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Mutations Affecting mRNA Processing and Fimbrial Biogenesis in the *Escherichia coli pap* Operon

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The *Escherichia coli pap* genetic determinant includes 11 genes and encodes expression of Pap pili on the bacterial surface. An RNase E-dependent mRNA-processing event in the intercistronic *papB-papA* region results in the accumulation of a *papA*-gene-specific mRNA in considerable excess of the primary *papB-papA* mRNA transcription product. We have introduced mutations in the intercistronic region and studied the effect in vivo of these mutations on the processing event, PapA protein expression, and the biogenesis of fimbriae on the bacterial surface. Our studies establish that mRNA processing is an important event in the mechanism resulting in differential gene expression of the major *pap* operon. The deletion of sequences corresponding to the major cleavage site abolished processing, reduced expression of PapA protein, and resulted in "crew-cut" bacteria with short fimbrial structures on the bacterial surface. Only a limited part of the intercistronic region appeared to be required as the recognized target for the processing to occur. Upstream sequences to a position within 10 nucleotides of the major RNase E-dependent cleavage site could be deleted without any detectable effect on *papB-papA* mRNA processing, PapA protein expression, or fimbria formation. Substitution mutations of specific bases at the cleavage site by site-directed mutagenesis showed that there were alternative positions at which cleavage could be enhanced, and tests with an in vitro processing assay showed that such cleavages were also RNase E dependent. Our findings are discussed in relation to other fimbrial operons and other known targets of the RNase E endoribonuclease.

Several genetic determinants encoding different fimbrial structures have been identified in pathogenic strains of *Escherichia coli*. Expression of fimbriae (also denoted pili) on the bacteria is thought to play an essential role in the pathogenic process leading to infection by enabling the bacteria to adhere to epithelial surfaces. A common property of many of these determinants is the organization of the genes into polycistronic operons. These operons determine the production of one major structural protein (the pilin), some minor pilins, and other proteins involved in the assembly process of the fimbriae (37). The major structural proteins of the different determinants are expressed at levels markedly higher than those of the other proteins of the operons. Differential mRNA stability and mRNA processing have been suggested to be mechanisms to achieve the observed differential expression of genes in some *E. coli* pili determinants (3, 7, 17, 24, 28, 39).

pap gene clusters are commonly found in uropathogenic *E. coli* isolates. Studies of *pap* genes (*papBAHCDJKEFG*) from *E. coli* J96 suggest that expression includes mRNA processing, differential mRNA stability, and partial termination or attenuation (3, 28, 39). The two first cistrons expressed from the *papB* promoter, *papB* and *papA*, are cotranscribed to mRNA (mRNA-BA). A minor fraction of transcription proceeds past a terminator signal in between the second and third cistrons (*papAH*) in a region where dyad symmetry in the nucleotide sequence would allow the mRNA to form a stem and loop structure (2). Differential expression of the first two genes (*papBA*), at least to some extent, results from endonucleolytic cleavage of the mRNA in the intercistronic *papB-papA* region. That cleavage is followed by rapid decay of the upstream *papB*-coding region and accumulation of the stable *papA*-coding mRNA (mRNA-A) as shown by direct analysis of the mRNA in half-life studies and by Northern (RNA) blotting analysis (3, 28). The endonucleolytic activity is RNase E dependent, as evidenced by the use of *rne* mutant *E. coli*, and the cleavage is the rate-limiting step both for *papB* mRNA degradation and for generation of mRNA-A (28). Accumulation of the translationally active mRNA-A leads to high-level expression of the major pilin, PapA, relative to that of a regulatory protein, PapB (28).

The enzyme activities involved in the control of mRNA stability are still not fully understood, even though the importance of these cellular processes has been duly recognized (see, e.g., Belasco and Brawerman [4] for recent reviews). Several activities are known to participate in the processing and degradation of RNA in *E. coli* (5, 11). The endoribonuclease RNase E, which is encoded by the *rne* gene (allelic to *ams*), seems to have a central role, since average mRNA half-lives are increased in strains deficient for this activity (1, 23, 26, 29). For some specific *E. coli* mRNAs the initiation of decay has been shown to be *rne* dependent (18, 25–28, 31, 42). It is feasible that the decay of most mRNAs is initiated by endonucleolytic cleavages, followed by exonucleolytic degradation. The rate of decay for a specific mRNA is then, to a large extent, determined by how well it is recognized as a substrate by endonuclease activities.

In spite of great efforts, it has not yet been possible to define what makes up an RNase E recognition site. Ehretsmann et al. (12), by mutational analysis of the RNase E cleavage site in the mRNA from gene 32 of phage T4 and by comparison of putative RNase E cleavage sites in other molecules, have suggested a consensus nucleotide sequence at the cleavage site. It is obvious that this consensus sequence, RAUUW (R = A or G, W = A or U), is not enough to explain the specificity of cleavage by the enzyme. It was also shown that a putative

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stem-loop structure located 3′ of the cleavage site in this molecule was required for the cleavage event. A hairpin structure 3′ of the site of cleavage seems to be a common feature of many of the putative RNase E substrates and has previously been suggested to be a part of the recognition motif (27).

RNA processing may be a mechanism for achieving differential expression of genes in several *E. coli* pilus determinants. Our aim was to establish the importance of RNA processing in the *pap* operon for pilus formation on the bacterial surface and to learn more about the genetic information recognized by the endonuclease. By genetic analysis we wanted to assess the role of the putative target sequences in the region where the *pap* transcript is processed. We constructed a set of *pap* operon derivatives with mutations in or near the known RNase E-dependent cleavage site in the intercistronic *papB-papA* region. We studied the effect of these mutations in vivo on fimbria formation, expression of PapA protein, and the RNA-processing event.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* HB101 (9) was used as host strain for the different mutant *pap* plasmids. For analysis of *pap* expression, strains were grown in 1.5% Casamino Acids medium supplemented with medium E (41) and 1 μg of thiamine per ml. During mRNA half-life experiments, 200 μg of rifampin per ml was added to the medium to inhibit transcription. The antibiotic carbenicillin at a concentration of 50 μg/ml was used for selection of plasmid-containing cells. The strains used for preparation of RNase E enzyme extracts (see below) were N3433 (*lacZ43 relA1 spoT1 thi-1*) and N3431 (*lacZ43 relA1 spoT1 thi-1 rne-3071*[Ts]), as described by Goldblum and Apririon (16).

Plasmid constructions. The *pap* plasmids with different mutations in the intercistronic *papB-papA* region used in this report are all derivatives of pPAP5 (19). Recombinant DNA work was performed according to methods described by enzyme manufacturers and/or by published standard procedures (33). The two plasmids pPAP283 and pPAP284 carry part of the *pap* operon ($papI^+B^+A$::$lacZ^+$) in operon fusion constructs with *lacZ*, and there are small deletions in the intercistronic region of *papB* and *papA* with one endpoint at nucleotide position 1597 (3). pPAP291 was constructed by replacing the *Apa*I-*Hin*dIII fragment of pPAP5 with that of pPAP283 (a 37-nucleotide-deletion *pap* mutant), and pPAP292 was constructed from pPAP284 (a 69-nucleotide-deletion *pap* mutant) in the same way. pPAP2005 was made by inserting an 8-bp *Xho*I linker (CCTCGAGG) in the *Dra*I site at position 1710 of pPAP5, and pPAN2 has the same linker inserted in the *Eco*RV site at position 1720 of pPAP5. pPAN5 was made by joining the 12-kb *Xho*I-*Eco*RI fragment of pPAP2005 to the 1.6-kb *Eco*RI-*Xho*I fragment of pPAP284. In a similar fashion, pPAN3 was constructed by joining the 12-kb *Xho*I-*Eco*RI fragment of pPAN2 to the 1.7-kb *Eco*RI-*Xho*I fragment of pPAP2005. As a net result, in pPAN3 11 nucleotides from the original *pap* DNA were replaced by the 8 nucleotides of the *Xho*I linker. The deletion and insertion borders of all these plasmids have been verified by DNA sequencing directly on plasmid templates.

Site-directed mutagenesis. The plasmids pPAN11, pPAN12, pPAN13, and pPAN14 were made by site-directed mutagenesis by Amersham's oligonucleotide-directed in vitro mutagenesis system (version 2) according to the manufacturer's recommendations. The following primers were used (with the altered nucleotides shown in boldface, italic letters): M1, 5′-TTATTTGT***TGG***GATCT GGT; M2, 5′-TTATTT***C***TATTGAT***T***TGGT; M3, 5′-GAGAATATTAT***CA***G TATTGATCT; and M4, 5′-AATATTATTTGT***TG***TGATCTGGT.

mRNA analysis. RNA extractions were done according to the previously described hot-acidic phenol method (2). Northern blot analysis was done as described by Nilsson and Uhlin (28) except that the *papA*-specific probe was a 200-bp *Eco*RV-*Hin*dIII restriction DNA fragment (positions 1720 to 1948 in the *pap* sequence) labelled with [α-^{32}P]dATP. Amersham's multiprime DNA-labelling system was used for the labelling reactions. Primer extension analysis was done essentially as described previously with the primer ATAGCTACCGCAC CGGCA, which is complementary to mRNA-A from positions 1780 to 1797 (2). To obtain a size standard, dideoxy DNA sequencing was performed with the same primer on a pPAN5 plasmid DNA template with the Sequenase DNA sequencing kit (U.S. Biochemicals). The extension products were analyzed on denaturing 6% polyacrylamide gels as described elsewhere (2). Radioactive signals on dried gels and nylon membranes where visualized with a PhosphorImager (model 400S; Molecular Dynamics) or by autoradiography on X-ray film. Quantitations were performed with the software ImageQuant (Molecular Dynamics).

Western blot. Immunoblots were made essentially as described by Forsman et al. (15) with a PapA-specific polyclonal antiserum.

Electron microscopy. Bacterial suspensions in buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$) were allowed to sediment on copper grids coated with thin films of 2% Formvar. After negative staining with 1% sodium silicontungstate (pH 6.0), the grids were examined with a JOEL 1003 microscope.

In vitro processing assay. *E. coli* N3433 and N3431 were grown in Luria-Bertani medium at 30°C to early logarithmic growth phase. Cells were collected in a Beckman centrifuge and washed once with buffer A (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol). For preparation of enzyme extracts by a modification of a previously described procedure (36), cells were resuspended in 20 ml of buffer A and disrupted with a Heat Systems sonicator, model XL2022. Cell debris and unbroken cells were removed by centrifugation at 5,000 × *g* for 30 min. Solid $(NH_4)_2SO_4$ was added to a final concentration of 0.6 M. A high-speed supernatant (S200 extract) was prepared by centrifugation in a Beckman SW60 rotor at 50,000 rpm for 90 min. The extracts were further fractionated by precipitation with 26% (wt/vol) ammonium sulfate. The pellets were suspended in 10 ml of cold buffer A and then dialyzed against the same buffer with 50% glycerol (wt/vol) and stored at −20°C (AS 26 fractions). As templates for RNA synthesis in vitro, we used PCR-derived DNA fragments corresponding to 209 nucleotides of the wild-type *papBA* intercistronic region and deletion and substitution mutants of this region. The fragments in addition contained sequences corresponding to the T7 promoter Φ10 at their 5′ ends. The following oligonucleotide primers were used: Pr-1, 5′-P_{T7}Φ10-ATTATGGCATTCCGGAGTTTCT-3′, and Pr-2, 5′-AAATAACAACCTCTTTTTCATTACTGAAC-3′. P_{T7}Φ10 corresponds to the nucleotides 5′-TAATACGACTCACTATAGG-3′, which constitute the Φ10 promoter sequence of phage T7 (34).

To produce internally labelled transcripts, in vitro transcription was carried out with an RNA transcription kit (Stratagene) in the presence of [α-^{32}P]ATP according to the manufacturer's instructions. After the reaction, the T7 transcripts were purified on a 7.5 or 10% polyacrylamide gel containing 7 M urea.

Assays with RNase E were performed at 30°C in 10 mM Tris-HCl buffer (pH 8.0)–100 mM NH_4Cl–1 mM $MgCl_2$–0.1 mM dithiothreitol–50 μg of *Saccharomyces cerevisiae* tRNA per ml in a total reaction volume of 25 μl by the previously described procedure (36) with some modifications. One thousand to 3,000 cpm of the substrate RNA (from in vitro transcription) was heated in this buffer for 2 min at 50°C and 10 min at 30°C and then chilled on ice. Two microliters of AS 26 fraction was added, and aliquots were withdrawn at timed intervals. When necessary, extracts were heated prior to the reaction. Samples were diluted by the addition of 2 volumes of 90% deionized formamide containing 20 mM Na_2EDTA, 0.1% xylene cyanole, and 0.1% bromophenol blue. Digestion products were heat denatured and analyzed by electrophoresis on a 10% polyacrylamide gel containing 7 M urea in Tris-borate-EDTA buffer. Bands were visualized on a PhosphorImager (Molecular Dynamics).

RESULTS

Effect of deletion and insertion mutations on *pap* mRNA processing. We have previously shown that mRNA-BA is endonucleolytically processed in the *papB-papA* intercistronic region in an RNase E-dependent manner. The major cleavage occurs at nucleotide position 1676, but minor cleavages are also observed at positions 1670, 1671, and 1675 (3, 28).

A series of deletion and insertion mutations in the intercistronic *papB-papA* region were created in the cloned *pap* determinant (Fig. 1B). In plasmids pPAP291, pPAP292, and pPAN5, the deletions start at position −79 relative to the major cleavage site and extend to positions −42, −10, and +35, respectively. All deletions carry an 8-bp *Xho*I linker at the point of the deletion. In pPAN3 the sequence between +36 and +47 was replaced by the 8-bp *Xho*I linker, and the same linker was used to create insertion mutations at positions +35 or +36 and +47 or +48 in pPAP2005 and pPAN2, respectively. To investigate if these genetic alterations in the vicinity of the major cleavage point would alter the processing of mRNA-BA to mRNA-A, we performed a Northern blot analysis with RNA isolated from steady-state growing cultures of *E. coli* HB101 carrying the different plasmids. The blots were hybridized with a 200-bp *papA*-specific restriction fragment probe described in Materials and Methods. As shown in Fig. 2, the accumulation of mRNA-A relative to that of mRNA-BA was unaffected in pPAP291, pPAP292, pPAN2, and pPAP2005. The deletion in pPAP292 left only 10 nucleotides upstream of the cleavage, and the results demonstrated that the recognition of mRNA-BA as a substrate required only a very limited region of the upstream sequences. When an additional 45 nucleotides were deleted so that the deletion also removed the sequence corre-

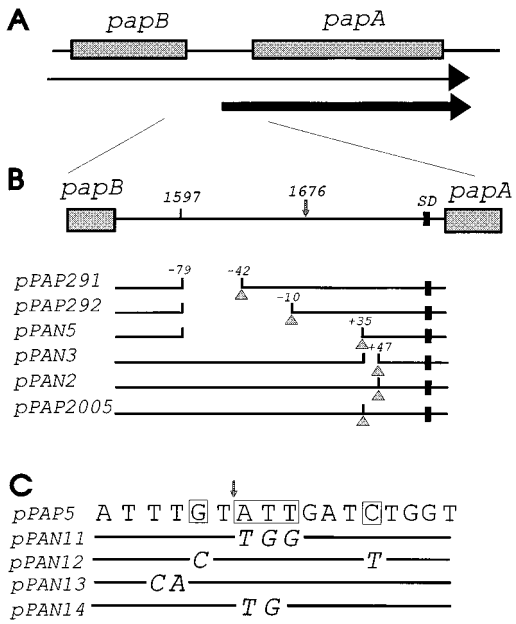

FIG. 1. (A) Transcriptional organization of the region encoding the first two cistrons (*papB* and *papA*, shown with shaded boxes) of the *papBAHCDJKEFG* operon from *E. coli* J96 (37). The horizontal arrows indicate the two major mRNA products (mRNA-BA and mRNA-A) corresponding to this region. (B) Physical map of the *papB-papA* intercistronic region showing the position of the RNase E-dependent cleavage (vertical arrow at position 1676 according to the numbering system of Båga et al. [2]) and the positions of the deletion and insertion mutations introduced in the *papB-papA* intercistronic region. The numbers indicating the deletion borders above the plasmid maps show positions relative to the major endonucleolytic cleavage point. Triangles under the maps show the positions of insertion of an 8-bp *Xho*I linker. SD, Shine-Dalgarno sequence. (C) The nucleotide sequence surrounding the RNase E-dependent cleavage site and the substitution mutations introduced by site-directed mutagenesis into *pap* plasmids. The boxed nucleotides mark sequence similarities among a selected panel of RNase E-dependent cleavage sites (4). The vertical arrow shows the RNase E-dependent cleavage site (position 1676).

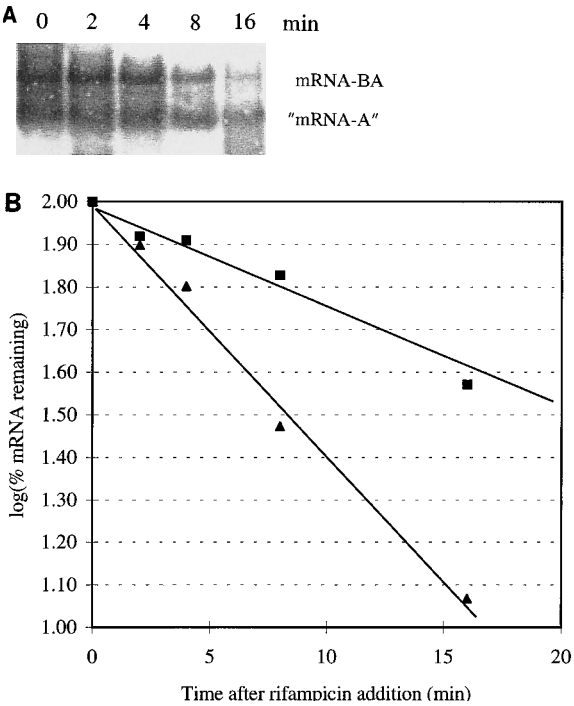

FIG. 3. (A) Chemical half-life determination of *papA*-specific mRNAs from HB101/pPAN5. Cells were withdrawn for RNA extraction from a steady-state growing culture at the indicated times after the addition of rifampin. (B) Diagram showing the kinetics of decay of the two *papA*-specific mRNA molecules expressed from HB101/pPAN5: mRNA-BA (▲) and mRNA-A-like transcripts (■).

sponding to the major cleavage site, the accumulation of the processing product was drastically decreased (Fig. 2, lane 3). A faint signal suggested that there was some accumulation of an mRNA approximately the same size as mRNA-A. While it hybridized to the *papA*-specific probe, we were not able to localize any major 5′ end by primer extension analysis (data not shown). However, the appearance of multiple weaker ends in the region suggested that this band on the Northern blot might represent multiple minor degradation and/or processing products of approximately the mRNA-A size.

It has been shown for some *E. coli* mRNAs that the 5′ leader sequence is important for mRNA stability (6, 8, 13); the deletion of such leader sequences causes a decrease in chemical

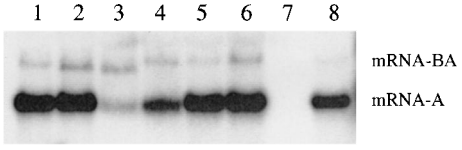

FIG. 2. Northern blot experiment with a *papA*-specific probe. RNA was isolated from steady-state growing cultures of *E. coli* HB101 harboring the mutant *pap* plasmids described in the legend to Fig. 1B. Lane 1, HB101/pPAP291; lane 2, HB101/pPAP292; lane 3, HB101/pPAN5; lane 4 HB101/pPAN3; lane 5, HB101/pPAN2; lane 6, HB101/pPAP2005; lane 7, HB010/pBR322; lane 8, HB101/pPAP5.

stability and thereby a decrease in mRNA accumulation. Since the deletion in pPAN5 also included 35 nucleotides corresponding to the mature or processed 5′ end of mRNA-A, the observed decreased accumulation of this mRNA could have been caused by an increased degradation, rather than decreased processing. We tested this possibility by determining the chemical half-life of the mRNA-A-like processed products in RNA isolated from HB101/pPAN5. As seen in Fig. 3A and B, we found that this mRNA was turned over with a half-life (about 15 min) very similar to that of the wild-type mRNA-A (about 20 min) (3, 28). The half-life of the processing-substrate mRNA-BA was approximately four times longer in this mutant than in the wild-type mRNA-BA (about 1.5 to 2 min) (3, 28). Taken together these results suggested decreased processing, rather than increased turnover, is the cause of the decreased accumulation of the mRNA-A-like molecules in this mutant. As reported before, a fourfold stabilization also of the wild-type mRNA-BA primary transcript was found in the *rne*(Ts) mutant strain after a shift to a nonpermissive temperature (28).

The substitution of 11 *pap* nucleotides with an 8-bp *Xho*I linker at position +35 relative to the processing site (pPAN3) also affected the accumulation of mRNA-A. However, the effect was less significant than that of the mutation in pPAN5 (Fig. 2, lane 4). In contrast, an insertion of the *Xho*I linker at either of the deletion borders of this plasmid, as with pPAN2 or pPAP2005, did not affect the processing (Fig. 2, lanes 5 and 6). Taken together, the results suggest that the loss of *pap* sequences per se and not a position effect was the cause of the decreased mRNA-A accumulation seen in pPAN3. Primer extension analysis of RNA from HB101/pPAP291, HB101/pPAP292, HB101/pPAN3, HB101/pPAN2, and HB101/

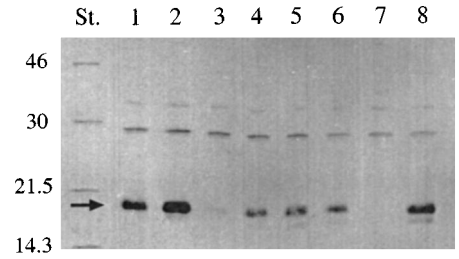

FIG. 4. Western blot analysis with a PapA-specific antiserum and protein extracts from steady-state growing cultures of *E. coli* HB101 harboring the mutant *pap* plasmids described in the legend to Fig. 1B. Lane 1, HB101/pPAP291; lane 2, HB101/pPAP292; lane 3, HB101/pPAN5; lane 4 HB101/pPAN3; lane 5, HB101/pPAN2; lane 6, HB101/pPAP2005; lane 7, HB010/pBR322; lane 8, HB101/pPAP5. The PapA-specific band is indicated by the horizontal arrow, and the molecular size standards (lane St.) are shown (in kilodaltons) along the left side.

pPAP2005 showed that the accumulation of mRNA-A from these strains was the result of processing primarily at the same major cleavage site as in the wild-type strain (data not shown).

Altered *pap* mRNA processing affects fimbrial biogenesis. We have previously shown that the functional half-life of *papA* translation is similar to the chemical half-life of mRNA-A (28), thereby arguing that mRNA-A is the primary source of PapA translation. After having established that the present deletion mutations in the processing determinant affected the accumulation of this mRNA, we could then directly assess the importance of the processing for pilus biogenesis. To see if protein expression and piliation of the bacteria were changed in the deletion mutants, we analyzed the expression of PapA protein by Western immunoblotting and examined the piliation of the bacteria by electron microscopy.

The Western blot analysis with a PapA polyclonal antiserum showed that the steady-state accumulation of PapA protein in HB101/pPAN5 was dramatically decreased (Fig. 4, lane 3). A slight reduction was also noted in the cases of HB101/pPAN3, HB101/pPAN2, and HB101/pPAP2005 (Fig. 4, lanes 4 to 6), while the level of expression in the other deletion mutant strains was roughly the same as in the wild-type strain, HB101/pPAP5. Electron microscopic analysis of the same set of strains was consistent with the Northern and Western blot data. The decreased expression of the major pilus protein, PapA, caused by decreased mRNA processing (pPAN5) resulted in a significant shortening of the pili on the bacterial surface and the cells had a "crew-cut" appearance (Fig. 5). The phenotypic properties of the different mutant derivatives indicated that gene expression from the rest of the operon was not particularly affected. All mutant strains were positive by a hemagglutination test, indicating that the adhesin protein PapG (i.e., the product of the most distal cistron) was still efficiently expressed on the surface of the bacteria.

Alternative cleavage positions revealed by site-directed mutagenesis. Several *E. coli* RNA molecules have been shown to be endonucleolytically processed in an RNase E-dependent manner (14, 18, 20, 21, 28, 30–32, 38). A comparison of the nucleotide sequences surrounding the sites of cleavage of several substrates reveals some common primary sequence determinants. The most striking common motif is an AUU just downstream of the cleavage, a G nucleotide at position −2 relative to the cleavage and a C at position +7. These shared positions might point to nucleotides important for the specific recognition of the substrate by the endonuclease and/or for the enzymatic cleavage of the substrate.

To test if these nucleotides were critically important, we constructed a set of mutant derivatives with nucleotide substitutions in these positions (Fig. 1C). In the plasmids pPAN11 and pPAN14 we changed the conserved AUU to UGG and UGU, respectively. In pPAN12, the G at −2 was changed to C and the C was changed at +7 to U. The well-characterized and rapidly turned over 9Sa (32) and RNA1 (38) cleavage sites share a CA dinucleotide at position −4, while the *pap* substrate has a UU in this position. In pPAN13, we changed this UU to CA, thereby making 7 of 7 nucleotides around the cleavage site in the *pap* substrate homologous to RNA1 and 6 of the 7 nucleotides homologous to the 9Sa cleavage site.

RNA isolated from steady-state growing *E. coli* HB101 carrying these mutant plasmids was analyzed with a *papA*-specific probe in a Northern blot experiment to see if these mutations had any effect on the processing of mRNA-BA to mRNA-A. As seen in Fig. 6A, we could detect accumulation of the processing product mRNA-A in all cases, and with the exception of a small decrease noted for pPAN14, there was no apparent decrease in the accumulation of mRNA-A. Therefore, we conclude that these mutational changes did not cause any dramatic negative effect on the kinetics of turnover of mRNA-BA to mRNA-A.

To see if the mutations had any qualitative effect on the processing, we performed primer extension experiments with end-labelled DNA primer (3). As seen in Fig. 6B (lanes 1, 2, and 4), the cleavage sites in pPAN11, pPAN12, and pPAN14 were shifted 5 nucleotides upstream relative to the wild-type site. The wild-type *pap* substrate is actually also cleaved at this position but to a lesser extent (3). In pPAN13 the cleavage at the major site was enhanced relative to the minor cleavage site at −5. In conclusion, the three mutations which made the *pap* substrate less similar to the shared sequence motif all seemed to abolish cleavage at the major cleavage site. Instead, cleavage occurred at the minor site with kinetics apparently similar to those of the major site in the wild-type substrate. In addition, the one mutation making the *pap* substrate more like the shared motif enhanced the cleavage at the major site relative to that at the minor site.

Western blot and electron microscopic analysis of the strains with site-directed mutations in the processing determinant (*E. coli* HB101 carrying pPAN11, pPAN12, pPAN13, or pPAN14) showed that these genetic alterations did not cause any dramatic change in the level of PapA expression or piliation (data not shown).

In order to test if processing still was RNase E dependent, we carried out in vitro processing analysis with RNA derived from templates corresponding to the *papB-papA* intercistronic region of the wild type (pPAP5) and of mutant derivatives (pPAN5, pPAN11, pPAN12, pPAN13, and pPAN14). For the analysis, we employed in vitro-synthesized RNA obtained from transcription with the T7 RNA polymerase and we used partially purified RNase E enzyme as described previously (35, 36). The in vitro processing reactions were done with RNase E enzyme extracts both from wild-type and from *rne*(Ts) strains of *E. coli* (see Materials and Methods for a description of the procedures). As shown in Fig. 7, RNase E-dependent cleavage of the 209-nucleotide-long RNA corresponding to the wild-type *papB-papA* intercistronic region resulted in discrete cleavage products of the expected lengths. Comparable results were obtained with RNA molecules derived from the different substitution mutants. Results from tests with pPAN13 and pPAN14 are shown in Fig. 7. Analysis with pPAN11 and pPAN12 gave similar results (data not shown). It was also clear that in all cases the processing was dependent on RNase E, as evidenced by the fact that virtually no detectable cleavage occurred with enzyme preparations from the *rne*(Ts) strain

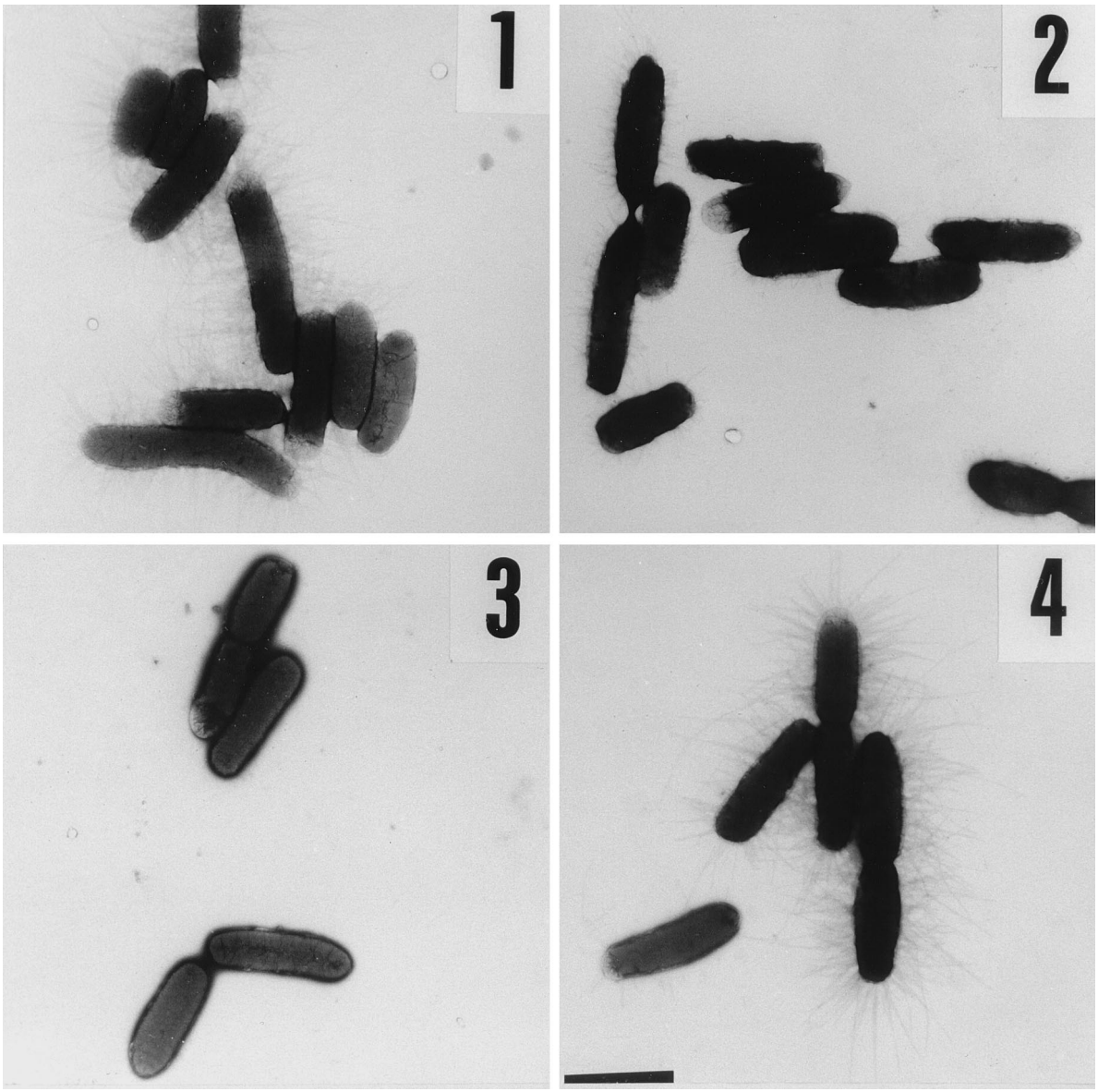

FIG. 5. Electron microscopic analysis of HB101/pPAP5 and HB101/pPAN5 showing the effects on pilus biogenesis caused by deletion of the RNAse E-dependent endonucleolytic cleavage site. Panel 1, HB101/pPAP292; panel 2, HB101/pPAN5; panel 3, HB101/pBR322; panel 4, HB101/pPAP5.

after heat treatment at a nonpermissive temperature (Fig. 7, lanes 11 to 13). The shorter RNA derived from the deletion mutant pPAN5 was a very poor substrate for RNase E in the in vitro processing reaction, and even after prolonged incubation most of the original transcript was undigested. This result was consistent with the recent findings that RNA derived from pPAN5 did not serve as a good substrate either for RNase E binding or for cleavage in vitro, while the corresponding wild-type (pPAP5) *pap* substrate did serve for both binding and cleavage (35). We conclude from these experiments that the processing events occurring in the case of *pap* transcripts from wild-type and substitution mutants are RNase E dependent. The sequence at the alternative cleavage site preferentially detected with pPAN11, pPAN12, and pPAN14 (Fig. 6B) also included the AUU motif found at the cleavage point at position 1676 (Fig. 1C). It was also evident that the most efficient endonucleolytic cleavages observed with the *pap* system in vivo and in vitro took place in the region lacking in the pPAN5 deletion mutant.

DISCUSSION

We analyzed mutations in the intercistronic *papB-papA* region that affected the processing of mRNA-BA to mRNA-A. We thereby established that the RNase E-dependent processing of mRNA-BA to mRNA-A in the *pap* operon has an important function in the expression of the pilus phenotype by allowing the cotranscribed *pap* genes to be expressed at different levels. A mutation causing decreased accumulation of PapA-coding mRNA led to a decreased PapA protein expression. Since PapA is the major structural protein of the pilus shaft, the mutation resulted in shorter pili on the bacterial surface.

The 9S precursor RNA of 5S ribosomal RNA is a well-

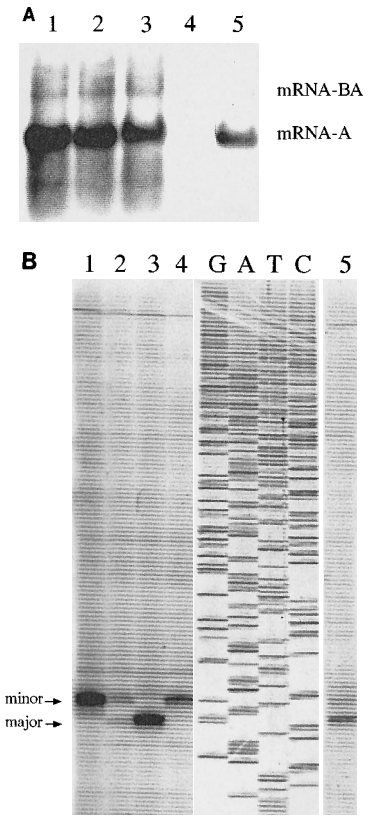

FIG. 6. (A) Steady-state level of *pap* mRNA examined by Northern blot analysis with a *papA*-specific probe. RNA was isolated from steady-state growing cultures of *E. coli* HB101 harboring the mutant *pap* derivatives described in the legend to Fig. 1C. Lane 1, HB101/pPAN11; lane 2, HB101/pPAN12; lane 3, HB101/pPAN13; lane 4 HB101/pBR322; lane 5, HB101/pPAN14. (B) Primer extension analysis of the mRNA-A 5′ ends from the mutant *pap* plasmids described in the legend to Fig. 1C. Arrows point to bands corresponding to major (position 1676) and minor (position 1671) 5′ ends in the processed wild-type *pap* transcript. Lane 1, HB101/pPAN11; lane 2, HB101/pPAN12; lane 3, HB101/pPAN13; lane 4 HB101/pPAN14; lane 5, HB101/pPAP5. DNA sequencing was performed with the same primer used in the primer extension reaction (see Materials and Methods).

established substrate for RNase E (32) and is used as a model system for studying RNase E recognition of its substrate. Cormack and Mackie (10) have chosen this substrate for an extensive deletion-mutational analysis to define the primary and secondary structure information required for RNase E cleavage. Their study indicated that genetic information upstream of the 9Sa cleavage site is needed for efficient cleavage, but downstream sequences and the 5S sequence are dispensable (10).

In contrast to what Cormack and Mackie (10) found for the 9S substrate, the upstream sequences were not required for efficient cleavage in the *pap* substrate. In our experiments, we deleted 67 nucleotides to a point only 10 nucleotides upstream of the major cleavage site (pPAP292) and we could not detect any effect on the cleavage rate or specificity. A small deletion downstream of the cleavage site (plasmid pPAN3) resulted in a somewhat decreased accumulation of the processing product mRNA-A and also a slight shortening of the pili. Since this deletion is only 27 nucleotides upstream of the *papA* ribosome-binding sequence, we cannot at this point discriminate between a decreased translational initiation (with concomitant increased mRNA-A turnover) and a true effect on the processing event per se.

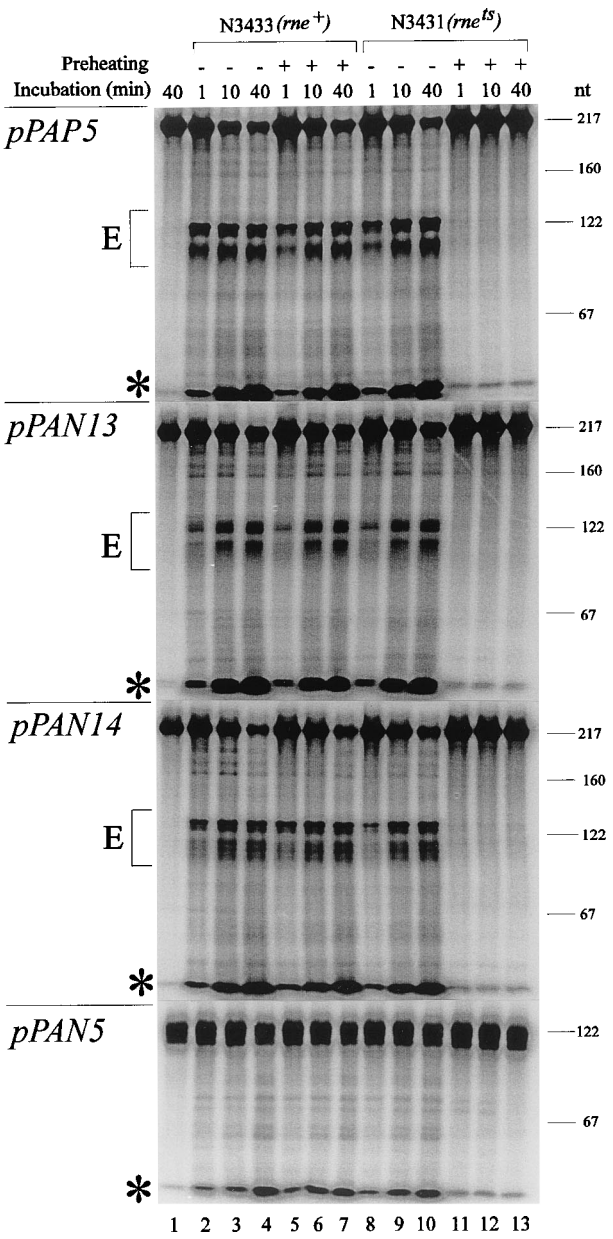

FIG. 7. RNase E-dependent in vitro cleavage of *pap* mRNA corresponding to the *papB-papA* intercistronic region of wild-type and mutant *pap* operons. Cleavage assays with different ^{32}P-labelled RNA molecules were performed at 30°C with enzyme extracts (AS 26 fraction) from the *rne*$^+$ strain N3433 (0.4 μg/ml; lanes 2 to 7) and from the *rne*(Ts) strain N3431 (0.6 μg/ml; lanes 8 to 13). Lane 1 was a sample of undigested substrate incubated in buffer. The enzyme used for samples run in lanes 5 to 7 and 11 to 13 had been preheated at 45°C for 15 min before the addition of the RNA substrate. Samples were withdrawn from incubation at the times indicated, and analysis by gel electrophoretic separation and detection by autoradiography were performed as described in Materials and Methods. Denatured end-labelled DNA fragments were run as markers in the gels, and their sizes in nucleotides (nt) are shown along the right side. The positions of RNase E cleavage products are indicated with the letter E and brackets on the left side. The positions of nucleotides migrating to the front of the gels are indicated with the asterisks.

Our mutational alterations of the *pap* nucleotide sequence at the cleavage site gave results that support the idea of a preferred sequence motif where cleavage may occur most efficiently. Substitution of AUU at the site of cleavage for UGG or UGU abolished cleavage at the major site almost com-

pletely. Instead, cleavage occurred at an alternative site a few nucleotides upstream of the major site. Cleavage at this position is also observed in the wild-type situation but to a lesser extent, and it appears to be a minor site. Changing a UU to a CA 3 and 4 nucleotides upstream of the major cleavage site made the region around the site almost identical to the 9Sa and RNA1 substrates. This mutation enhanced cleavage at the major site relative to that at the minor site.

In another study, Mackie and Genereaux (22) assessed the importance of internal stem-loop structures for RNase E-dependent cleavage in the mRNA for *E. coli* S20 ribosomal protein. They showed that no single stem-loop structure in the template mRNA is absolutely required for cleavage to occur. In addition, changes of primary or secondary structure 5′ or 3′ of the cleavage site have only moderate effects on cleavage specificity but can strongly change the rate of cleavage. Destabilization of stem structures in the mRNA template close to the cleavage site (5′ and 3′) also revealed cryptic RNase E-dependent cleavage sites. Mackie's and Genereaux's data favor the hypothesis that RNase E is a single-strand-specific enzyme and that secondary structure determinants could be important for keeping the structure in a single-stranded conformation and thus accessible for cleavage. Our findings with the *pap* transcript are not inconsistent with such a hypothesis, and it is possible to fold the *pap* mRNA into a model with stem-loop structures, although the actual secondary structure of the mRNA in vivo is not known. It is also feasible that the stability of the mRNA-A processing product is influenced by formation of a stem structure at the 5′ end (13).

In the *sfa*, CFA/I, and F1845 determinants, the high-level expression of the major pilin protein relative to that of other pilus proteins seems to be achieved by mechanisms similar to that of *pap* expression. The transcriptional organization of *sfa* and CFA/I is very similar to that of *pap*. In both cases there seem to be putative cleavage sites in regions with an AUU sequence motif which is commonly seen in RNase E substrates. It is not yet shown that the cleavages in these two systems are RNase E dependent. However, it is reasonable to believe that there exists a common RNase E-dependent mechanism for achieving differential expression of the pilus genes in *sfa*, *cfa*, and *pap* and possibly other pili determinants. In the F1845 determinant the major pilin is also the adhesin and is encoded by the *daaE* gene (7). The transcriptional organization of the F1845 operon is different from those of *sfa* and *pap* in that the *daaE* gene is the most promoter-distal gene of the operon. *daaE* mRNA accumulates as the result of a processing event which is independent of RNase E and RNase III, according to studies with mutant *E. coli* strains (7). The *daaE* sequence around the putative cleavage site is also quite G/C rich, and unlike the sequences of *sfa*, *cfa*, and *pap*, no AUU motifs are present. Therefore, a distinct but similar mechanism seems to be acting in the *daaE* system.

The *pap* operon is considered to be important for the bacterial ability to infect the urinary tract by allowing specific adhesion to the uroepithelium. The interaction between the adhesin molecule PapG on the tip of each pilus and the glycolipid receptor on the epithelium is considered to be relatively weak, but contact between many pili on the same bacterium and the receptors on the epithelium is thought to result in a strong interaction. It is also known that the adhesive capacity of the bacteria is dependent on pili as carriers of this type of adhesin in wild-type isolates of uropathogenic *E. coli* with complete O antigen lipopolysaccharide (40). Therefore, it is likely that the polyvalent interaction made possible by the length and flexibility of the pili is important for this receptor contact and for the infection process. Since the efficiency of the RNase E-dependent processing event to a large extent influences the expression of PapA and thereby the lengths of the pili, it would be possible for the cell to control the lengths of pili by changing the rate of processing.

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