectors, each containing a *PpilE*::*cat* cassette (with wild-type or mutated promoters) subcloned into a suitable region of target DNA (Fig. 5). Because the efficiency of recombination of a heterologous sequence into the gonococcal chromosome depends on the size of the heterologous region relative to the flanking homologous sequences (3), we decided that at least 1 kb of DNA homologous to the target region was required both upstream and downstream of the *PpilE*::*cat* cassettes to ensure efficient recombination. The target sequence chosen for incorporation into these vectors was a region of the *iga* gene (encoding immunoglobulin A [IgA] protease), being a large nonessential gene (at least for growth in vitro) of known sequence (33). Consequently, oligonucleotide primers 968 and 970 were designed to amplify, by PCR, a 2.9-kb region of *iga* from the chromosome of MS11-A. This region corresponded to nt 505 to 3450 of the published sequence (33) and was flanked by *Hin*dIII and *Acc*I sites incorporated into the primers, with a unique *Bgl*II site 1.3 kb downstream of the *Hin*dIII site. The *PpilE*::*cat Bam*HI cassettes were subcloned into this *Bgl*II site.

As neither the *pilE* promoter nor the 2.9-kb *iga* fragment contained a copy of the gonococcal transformation uptake signal (11), low transformation frequencies (10^{-7} per μg of linearized plasmid DNA) were obtained for these initial constructs. To overcome this problem, a 300-bp *Sph*I-*Bam*HI DNA fragment containing a region upstream of the gonococcal *recA* gene, including two copies of the transformation uptake signal, was subcloned from pJKD1499 into each of the vectors, as shown in Fig. 5. When 1 μg of linearized pJKD1502 ($P1^+$ $P2^+$ $P3^+$) was transformed into MS11-A, approximately 10^5 Cm^r colonies were obtained on plates containing 10 μg of chloramphenicol per ml. Similar numbers of Cm^r recombinants were observed for all the constructs containing an intact P1 promoter (pJKD1518, pJKD1520, and pJKD1521), regardless of the status of P2 and P3. However, no transformants were obtained under similar conditions for any of the plasmids with a mutated P1 promoter (pJKD1519, pJKD1522, and

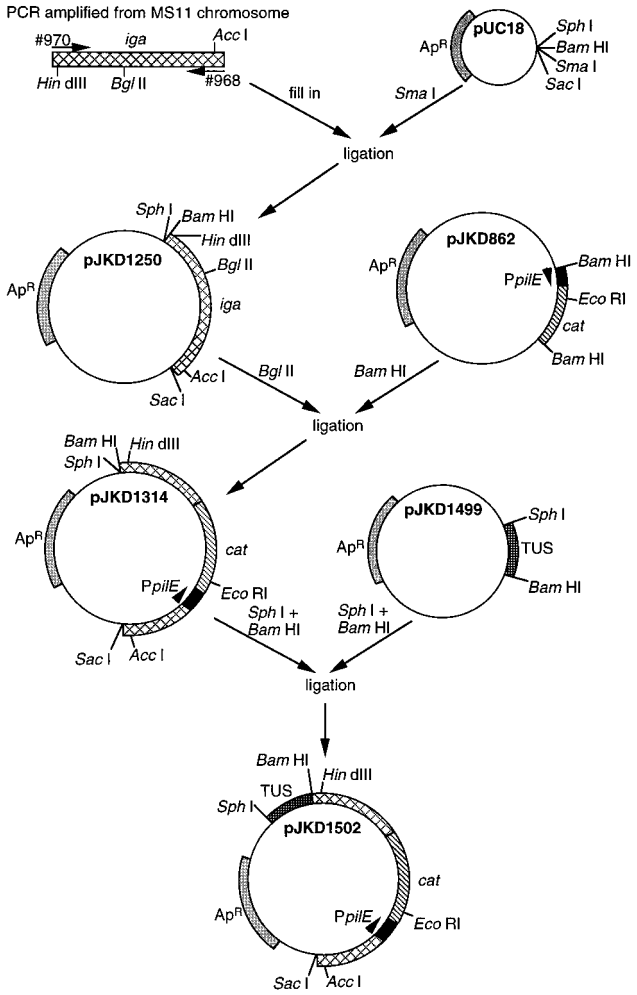

FIG. 5. Construction of pJKD1502, containing the wild-type *PpilE*::*cat* fusion, flanked by regions of the gonococcal *iga* gene. pJKD1499 is a derivative of pUC18 containing the gonococcal transformation uptake signal (TUS) within a 300-bp *Sph*I-*Bam*HI fragment. Similar plasmids containing promoter-mutated *PpilE*::*cat* cassettes were constructed by the same strategy. These plasmids were pJKD1518 ($P1^+$ $P2^-$ $P3^-$), pJKD1519 ($P1^-$ $P2^+$ $P3^-$), pJKD1520 ($P1^+$ $P2^-$ $P3^+$), pJKD1521 ($P1^+$ $P2^+$ $P3^-$), pJKD1522 ($P1^-$ $P2^-$ $P3^+$), and pJKD1528 ($P1^-$ $P2^+$ $P3^+$).

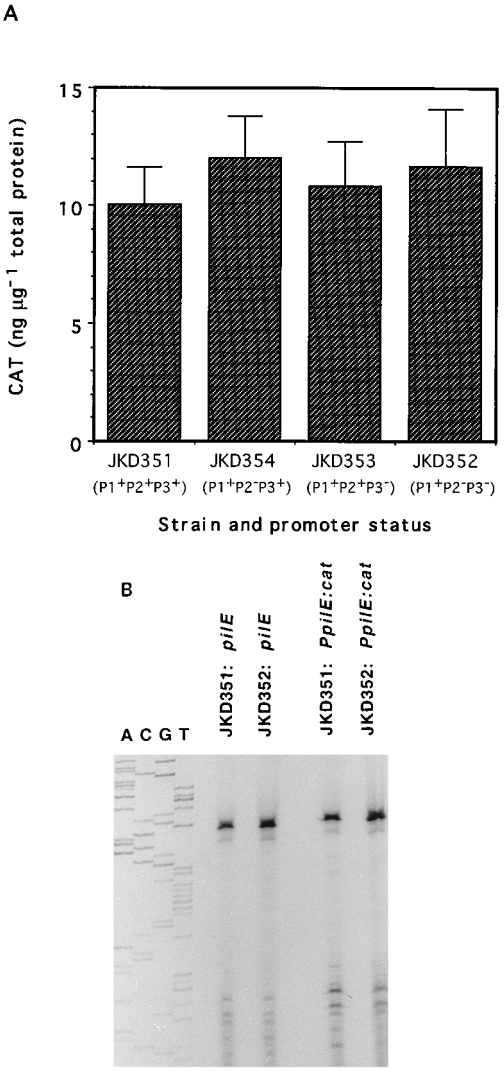

FIG. 6. Determination of the effect of P2 and P3 promoter mutations on the expression of *PpilE*::*cat* in *N. gonorrhoeae*. (A) CAT levels present in whole cell extracts were determined for the gonoccocal reporter strains JKD351, JKD352, JKD353, and JKD354. The results shown are averages for four independent experiments. The error bars represent one standard deviation. (B) Primer extension analysis of RNA prepared from gonococcal reporter strains JKD351 and JKD352. Reactions to generate *pilE*-specific transcription products were primed with radiolabelled 935, whereas *PpilE*::*cat*-specific products were generated with radiolabelled 1768. The sequencing ladder, which is the complement of the coding strand, was generated by using pJKD862 DNA primed with 1768. All reaction mixes were electrophoresed through an 8% polyacrylamide sequencing gel.

pJKD1528), nor were they obtained when selection was made with chloramphenicol at 8µg ml^{-1} or on gradient plates (0 to 15 µg ml^{-1}), indicating that P2 and P3 were not active promoters in MS11-A under the conditions required for the growth of transformants.

Chromosomal DNA was prepared from a piliated, Cm^r colony from each of the successful transformations, and the appropriate *PpilE*::*cat* cassette was shown to be inserted into the *iga* gene in each case by a combination of Southern hybridization and PCR followed by DNA sequence analysis of the PCR products (data not shown). The reporter strains generated by this procedure were JKD351 ($P1^+$ $P2^+$ $P3^+$), JKD352 ($P1^+$ $P2^-$ $P3^-$), JKD353 ($P1^+$ $P2^+$ $P3^-$), and JKD354 ($P1^+$ $P2^-$ $P3^+$).

P1 appears to be responsible for *pilE* transcription in MS11-A growing exponentially in GC broth. The results of the primer extension experiment with RNA prepared from MS11-A (Fig. 1A), combined with the observed lack of Cm^r transformants for the P1-mutated *PpilE*::*cat* reporters, provided strong evidence that P1 was the only *pilE* promoter with significant activity in MS11-A grown in GC broth or on plates. The fact that P1 transcription under these conditions was not influenced by the presence of P2 and P3 consensus sequences was shown by the CAT levels detected in total cell extracts prepared from mid-exponential-phase cultures of reporter strains JKD351, JKD352, JKD353, and JKD354 (Fig. 6A). In addition, Fig. 6B shows that the sizes of the *pilE*-specific and *PpilE*::*cat*-specific primer extension products obtained from JKD351 ($P1^+$ $P2^+$ $P3^+$) and JKD352 ($P1^+$ $P2^-$ $P3^-$) were the same.

The immunological detection of a σ^{54} analog in both whole cell extracts and purified preparations of RNAP from gonococcal strain F62 has been reported (23), although the protein

reacting with the monoclonal antibody was larger than expected. No σ^{54}-dependent *pilE* transcription in gonococcal strain MS11-A was detected in this study. This may have been due to insufficient levels of a functional activator protein under these growth conditions. However, unlike the situation observed when the gonococcal *pilE* gene was expressed in *E. coli*, there was no evidence that in the PpilE::cat reporter strains derived from MS11-A, RNAP-σ^{54} binding to the P3 consensus sequence had an inhibitory effect on P1 transcription. There are several possible explanations for this. First, the σ^{54} *pilE* promoter may be redundant in gonococci. This is unlikely, as not only the promoter sequence but also the putative UAS are highly conserved among gonococcal strains, and in addition, the promoter is functional in *P. aeruginosa* (Fig. 4). An alternative explanation is that the cells did not contain significant levels of the gonococcal σ^{54} analog or that the σ factor has a low affinity for the promoter in gonococci grown under these conditions, possibly due to the DNA topology.

The *rpoN* gene has been shown to form part of an operon in several bacterial species, including *E. coli* (22), *Klebsiella pneumoniae* (25), *Alcaligenes eutrophus* (41), and *P. aeruginosa* (20). In each case, the products of genes cotranscribed with *rpoN* appear to either negatively regulate σ^{54} activity or act as a coinducer of some σ^{54}-dependent genes. The activity of the alternative σ factor, σ^{F}, in *Salmonella typhimurium* and *Bacillus subtilis* has been shown to be regulated by an anti-σ factor which binds directly to σ^{F}, disturbing its ability to form a complex with RNAP (7, 27, 29). It is possible that the activity of the gonococcal σ^{54} analog is tightly regulated by one of these mechanisms or, alternatively, that MS11-A is an *rpoN* mutant. Experiments are under way in our laboratory to try to resolve this question.

It is interesting that the P1 promoter is apparently so efficient, considering that it lacks a consensus -35 box. One possible explanation is that it is subject to activation, perhaps via the putative activator PilA, which is a homolog of FtsY in *E. coli* (39). Alternatively, the AT-rich sequence upstream of the -35 region, a region likely to be inherently flexible (32), may be acting as an extra RNAP recognition site. Such upstream promoter elements have recently been shown to increase the strength of σ^{70} promoters in *E. coli* (5). It appears that P1 promoter activity is independent of the DNA-bending protein IHF (9), at least in *E. coli*, as a *PpilE*::*cat* reporter containing P1 only is expressed in both a *himA* and a *himD* mutant at the same level as in the wild-type parent strain (unpublished data).

Another question arising from these results is whether the second σ^{70} promoter, P2, is functional in *N. gonorrhoeae*. If so, it must be subject to repression during growth in GC broth but may be active under other conditions. It will be interesting to determine whether *pilE* transcription from P2 and/or P3 can be detected in gonococcal strain MS11-A grown under different in vitro conditions, or whether the activity of these promoters requires an environmental signal detected only in the human host.

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