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Transposition of the Endogenous Insertion Sequence Element IS*1126* Modulates Gingipain Expression in *Porphyromonas gingivalis*

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We have previously reported on a Tn*4351*-generated mutant of *Porphyromonas gingivalis* (MSM-3) which expresses enhanced arginine-specific proteinase activity and does not utilize hemin or hemoglobin for growth (C. A. Genco et al., Infect. Immun. 63:2459–2466, 1995). In the process of characterizing the genetic lesion in *P. gingivalis* MSM-3, we have determined that the endogenous *P. gingivalis* insertion sequence element IS*1126* is capable of transposition within *P. gingivalis*. We have also determined that IS*1126* transposition modulates the transcription of the genes encoding the lysine-specific proteinase, gingipain K (*kgp*) and the arginine-specific proteinase, gingipain R2 (*rgpB*). Sequence analysis of *P. gingivalis* MSM-3 revealed that Tn*4351* had inserted 60 bp upstream of the *P. gingivalis* endogenous IS element IS*1126*. Furthermore, *P. gingivalis* MSM-3 exhibited two additional copies of IS*1126* compared to the parental strain A7436. Examination of the first additional IS*1126* element, IS*1126*$_1$, indicated that it has inserted into the putative promoter region of the *P. gingivalis kgp* gene. Analysis of total RNA extracted from *P. gingivalis* MSM-3 demonstrated no detectable *kgp* transcript; likewise, *P. gingivalis* MSM-3 was devoid of lysine-specific proteinase activity. The increased arginine-specific proteinase activity exhibited by *P. gingivalis* MSM-3 was demonstrated to correlate with an increase in the *rgpA* and *rgpB* transcripts. The second additional IS*1126* element, IS*1126*$_2$, was found to have inserted upstream of a newly identified gene, *hmuR*, which exhibits homology to a number of TonB-dependent genes involved in hemin and iron acquisition. Analysis of total RNA from *P. gingivalis* MSM-3 demonstrated that *hmuR* is transcribed, indicating that the insertion of IS*1126* had not produced a polar effect on *hmuR* transcription. The hemin-hemoglobin defect in *P. gingivalis* MSM-3 is proposed to result from the inactivation of Kgp, which has recently been demonstrated to function in hemoglobin binding. Taken together, the results presented here demonstrate that the introduction of Tn*4351* into the *P. gingivalis* chromosome has resulted in two previously undocumented phenomena in *P. gingivalis*: (i) the transposition of the endogenous insertion sequence element IS*1126* and (ii) the modulation of gingipain transcription and translation as a result of IS*1126* transposition.

The gram-negative anaerobe *Porphyromonas gingivalis* has been implicated as a major pathogen associated with the induction and/or progression of adult periodontal disease (5). This organism is armed with a number of putative virulence factors; of these, the cysteine proteinases have received considerable attention due to their ability to degrade and inactivate host defense proteins (iron binding proteins, immunoglobulins, and complement components), structural proteins (collagen, fibronectin, and fibrinogen), and plasma protein inhibitors (10, 35). The majority of the *P. gingivalis* proteinase activity is due to the production of cysteine proteinases referred to as gingipains, which cleave synthetic and natural substrates after arginine and lysine residues.

The genes encoding arginine specific gingipains (*rgpA* and *rgpB*) have been characterized (26, 33, 35, 36). The translated portion of *rgpA* encodes a prepropeptide, catalytic, and hemagglutinin domain, and the initial polyprotein is apparently subject to posttranslational processing. Although the *rgpA* and *rgpB* genes share a strong degree of similarity, the *rgpB* gene does not possess the hemagglutinin domain present in the C-terminal region of the *rgpA* gene. Nakayama et al. (27) have suggested that *rgpA* and *rgpB* may have been generated through the duplication of an ancestral *rgp* gene, with insertion of the hemagglutinin domain into one copy of the two resulting genes and homologous recombination between the proteinase domains of *rgpA* and *rgpB. P. gingivalis* has been demonstrated to undergo nonreciprocal recombination, further supporting this scenario (27).

The gene encoding the lysine-specific gingipain (*kgp*) has also been characterized from a number of different *P. gingivalis* strains (2, 29, 32). Like *rgpA*, the initial translation product of *kgp* is composed of four functional regions: the signal peptide, the NH_2-terminal prosequence, the mature proteinase domain, and the COOH-terminal hemagglutinin domain (29). Sequence comparison reveals that *kgp* is nearly identical to *rgpA* at the C terminus and suggests that a recombinational rearrangement event (i.e., transposition or gene conversion) may have occurred in this region.

Transposition of IS elements can lead to inactivation of genes, to the transcriptional activation of dormant genes, or to genomic rearrangement, all of which can contribute to the

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TABLE 1. Probes and oligonucleotides used for Southern blot, Northern blot, and RT-PCR analyses

Probe designation	Description[a]
rgpA/rgpB	PCR fragment corresponding to bp 649 to 1,177 of *rgpB*; recognizes both *rgpA* and *rgpB* (528 bp)
hmuR	PCR fragment corresponding to bp 8 to 493 of *hmuR* (505 bp: forward and reverse primers used each contain 10 nonspecific bases)
prtT	PCR fragment corresponding to bp 914 to 1,200 of *prtT* (286 bp)
1126_1	17-bp oligonucleotide derived from IS112_1 flanking sequences; 5′-CTCATGCTCGACTGACT-3′
1126_2	18-bp oligonucleotide derived from IS1126_2 flanking sequences; 5′-CCTACAAATTGGGATTGC-3′
kgp	33-bp oligonucleotide probe 5′-CATACGAACCGGCGTATTATACAAGTCGCCATG-3′

[a] The length of the DNA fragment is indicated in parentheses.

genetic diversity of bacterial populations (8, 31, 34, 44). To date, three endogenous insertion sequence elements have been characterized in *P. gingivalis*. PGIS2 was recently identified by our laboratory and has been demonstrated to be capable of transposition within *P. gingivalis* (44). IS*195* is an insertion sequence-like element recently reported by Lewis and Macrina (20) that is associated with protease genes in *P. gingivalis*. IS*195* was found flanking the *kgp* genes in *P. gingivalis* strains HG66 and 381 and within a *prtP* gene (*kgp* homolog) from *P. gingivalis* W83. The *P. gingivalis* insertion sequence IS*1126* was originally described by Maley et al. (24); however, transposition within the *P. gingivalis* genome was not demonstrated by these investigators. Barkocy-Gallagher et al. (2) have demonstrated that an incomplete copy of IS*1126* is found directly 3′ of the *prtP* gene in *P. gingivalis* W12. Aduse-Opoku et al. (1) have recently reported that located in the 3′ end of the *tla* gene (which is homologous to the 3′ portion of the *rgpA* gene), is a copy of a vestigial IS*1126* in which an essential region of the transposase gene is deleted. These observations suggest that recombination within the gene locus encoding the arginine- and lysine-specific proteinases may have occurred via an IS*1126*-mediated transposition event. In this study, we demonstrate for the first time the transposition of IS*1126* within *P. gingivalis*. We also show that IS*1126* transposition modulates the transcription of the genes encoding gingipain K (*kgp*), gingipain R1, and gingipain R2 (*rgpB*).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. gingivalis* A7436, W50, HG66, ATCC 33277 (12), and MSM-3 (11), and *Escherichia coli* XL1-Blue MR and JM109 were used in these studies. *P. gingivalis* A7436, W50, HG66, and 33277 were maintained on anaerobic blood agar (ABA) plates (Remel, Lenexa, Kans.). *P. gingivalis* MSM-3 was maintained on ABA plates supplemented with 1 μg of erythromycin per ml. All *P. gingivalis* cultures were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc.) with 85% N_2, 5% H_2, and 10% CO_2 for 3 to 5 days. After incubation at 37°C, cultures were inoculated in Anaerobe Broth MIC (Difco) or TSB (see below) and then incubated at 37°C (under anaerobic conditions) for 24 h. *E. coli* strains were typically maintained in Luria-Bertani media and incubated aerobically with shaking.

P. gingivalis MSM-3 is a hemin-hemoglobin utilization mutant isolated after transpositional mutagenesis of *P. gingivalis* A7436 with the *Bacteroides fragilis* transposon Tn*4351* (11). *P. gingivalis* MSM-3 cultures grown by continuous passage and those recovered from subcutaneous chambers implanted in BALB/c mice (11) maintain their nonpigmented phenotype and erythromycin resistance, indicating that there is no apparent reversion of the mutation. Cultures passaged continuously also maintain increased levels of arginine-specific proteinase activity, as well as a decreased lysine-specific proteinase activity.

Enzyme activity assay. The amidolytic activity of whole cultures was determined with either *N*-benzoyl-L-arginine-*p*-nitroanilide (BApNA) or *N*-carbobenzoxy-L-lysine-*p*-nitroanilide (z-KPNA). Samples were preincubated in 0.2 M Tris-HCl–0.1 M NaCl–5 mM $CaCl_2$–10 mM cysteine (pH 7.6) for 5 min at 37°C and assayed for amidase activity with 2 mM substrate. The formation of *p*-nitroaniline was monitored spectrophotometrically at 405 nm.

Isolation of genomic DNA. *P. gingivalis* cells were pelleted and suspended in 15 ml of 10 mM NaCl–20 mM Tris-HCl (pH 8.0)–100 μg proteinase K per ml–0.5% (wt/vol) sodium dodecyl sulfate (SDS). Cells were gently mixed and incubated for 6 h or overnight at 50°C. Genomic DNA was extracted by gentle inversion with an equal volume of phenol-chloroform for 10 min at room temperature. The mixture was centrifuged at 4,000 rpm and at 10 to 12°C for 20 min, and the upper aqueous layer was removed. The DNA sample was precipitated with 3.0 M sodium acetate (pH 5.5), and two volumes of ethanol were added to the aqueous phase. The DNA was spooled out at the aqueous ethanol interphase by using a sterile glass rod. The DNA was washed with 70% (wt/vol) ethanol, touched to the side of a sterile tube to drain the ethanol, air dried, and dissolved in 5 ml of TE buffer. *P. gingivalis* A7436 and MSM-3 genomic DNA were partially digested, ligated, and packaged by using the SuperCos1 Cosmid Vector Kit, as described by the manufacturer (Stratagene, Inc.).

Southern blot analysis. Agarose gels were blotted against nylon membranes as described by Sambrook et al. (38). After blotting, nylon membranes were prehybridized for 30 min at 65°C and then hybridized for 2 h (65°C) in Rapid hybridization buffer (Amersham Life Sciences) containing the appropriate probe (see Table 1 and Results). Probes were labeled by using ^{32}P as described by the Prime-a-Gene labeling system (Promega). After hybridization, membranes were washed twice with 2× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min at 65°C and exposed to X-ray film.

Northern blot analysis. For total RNA isolation, *P. gingivalis* strains were grown in 125 ml of TSB broth (30 g of Trypticase soy broth, 5 g of yeast extract, 0.5 g of cysteine, and 1 mg of menadione per liter supplemented with 1.5 μM hemin). Total RNA was prepared by using the Purescript kit (Gentra).

Northern blot analysis was conducted by electrophoresis of RNA samples in a 1% agarose gel containing 2.2 M formaldehyde, followed by capillary transfer to a Hybond-N membrane. Filters were hybridized with probes specific for *rgpA* and *rgpB* (*rgpA/B*) (labeled with [^{32}P]dCTP by using the High Prime labeling system [Boehringer Mannheim]) and a *kgp* oligonucleotide probe 5′ labeled with [^{32}P]ATP and polynucleotide kinase (Table 1). Hybridization was conducted in a mixture containing 1 M NaCl, 1% SDS, and 10% dextran sulfate at 65°C for the *rgpA/B* probe and at 55°C for the *kgp* probe. Nonspecific radioactivity was removed by two washes at room temperature in 30 mM NaCl–3.0 mM sodium citrate and two washes at 65°C or 55°C (*kgp* probe) in a mixture containing 30 mM NaCl, 3 mM sodium citrate, and 0.1% SDS. Membranes were exposed to X-ray films, and autoradiographs were scanned by using the Eagle Eye II still video system (Stratagene).

RT-PCR. *P. gingivalis* cultures were grown to the mid-logarithmic phase in basal medium (BM) or BM supplemented with hemin (1.5 μM) (11). Total RNA was isolated by using the RNagents kit (Promega). Samples were initially treated with DNase prior to reverse transcriptase PCR (RT-PCR). To 1.0 μg of total RNA was added 1 μl of 10× DNase I (Promega) and 1 U of DNase I in diethyl pyrocarbonate (DEPC)-treated water (final volume, 10 μl). Samples were incubated at room temperature for 15 min. DNase I was inactivated by the addition of 1 μl of 25 mM EDTA to the reaction mixture. The samples were then heated to 65°C for 10 min and placed on ice. To this was added 25 μl of 2× reaction mix, 100 ng of each primer, 1 μl of RT-*Taq* mix, and DEPC-treated water to a final volume of 50 μl. The samples were overlaid with mineral oil and placed in a Thermacycler. cDNA synthesis was performed at 50°C for 30 min, followed by predenaturation at 94°C for 2 min. PCR amplification was carried out by using the following parameters: denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and elongation at 72°C for 2 min for 35 cycles. Primers were designed to amplify a 505-bp fragment for the *hmuR* gene and a 286-bp fragment for *prtT* gene (Table 1).

DNA sequencing and computer analysis. DNA sequencing was performed by using the PRISMTM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, Calif.) and 373A DNA sequencer (Applied Biosystems). Computer analysis was performed as outlined by the Intelligenetics Suite and Blast programs.

GenBank accession number. The partial sequence of *hmuR* was deposited into GenBank under accession number U87395 (*hmuR* was previously designated *hemB*). The remainder of A7436 *hmuR* was sequenced as described above.

RESULTS

Characterization of the insertion site of Tn*4351* in *P. gingivalis* MSM-3. We previously reported on the initial characterization of a *P. gingivalis* hemin uptake mutant, *P. gingivalis* MSM-3, isolated after transpositional mutagenesis of *P. gingi-*

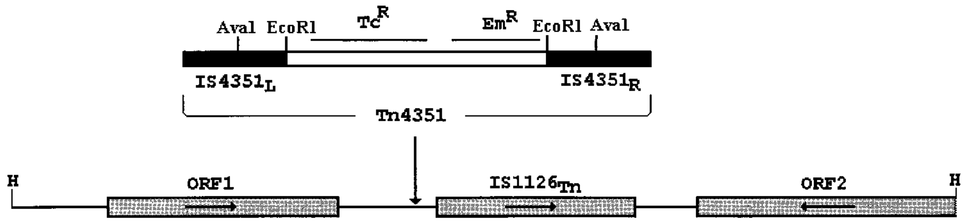

FIG. 1. Genetic organization of DNA flanking Tn*4351* target sequence in *P. gingivalis* MSM-3 genome. Tn*4351* contains a tetracycline resistance gene (Tc^r) and an erythromycin-clindamycin resistance gene (Em^r) flanked by direct repeat insertion sequence IS*4351*. The Tn*4351* insertion was located 60 bp upstream from an IS*1126* element (designated IS*1126*$_{Tn}$). Arrows in ORF1, IS*1126*$_{Tn}$, and ORF2 indicate the direction of transcription. H, *Hin*dIII.

valis A7436 with the *B. fragilis* transposon Tn*4351* (11). Southern blot analysis of *Hin*dIII-digested *P. gingivalis* MSM-3 genomic DNA with a Tn*4351*-specific probe revealed a 5-kb fragment (data not shown) containing the partial *ermF* gene, the entire tetracycline-resistance gene, and IS*4351* attached to the chromosomal junction fragment (data not shown). Using the tetracycline gene as a selective marker, this fragment was cloned from *P. gingivalis* MSM-3 into plasmid pGEM3Zf(−). An *Ava*I-*Ava*I fragment which contains a portion of the IS*4351* sequence (Fig. 1) attached to the *P. gingivalis* MSM-3 chromosomal junction fragment, and the multiple cloning site of pGEM3Zf(−) was purified and used as a probe to screen a *P. gingivalis* A7436 cosmid library for wild-type sequences containing the insertion site. Cosmid DNA from positive colonies was digested with *Hin*dIII and analyzed by Southern blot hybridization by using the previous *Ava*I-*Ava*I restriction fragment as a probe. A 5.3-kb DNA fragment was identified and subjected to nucleotide sequence determination.

Computer analysis of the nucleotide sequence of the 5.3-kb fragment demonstrated that, in MSM-3, Tn*4351* had inserted in a noncoding region 60 bp upstream from the *P. gingivalis* IS*1126* element (Fig. 1). The nucleotide sequence of the element in *P. gingivalis* MSM-3 was 98% identical to the IS*1126* purified from *P. gingivalis* W83, as previously reported by Maley and Roberts (23). The IS*1126* element in MSM-3 (designated IS*1126*$_{Tn}$) was 1,334 bp in length with 12-bp imperfect repeats at either end. When compared to the previously reported sequence of IS*1126* (23), a 4-bp deletion in the major open reading frame (ORF), presumably representing the IS*1126* transposase, was noted. Also identified in this 5.3-kb region were two long ORFs (Fig. 1). ORF1 contained 1,347 bp coding for a putative 449-amino-acid protein. The protein encoded by ORF2 exhibited 45% identity to the polynucleotide phosphorylase genes of both *E. coli* and *Photohabdus* spp. (4, 37).

***P. gingivalis* MSM-3 contains two additional copies of IS*1126*.** The insertion of Tn*4351* upstream of the *P. gingivalis* IS*1126* element led us to postulate that IS*1126* could transpose and that this could be responsible for the mutation in MSM-3. To explore this possibility, Southern blot analysis was performed with *P. gingivalis* A7436 and MSM-3 genomic DNA digested with *Bam*HI and probed with a fragment isolated from IS*1126*. Since IS*1126* does not contain a *Bam*HI site, a single hybridizing fragment was assumed to represent a single copy of the element. However, it is possible that there may be two or more comigrating fragments which hybridize with the IS1126 probe. Likewise, it is possible that the hybridizing bands may represent vestigial copies of IS*1126*. As shown in Fig. 2, two additional bands of 4 and 5 kb were observed in *P. gingivalis* MSM-3 compared to the wild-type strain *P. gingivalis* A7436. Seven additional independently isolated Tn*4351*-generated transconjugants were examined and exhibited an IS*1126* banding pattern identical to that of *P. gingivalis* MSM-3 (Fig. 2). These Tn*4351*-generated transconjugants were also nonpigmented on ABA plates. These observations suggest that the insertion of Tn*4351* may be site specific. In addition, these results suggest that introduction of Tn*4351* into *P. gingivalis* may have resulted in the duplication and transposition of the endogenous IS element IS*1126*. We also examined genomic DNA from three other *P. gingivalis* strains to determine the number of IS*1126* elements present. The hybridization patterns indicate that strains HG66, ATCC 33277, and W50 were different from A7436 and from each other. The variation in number and size of IS*1126*-bearing restriction fragments among different strains is in agreement with previous studies (22) and suggests the mobile nature of IS*1126* within the *P. gingivalis* chromosome.

Examination of the IS*1126* insertion sites in *P. gingivalis* MSM-3. To examine the insertion site of the first additional IS*1126* element (designated IS*1126*$_1$), DNA from *P. gingivalis* MSM-3 was digested with *Bam*HI, and a 5-kb fragment was isolated, cloned into pGEM3Zf, and transformed into *E. coli* JM109. Sequence analysis revealed that IS*1126*$_1$ had inserted 185 bp upstream of the start codon of the signal peptide of the

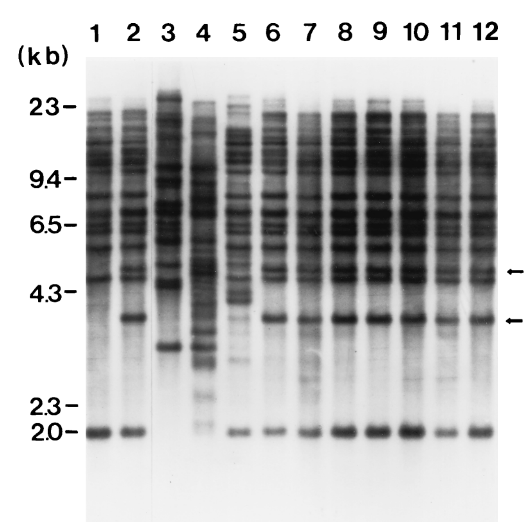

FIG. 2. Southern blot hybridization analysis of *P. gingivalis* chromosomal DNA probed with insertion sequence IS*1126*. Marker DNAs are indicated at left in kilobases. Chromosomal DNA from *P. gingivalis* A7436 (lane 1), MSM-3 (lane 2), HG66 (lane 3), 33277 (lane 4), W50 (lane 5), and seven independently isolated Tn*4351*-generated transconjugants (lanes 6 to 12) were isolated, digested with *Bam*HI, and electrophoretically separated on a 0.8% agarose gel. Arrows on the right indicate two additional copies of IS*1126*. The Southern blot was probed with a [^{32}P]dCTP-labeled, 526-bp *Sac*I-*Hinc*II fragment purified from IS*1126*$_{Tn}$.

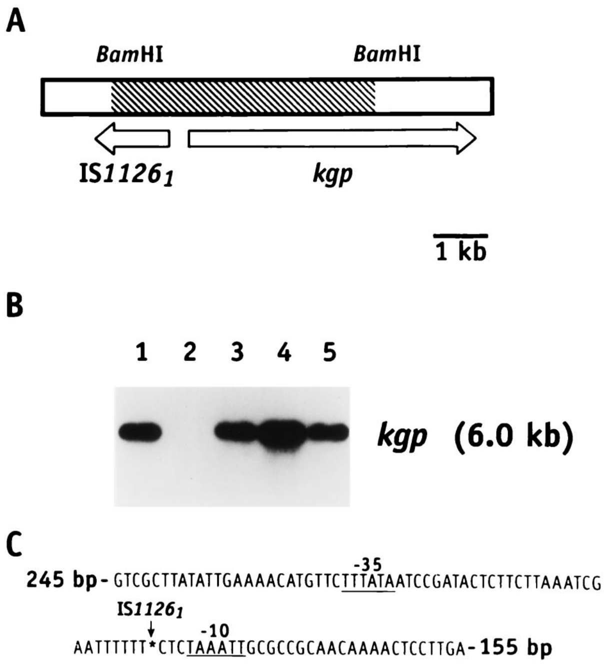

FIG. 3. Localization of IS*1126*$_1$ upstream of *kgp* and Northern blot analysis of *kgp* transcript in *P. gingivalis*. To identify the insertion site of IS*1126*$_1$, *P. gingivalis* MSM-3 chromosomal DNA was digested with *Bam*HI and a 5-kb fragment was isolated, cloned, and sequenced (represented by hatched area). (A) The insertion of IS*1126* was located 185 bp upstream of the start codon of *kgp*. Arrows represent the size and direction of transcription of IS*1126*$_1$ and *kgp*. (B) Northern blot analysis of total RNA from *P. gingivalis* A7436 (lane 1), MSM-3 (lane 2), W50 (lane 3), 33277 (lane 4), and HG66 (lane 5). Equal amounts of total RNA were loaded and confirmed by equal staining intensity of the rRNA bands stained with ethidium bromide (data not shown). (C) Nucleotide sequence of IS*1126*$_1$ insertion site. Putative −35 and −10 promoter boxes are denoted and underlined. Actual site of insertion of IS*1126*$_1$ within the sequence is indicated by an arrow. Numbers represent the position of the bases in relation to the *kgp* start codon.

P. gingivalis kgp gene (29) (Fig. 3A). DNA sequence analysis revealed that the entire *kgp* gene was intact in *P. gingivalis* MSM-3. The absence of IS*1126*$_1$ in the corresponding region of the parental strain was verified by using an oligonucleotide constructed from MSM-3 genomic DNA which flanks IS*1126*$_1$ (Table 1). A *P. gingivalis* A7436 cosmid library was screened with this probe, and DNA sequence analysis of positive clones revealed that IS*1126*$_1$ was not present in the corresponding region of the A7436 genome (data not shown).

IS*1126*$_1$ insertion shuts down *kgp* transcription and corresponding Lys-specific cysteine proteinase activity. To examine the consequence of the insertion of IS*1126*$_1$ 5′ to the *kgp* gene, we examined RNA from cultures of *P. gingivalis* for the presence of a *kgp* transcript. Total RNA from *P. gingivalis* A7436 and MSM-3, as well as two additional *P. gingivalis* laboratory strains (W50 and 33277), was isolated and probed with a *kgp*-specific oligonucleotide (Table 1). The *kgp* transcript (6 kb) was detected in the *P. gingivalis* parental strain A7436, as well as in additional *P. gingivalis* laboratory strains W50 and 33277 (Fig. 3B). However, we did not detect a *kgp* transcript in RNA obtained from *P. gingivalis* MSM-3 (Fig. 3B).

Promoter sequences for *kgp* have not been previously identified; however, the insertion site of IS*1126*$_1$ proximal to the *kgp* start codon and the absence of a *kgp* transcript in *P. gingivalis* MSM-3 suggested that the site of insertion may represent the putative *kgp* promoter. We thus examined the IS*1126*$_1$ insertion site for putative −35 and −10 sequences. Interestingly, a region located 220 bp upstream of the *kgp* start codon (TTTATA) was found to exhibit 67% homology with the *E. coli* consensus −35 sequence, while a region located 182 bp upstream of the *kgp* start codon (TAAATT) exhibited 83% homology to the −10 sequence (Fig. 3C). The IS*1126*$_1$ insertion was located 3 bp upstream of the putative −10 sequence (Fig. 3C). These findings suggest that IS*1126*$_1$ has inserted into the *kgp* promoter region, resulting in disruption of *kgp* transcription.

To confirm that the absence of *kgp* transcription resulted in translational effects, *P. gingivalis* MSM-3 and A7436 were assayed for the presence of lysine-specific proteinase activity. In agreement with our previous studies (11), we found that *P. gingivalis* MSM-3 exhibited enhanced arginine-specific proteinase activity compared with A7436. However, in agreement with the transcriptional studies, MSM-3 was found to possess virtually no lysine-specific proteinase activity when compared to the parental strain A7436. Lysine- and arginine-specific proteinase activities of *P. gingivalis* were as follows. For strain MSM-3 the BApNA and z-Lys-pNA activities were 121.7 and 1.085 U, respectively, while for strain A7436 the BApNA and z-Lys-pNA activities were and 34.8 and 26.120 U, respectively. These activities are defined as the amount which gives an optical density of 1.0/min and are derived from the results of three separate experiments.

Enhanced *rgpA* and *rgpB* transcription in *P. gingivalis* MSM-3. To determine if the enhanced arginine-specific proteinase activity correlated with increased transcription of *rgpA* and/or *rgpB*, Northern blot analysis of *P. gingivalis* A7436 and MSM-3 total RNA was performed. Northern blot analysis with a probe which recognizes sequences present in both *rgpA* and *rgpB* (Table 1) revealed two transcripts representing *rgpA* and *rgpB* in both *P. gingivalis* A7436 and MSM-3 (Fig. 4). Densitometry scans of the Northern blot depicted in Fig. 4 indicated that the levels of the *rgpA* and *rgpB* transcripts detected in *P. gingivalis* MSM-3 were increased compared to the parental strain A7436 (Fig. 4C). Densitometry scans of the Northern blot depicted in Fig. 4A revealed that the relative band intensity representing the *rgpA* transcript in *P. gingivalis* MSM-3 was approximately 2.5-fold of that observed in *P. gingivalis* A7436. The relative band intensity representing the *rgpB* transcript was also higher in *P. gingivalis* MSM-3 compared to the *rgpB* transcript in strain A7436. Thus, the increased arginine-specific proteinase activity in *P. gingivalis* MSM-3 results from increased transcription of the *rgpA* and *rgpB* genes. We also observed both *rgpA* and *rgpB* transcripts in two additional *P. gingivalis* laboratory strains (W50 and 33277).

Examination of the second additional IS*1126* element. In some instances, insertion of an IS element can transcriptionally activate expression of an adjacent gene by virtue of readthrough transcription from a promoter within the element (34). To determine if the second additional IS*1126* element (designated IS*1126*$_2$) had inserted proximal to the *rgpA* or *rgpB* genes and had resulted in the increased transcription of these genes, we examined the site of insertion of IS*1126*$_2$. A 4-kb *Bam*HI restriction fragment was cloned from *P. gingivalis* MSM-3, and the nucleotide sequence of IS*1126*$_2$ and its junction fragments were analyzed. Analysis of IS*1126*$_2$ indicated that it was identical to the IS*1126* element isolated from *P. gingivalis* W83 (23) with the restoration of the 4-bp 5′-GAAG-3′ deletion observed in IS*1126*$_{Tn}$ (Fig. 5A). Examination of the DNA flanking IS*1126*$_2$ revealed that IS*1126*$_2$ was located 322 bp downstream from the *P. gingivalis prtT* gene (Fig. 5B). The *prtT* gene encodes for a streptopain-related cysteine proteinase which was originally cloned from *P. gingivalis* ATCC 53977 but does not share homology with *kgp*, *rgpA*,

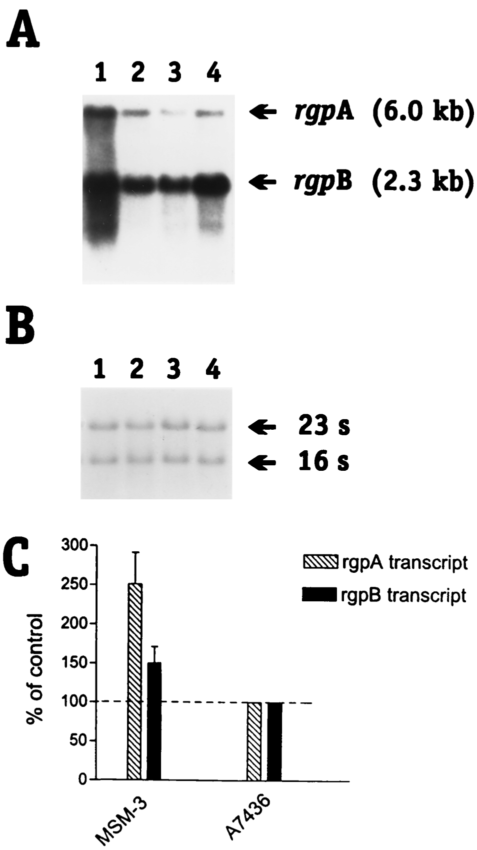

FIG. 4. Northern blot analysis of *rgpA* and *rgpB* transcripts in *P. gingivalis*. (A) Total RNA from *P. gingivalis* was hybridized with a *rgpA-rgpB*-specific probe (a 528-bp DNA fragment corresponding to bp 649 to 1,177 from the *rgpB* gene). Upper bands correspond to *rgpA*, and the lower bands correspond to *rgpB*. Lanes 1 to 4 correspond to *P. gingivalis* MSM-3, A7436, W50, and 33277, respectively. (B) Equal amounts of total RNA were loaded and confirmed by equal staining intensity of the rRNA bands stained with ethidium bromide. 23S and 16S refer to the rRNA bands. (C) Densitometry scan of the hybridizing bands. Data represents the mean ± the standard deviation of three separate experiments and are expressed as the percentage of the control value, arbitrarily set at 100%.

or *rgpB* (22, 30). Northern blot analysis of *P. gingivalis* MSM-3 and A7436 with a probe specific for *prtT* (Table 1) showed that similar transcript levels of *prtT* were present in both strains, thus indicating that the insertion of IS*1126*$_2$ did not alter the transcription of this proximal gene (data not shown).

To confirm that IS*1126*$_2$ was not present in the corresponding region of the parental strain A7436, a radiolabeled oligonucleotide corresponding to the MSM-3 genomic DNA sequences which flank IS*1126*$_2$ (Table 1) was used to screen a *P. gingivalis* A7436 cosmid library. A *Hin*dIII-generated fragment of approximately 7 kb from two independent clones was subcloned, and nucleotide sequence analysis confirmed that IS*1126*$_2$ was not present in the corresponding region of the wild-type genome (data not shown).

Located 677 bp downstream of the IS*1126*$_2$ insertion site in *P. gingivalis* MSM-3, a small ORF (*orfA*) of 428 bp was identified. This ORF was identical to an ORF recently identified by Karunakaran et al. (18) in *P. gingivalis* ATCC 53977. Further downstream of *orfA*, a 1.9-kb ORF was fortuitously identified (Fig. 5). This ORF exhibited homology to the *Yersinia enterocolitica hemR* gene, which is a member of the hemin uptake operon of *Y. enterocolitica* (39), and to several genes whose products have been shown to be TonB-dependent outer membrane receptors involved in the acquisition of iron. These include the *E. coli fepA*, *fhuA*, *cirA*, *btuB*, and *fhuE* genes (7, 13, 16, 21, 39), the *V. cholerae irgA* gene (13), and the *Pseudomonas aeruginosa pfeA* gene (6). Furthermore, we found that a region of the translated ORF exhibited extensive homology to TonB box IV, which has been postulated to be the domain of the TonB-dependent receptors that physically interact with the TonB protein (40). Based upon this homology, we postulated that this gene may be a TonB-dependent outer membrane receptor which functions in the acquisition of hemin and hemoglobin in *P. gingivalis*, and thus we designated this ORF *hmuR*.

Karunakaran et al. (18) also recently reported upon the identification of the *hemR* gene from *P. gingivalis* 53977. The amino-terminal region of *hmuR* exhibited extensive homology to the initial 516 bases of the *P. gingivalis hemR* gene, suggesting that *hmuR* may be a *hemR* homolog (Fig. 6). The carboxy terminus of *hmuR* exhibits identity to genes involved in hemoglobin binding and utilization, while the carboxy terminus of *hemR* exhibits extensive identity with the *prtT* gene of *P. gingivalis* (41). *P. gingivalis hemR* also exhibits homology to genes involved in hemin and iron acquisition from a number of microorganisms and has been postulated to encode for a TonB-dependent outer membrane receptor (18).

Transcription of *hmuR* is not altered in *P. gingivalis* MSM-3. To determine if the transcription of *hmuR* in *P. gingivalis* MSM-3 was altered by the insertion of IS*1126*$_2$, total RNA from *P. gingivalis* A7436 and MSM-3 were examined by both Northern blot analysis and RT-PCR. Northern blot and RT-PCR analysis with a probe specific for an 505-bp internal fragment of *hmuR* (Table 1) revealed that similar levels of the *hmuR* transcript were detected in *P. gingivalis* A7436 and MSM-3 (Fig. 7 and data not shown). Since transcription of *prtT* was shown to be unaffected by the insertion of IS*1126*$_2$ (data not shown), amplification of the *prtT* transcript was used as a positive control for these experiments. As anticipated, a *prtT* transcript was detected in *P. gingivalis* A7436 and MSM-3 (Fig. 7). These results indicate that the insertion of IS*1126*$_2$ upstream of *hmuR* did not produce a polar effect on *hmuR* transcription. Thus, the hemin utilization defect observed in *P. gingivalis* MSM-3 is not attributed to transcriptional inactivation of *hmuR*.

DISCUSSION

Transposition of IS*1126*. *P. gingivalis* IS*1126* was originally described by Maley and Roberts (24). During experiments involving the transfer of the *Bacteroides-E. coli* shuttle vector pNJR12 into *P. gingivalis* W83, these investigators found that IS*1126* had transposed into pNJR12 (24). However, transposition of IS*1126* in *P. gingivalis* was not demonstrated in this study. We have demonstrated for the first time the transposition of IS*1126* within *P. gingivalis*. We also demonstrated that IS*1126* transposition modulates the transcription of the genes encoding gingipain K (*kgp*) and gingipains R (*rgpA* and *rgpB*). Transposition of IS*1126* in *P. gingivalis* was observed after introduction of the *Bacteroides* transposon Tn*4351*, suggesting that the introduction of Tn*4351* into *P. gingivalis* may have resulted in IS*1126* duplication and transposition. This was observed in several independently isolated Tn*4351*-generated transconjugants, suggesting that IS1126 transposition in *P. gingivalis* may be site specific. It is also possible that transposition of IS*1126* may have occurred spontaneously during laboratory passage. However, Southern blot hybridization analysis of genomic DNA from 15 independent passages of *P. gingivalis* MSM-3 demonstrated that laboratory passage did not result in

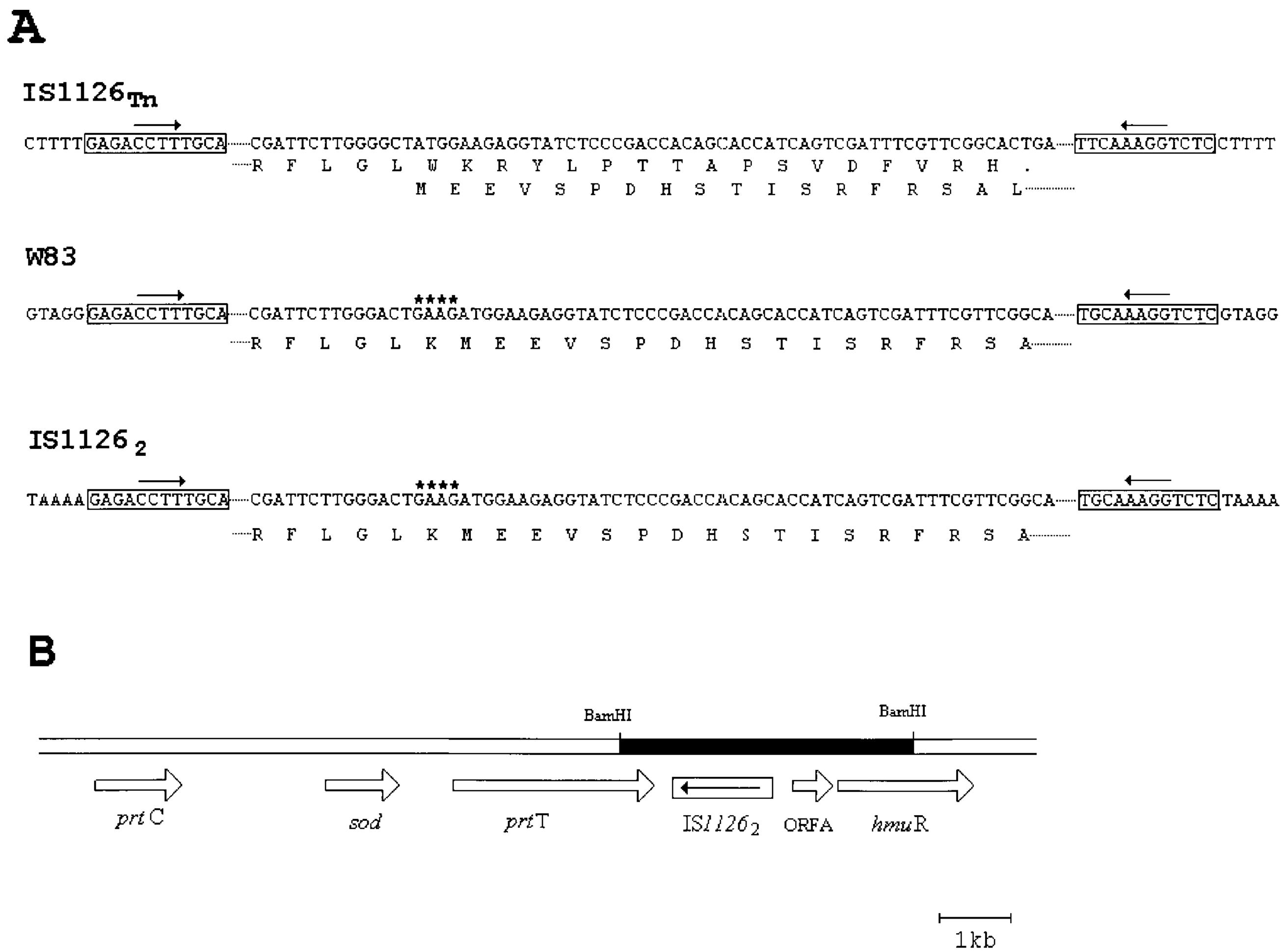

FIG. 5. Nucleotide sequence of the IS*1126* elements isolated in this study and the transposition site of IS1126_2. (A) Comparison of the nucleotide sequences and the deduced amino acid sequences of different IS*1126* elements. Partial nucleotide and amino acid sequences of IS1126_{Tn} were compared with those of IS*1126* from *P. gingivalis* W83 and IS1126_2. Boxes represent the 12-bp terminal inverted repeats of IS*1126*. The 5-bp nucleotide sequences flanking inverted repeats are duplicated target sequences generated after IS*1126* transposition. Asterisks denote the 4-bp 5′-GAAG-3′ deletion found in IS1126_{Tn} which resulted in premature termination of IS*1126* transposase synthesis. (B) Location of the duplicated copy of IS1126_2 found in *P. gingivalis* MSM-3 genome. A 4-kb *Bam*HI restriction fragment (shaded) was cloned directly from MSM-3 chromosomal DNA into pGEM3Zf, and the nucleotide sequence was determined. Large arrows represent the size and orientation of the *prtT*, *sod*, *prtC*, and *hmuR* genes and of *orfA*. Small arrow in IS1126_2 indicates the direction of IS*1126* transposase gene. *prtT*, cysteine protease gene; *sod*, superoxide dismutase gene; *prtC*, putative collagenase gene.

the transposition of IS*1126* (data not shown). Thus, these results lead us to conclude that IS*1126* transposition was mediated by Tn*4351*; however, this needs to be definitively proven. Recently, we identified a new IS element in *P. gingivalis* designated PGIS2 and reported its transposition following the introduction of Tn*4351* (44). Though the precise mechanism of IS transposition within *P. gingivalis* has not yet been elucidated, our results indicate that the transposition of endogenous IS elements is associated with the introduction of Tn*4351* into the *P. gingivalis* genome. The complexity of *P. gingivalis* genomic rearrangements after Tn*4351* transposition and the apparent site specificity of insertion will thus restrict its use for further transpositional mutagenesis for *P. gingivalis*.

Lewis and Macrina (20) recently described a new *P. gingivalis* insertion sequence, IS*195*. These investigators identified a naturally occurring variant of *P. gingivalis* W83 carrying IS*195* within the coding region of *prtP* gene (*kgp* homolog). IS*195* was also present downstream of the *prtP* gene in *P. gingivalis* HG66 and 381. Comparison of the nucleotide sequences of *rgpA* and *kgp* indicates that a majority of the C-terminal sequences of these genes are identical. It has been suggested that recombinational rearrangement, such as transposition or gene conversion, may have occurred in this nucleotide region between *kgp* and *rgpA*. At least two other DNA regions on the *P. gingivalis* chromosome that may encode for other hemagglutinins share homology with this region (14), and this suggests that these DNA regions may have also taken part in this recombinational event. It is also possible that these DNA regions may have been supplied from the chromosomal DNA of other *P. gingivalis* cells (horizontal gene transfer). Gene conversion type recombination has been observed in *P. gingivalis* (26), and thus it is reasonable to postulate that recombination between *P. gingivalis rgpA* and *kgp* could occur by such a mechanism. Our results suggest that, in addition to gene conversion, the transposition of endogenous IS elements may facilitate recombinational rearrangements in *P. gingivalis* and that recombination within *kgp* and *rgpA* genes could have occurred via a transposition event mediated by *P. gingivalis* IS*1126*.

IS*1126* transposition modulates gingipain expression. Although it is well established that transposition of IS elements

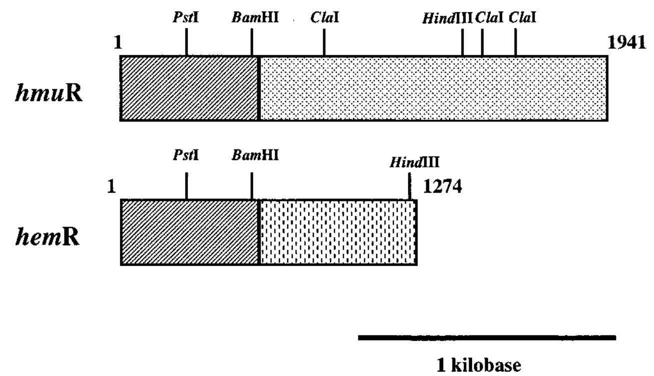

FIG. 6. Comparison of the genetic organization of the *hmuR* and *hemR* genes of *P. gingivalis*. The *hmuR* gene was cloned and sequenced from *P. gingivalis* A7436, while the *hemR* gene was cloned and sequenced from *P. gingivalis* 53977 (18). Hatched boxes denote the regions of identity between the two genes. Restriction sites are noted.

can inactivate a targeted gene, in this study we report for the first time that IS*1126* transposition can modulate gingipain expression in *P. gingivalis*. The location of the IS1126_1 insertion in *P. gingivalis* MSM-3 indicates that IS1126_1 has inserted into a putative *kgp* promoter region. Directly associated with and flanking the area of IS1126_1 insertion are regions which exhibit extensive homology to consensus bacterial −35 and −10 sequences, suggesting that this area corresponds to the putative *kgp* promoter. Insertion into the putative promoter or ribosomal binding site would disrupt the transcription of *kgp* with concomitant disruption of lysine-specific cysteine proteinase activity.

The increased transcription of the *rgpA* and *rgpB* genes may be due to the absence of *kgp* in the *P. gingivalis* proteinase population. The Kgp protease appears to be the major lysine-specific protease expressed in *P. gingivalis*, and its absence could serve as an intracellular stress signal, signaling the organism to upregulate the transcription of other gingipains, such as *rgpA* and *rgpB*. This scenario is supported by recent studies by Tokuda et al. (43), which suggest that *kgp* and *rgp* transcription may be coordinately linked. Alternatively, the increased *rgpA* and *rgpB* transcription may result from additional but uncharacterized IS*1126* elements which may have transposed to different chromosomal loci but whose movements have not led to the generation of novel hybridizing bands due to preexisting IS*1126* elements in this region.

Recent studies by Kuboniwa et al. (18) have demonstrated that Kgp can bind human hemoglobin and that binding is mediated through Kgp domains which are distinct from the proteinase domain. We have also demonstrated that Kgp can bind human hemoglobin and that binding is to the hemagglutinin domains of the protein (9). Okamoto et al. (28) recently reported that *P. gingivalis kgp*-deficient mutants are nonpigmented and are markedly decreased in their ability to bind hemoglobin. Although these mutants could not bind hemoglobin, these investigators failed to demonstrate if the *kgp*-deficient mutants were capable of growing with hemin and/or hemoglobin as sole iron sources. The phenotype of the *kgp* mutants described by these investigators is similar to the phenotype of *P. gingivalis* MSM-3, the mutant we describe in this study which resulted from IS*1126* insertional inactivation of the *kgp* gene. In previous studies, we determined that *P. gingivalis* MSM-3 grew poorly with hemin or hemoglobin as the sole iron sources (10). Hemoglobin binding assays demonstrated that *P. gingivalis* MSM-3 bound less hemoglobin compared to the parental strain (41). Thus, the decreased ability of *P. gingivalis* MSM-3 to utilize hemin and hemoglobin as sole iron sources may result from disruption of the *kgp* gene. The observation that *P. gingivalis* MSM-3 did not exhibit a total decrease in hemoglobin binding may be due the presence of multiple hemoglobin receptors in *P. gingivalis*, including HmuR, and as has been described for other gram-negative organisms (3). We should stress that the exact role of Kgp in hemin-hemoglobin transport in *P. gingivalis* remains to be defined. Aduse-Opoku et al. (1) have reported on the identification of the *tla* gene which is required by *P. gingivalis* for growth with low levels of hemin. These investigators found that a *P. gingivalis tla* mutant produced significantly lower arginine- and lysine-specific protease activities and, on the basis of these results, suggested that a regulatory link exists between *tla* and other members of this gene family. Taken together, the results reported in the present study, as well as those of other investigators (1, 19, 27, 28), indicate that the gingipains may function in hemin-hemoglobin utilization and that expression of the genes encoding these proteins may be coordinately regulated by hemin.

Identification of *hmuR*. In this study we have also identified a novel *P. gingivalis* gene, *hmuR*, which exhibits a high degree of homology to genes encoding TonB-dependent outer membrane receptors. In most organisms, the energy for the transport of ligands across the outer membrane is provided by the TonB protein. The transport of hemin in *Shigella dysenteriae* (25), *Haemophilus influenzae* (17), and *Yersinia enterocolitica* (42) requires the TonB protein. The TonB protein interacts with respective ligands at several unique sites termed TonB boxes. The protein encoded by *hmuR* exhibits extensive homology to other TonB-dependent ligands at TonB box IV, the domain of the receptor believed to physically interact with the TonB protein (40). Although we have previously demonstrated that hemin transport in *P. gingivalis* occurs via an energy-dependent process (12) and have postulated the existence of a TonB homolog in *P. gingivalis*, a *P. gingivalis* TonB homolog has not yet been identified. Our results also indicate that the defect in the ability of *P. gingivalis* MSM-3 to utilize hemin for growth is not a result of transcriptional inactivation of *hmuR* by IS*1126* insertion. However, whether or not HmuR is translated in MSM-3 remains to be determined. Nonetheless, a *P. gingivalis hmuR* mutant was demonstrated to grow poorly with

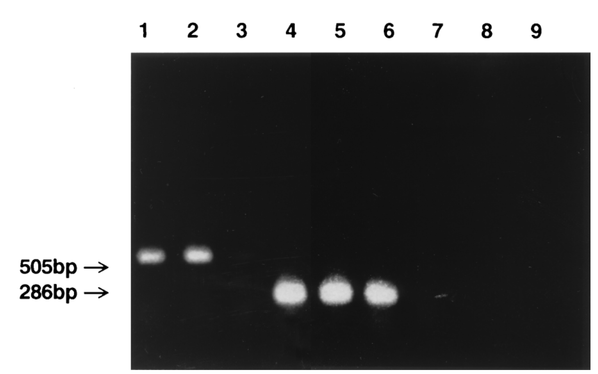

FIG. 7. Transcription of *hmuR* in *P. gingivalis* strains. Cultures were grown in BM without hemin for 16 h. Lanes: *hmuR* amplified from A7436 (lane 1), *hmuR* amplified from MSM-3 (lane 2), *hmuR* amplified from W50 (lane 3), *prtT* amplified from A7436 (lane 4), *prtT* amplified from MSM-3 (lane 5), *prtT* amplified from W50 (lane 6). Negative controls included *hmuR* amplified from A7436 RNA by using *Taq* polymerase (lane 7), *hmuR* amplified from MSM-3 RNA by using *Taq* polymerase (lane 8), and *hmuR* amplified from W50 RNA by using *Taq* polymerase (lane 9).

hemin or hemoglobin as the sole sources of iron (41) and the identification of the *hmuR* gene in this study was fortuitous.

Interestingly, we found that *hmuR* was nearly identical at the 5′ end with *P. gingivalis hemR* (18). Our studies demonstrate that the 3′ end of *hmuR* exhibits identity to genes involved in hemoglobin binding and/or utilization. Karunakaran et al. (18) have shown that the 3′ region of *hemR* exhibits identity to the *prtT* gene of *P. gingivalis*. The differences in the 3′ regions of *hmuR* and *hemR* may have resulted from (i) a rearrangement event that mediated the insertion of a portion of *prtT* into *hemR* via homologous recombination, and *hmuR* is representative of the ancestral gene, or (ii) a rearrangement event in which the region homologous to *prtT* was deleted from *hmuR*. Either scenario is reminiscent of the proposed genomic rearrangements in the *P. gingivalis* proteinase and hemagglutinin genes (26).

Conclusions. There is increasing evidence that gingipains are major virulence factors of *P. gingivalis* and may be directly responsible for the clinical features of adult periodontal disease such as gingival crevicular fluid production, neutrophil accumulation, and bleeding (10). Since the majority of *P. gingivalis* strains examined in one study appear to produce gingipains R and gingipain K (32), it has been postulated that the involvement of these proteinases in virulence may be due to differential regulation and enhanced expression in virulent strains. The results presented here indicate that transposition of *P. gingivalis* IS elements can modulate the expression of gingipain K and, indirectly, gingipains R. Taken together, these results suggest that transposition of IS elements (those which have been described and those remaining to be identified) within the *P. gingivalis* genome and that the subsequent modulation of gingipain expression may be common events which serve to alter the virulence potential of *P. gingivalis*.

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