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Analysis of Involvement of the RecF Pathway in *p44* Recombination in *Anaplasma phagocytophilum* and in *Escherichia coli* by Using a Plasmid Carrying the *p44* Expression and *p44* Donor Loci

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Anaplasma phagocytophilum*, the etiologic agent of human granulocytic anaplasmosis, has a large paralog cluster (approximate 90 members) that encodes the 44-kDa major outer membrane proteins (P44s). Gene conversion at a single *p44* expression locus leads to P44 antigenic variation. Homologs of genes for the RecA-dependent RecF pathway, but not the RecBCD or RecE pathways, of recombination were detected in the *A. phagocytophilum* genome. In the present study, we examined whether the RecF pathway is involved in *p44* gene conversion. The recombination intermediate structure between a donor *p44* and the *p44* expression locus of *A. phagocytophilum* was detected in an HL-60 cell culture by Southern blot analysis followed by sequencing the band and in blood samples from infected SCID mice by PCR, followed by sequencing. The sequences were consistent with the RecF pathway recombination: a half-crossover structure, consisting of the donor *p44* locus connected to the 3′ conserved region of the recipient *p44* in the *p44* expression locus in direct orientation. To determine whether the *p44* recombination intermediate structure can be generated in a RecF-active *Escherichia coli* strain, we constructed a double-origin plasmid carrying the *p44* expression locus and a donor *p44* locus and introduced the plasmid into various *E. coli* strains. The recombination intermediate was recovered in an *E. coli* strain with active RecF recombination pathway but not in strains with deficient RecF pathway. Our results support the view that the *p44* gene conversion in *A. phagocytophilum* occurs through the RecF pathway.

Human granulocytic anaplasmosis (HGA; formerly human granulocytic ehrlichiosis or HGE) is a significant, emerging tick-borne infectious disease, first reported in 1994 (9). The disease had been increasingly recognized in the United States and Europe, and HGA was designated as a nationally notifiable disease for the United States in 1998 (14). HGA is a potentially fatal systemic disease characterized by fever, headache, myalgia, anorexia, and chills and is frequently accompanied by leukopenia, thrombocytopenia, anemia, and elevations in serum hepatic aminotransferases (2). The etiologic agent, isolated from HGA patients in 1995 (15), is an obligate intracellular rickettsial pathogen that was recently reclassified with other related *Ehrlichia* spp. as *Anaplasma phagocytophilum* (10).

The *p44* multigene family of *A. phagocytophilum* encodes immunodominant 44-kDa major outer membrane proteins, P44s (4, 11, 25–27, 39, 42–44). P44 plays critical roles in infection. For example, anti-P44 antibodies can prevent *A. phagocytophilum* infection in cell culture (39) and partially protect mice from experimental infection with *A. phagocytophilum* (20), and a recombinant P44 protein induces proinflammatory cytokines in human leukocytes in vitro (21). The *p44* gene family has a central hypervariable region of approximately 280 bp. This region is flanked by 50- to 500-bp sequences from each of 5′ and 3′ conserved regions (see Fig. 1A). To date, 88 individual *p44* paralogs or orthologs had been identified by their signature hypervariable nucleotide sequences. Many of *p44*s are considered functional pseudogenes (silent storage copies) rather than nonfunctional pseudogenes on the way to elimination since, despite truncation of 5′- and/or 3′-terminal sequences, some express the full-length *p44* transcripts and P44 proteins (26, 42).

Several studies reported that diverse *p44* paralogs are expressed in patients, in animal models of infection (mouse and horse), and in ticks (4, 11, 25, 26, 40, 42, 44). This antigenic variation system is expected to allow *A. phagocytophilum* to avoid and escape host immune recognition and to allow adaptation to new environments, especially during tick transmission of *A. phagocytophilum* (11, 33, 34, 44). A single polymorphic *p44* expression locus that consists of four tandem genes—*tr1*, *omp-1X*, *omp-1N* (corresponding to *p44ESup1* described by Barbet et al. [4]), and *p44E* (any *p44* species at the *p44* expression locus, corresponding to *msp2* described by Barbet et al. [4])—was identified in several *A. phagocytophilum* strains (4, 26). Recently, the successful development of an isogenic clone from *A. phagocytophilum* HZ strain allowed us to demonstrate the nonreciprocal recombination (gene conversion) of paralogous *p44*s at this *p44* expression locus (27). Although expression locus is expected to be the primary locus for diverse *p44* gene expression in *A. phagocytophilum* (4, 26), the molecular mechanisms of *p44* gene conversion, and thus antigenic variation, are largely unknown.

Several bacterial pathogens such as *Borrelia burgdorferi* and *Neisseria gonorrhoeae* exhibit antigenic variation by gene conversion (the nonreciprocal transfer of DNA sequences between homologous genes) within their hosts (5). Only a few studies, however, have described recombination mechanisms responsible for antigenic switching in bacteria. RecA-dependent RecF-mediated

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recombination was suggested to mediate *N. gonorrhoeae* pilin antigenic variation (32). Three RecA-dependent homologous recombination pathways, RecF, RecE, and RecBCD, have been identified in *Escherichia coli* (22, 23). Several studies using *E. coli* mutant strains and plasmids showed that RecF pathway recombination is nonreciprocal without crossover, but RecBCD and RecE pathway recombination can be reciprocal and associated with crossover (36). We previously reported that *A. phagocytophilum* lacks homologs of genes required for RecBCD or RecE recombination pathways but has homologs of most of the genes involved in RecF recombination (26, 27). The *p44* recombination is apparently nonreciprocal (i.e., gene conversion), in that the donor *p44* is copied at the *p44* expression locus and previous resident *p44E* vanishes after conversion (26, 27); this gene conversion occurs without crossover, preserving the entire donor region and noncoding regions flanking *p44E*; this gene conversion is nonsegmental, in that the *p44* hypervariable region is identical between donor *p44* and *p44E* (11, 26, 27), although Barbet et al. proposed that *p44* gene conversion is segmental (4). Our analysis using cloned *A. phagocytophilum* population is consistent with our previous observation that both 5′ and 3′ conserved regions of *p44E* flanking the hypervariable region contain nucleotide sequence variations (11, 26, 27). This analysis is also in agreement with our previous prediction that instead of using the entire conserved regions for gene conversion, various lengths (50 to 200 bp) of partial 5′ and 3′ conserved region sequences at the border of the hypervariable region are used for gene conversion in the *p44* expression locus (27). Kobayashi (22) proposed a novel successive two-half-crossover (no crossover) model as the mechanism of RecF pathway gene conversion since two types of half-crossover intermediates could be isolated as only one product of possible crossover using a double-origin plasmid in the *E. coli* RecF active strain (22, 38, 41). A similar mode of gene conversion without crossover during yeast meiosis, called the synthesis-dependent strand annealing model, can be also explained by the successive two-half-crossover model (1). For obligatory intracellular bacteria including *A. phagocytophilum*, there is no useful genetic system and no naturally isolated mutant, making the genetic analysis of *p44* recombination mechanisms impossible. In the present study, therefore, we first examined whether the half-crossover intermediate structure is formed between the donor *p44* and recipient *p44E* in *A. phagocytophilum*. Second, we constructed a double-origin plasmid carrying a donor *p44* locus and a recipient *p44E* locus with the antibiotic selection markers to investigate whether the *p44* half-crossover occurs in the RecF pathway active *E. coli* strain. This study is the first analysis of the recombination pathway and intermediate structure involved in the *A. phagocytophilum p44* antigenic variation.

MATERIALS AND METHODS

Southern blot analysis of *A. phagocytophilum* genomic DNA digested by SacI and sequencing of the 5.3-kb DNA fragment. The genomic DNA of *A. phagocytophilum* HZ was extracted from organisms purified from HL-60 culture by Sephacryl S1000 chromatography (26). Total DNA (8 μg) was digested by restriction enzyme SacI and loaded into each well of a 0.7% agarose gel. The *p44-18* (P1) probe was amplified by the primer pair p1263960 (5′-CGTGGAG ATTTCTAATTCCGG-3′) and p1263637 (5′-TTCAGGGGTGAGCTTCTTA G-3′). The probe (P2) *p44E* upstream intergenic region was amplified by using the primer pair p1289877 (5′-TGGACGAGAAGAATGGGATC-3′) and p1290352 (5′-TCTTCGTCTCCTCACTTCAG-3′). PCR for preparing Southern blot probes was performed in a 50-μl reaction mixture, containing 10 ng of genomic DNA of *A. phagocytophilum* HZ, 10 pmol of each primer, a 0.2 mM concentration of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, and 5 U of *Taq* DNA polymerase. PCR was performed with 2 min of denaturation at 94°C, followed by 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 58°C, and 1 min of extension at 72°C. The probes were labeled by PCR amplification using a biotinylation kit according to the manufacturer's instructions (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). DNA transfer, hybridization, and detection were carried out by using an AP Chemiluminescent Blotting Kit (Kirkegaard & Perry).

The 5.3-kb region DNA fragments digested by SacI were recovered from 0.7% agarose gel by using QIAEX II gel extraction kit (QIAGEN, Valencia, CA). The intermediate sequence was PCR amplified with extracted DNA as a template with the primer pair p1266002 (5′-CAATCTTACCCCTAGTGAGCAGT-3′) and p1292839 (5′-TGTACAGCTTGTAGCCGGTAAT-3′) and the primer pair p1265827 (5′-TTACCTATTACAGTATCACCATGC-3′) and p1292327 (5′-AA CAAGATATGCATCCGCAAATC-3′). The first PCR product was 1:100 diluted, and 5 μl was used for the second PCR. The products of the second PCR were sequenced. Sequence assembling, alignments, and analysis were performed by using the SeqMan, MegAlign, and MapDraw programs (DNAStar, Inc., Madison, WI).

Analysis of *A. phagocytophilum* recombination intermediate structure in an infected SCID mouse. Ten 4-week-old ICR SCID male mice (Taconic Farm, Inc., Germantown, NY) were each inoculated intraperitoneally with the cloned *A. phagocytophilum* HZ strain (27). The blood sample of SCID mouse 9 was used to investigate recombination based on the previous temporal *p44E* sequence population analysis study (27). The nested PCR was performed with primers specific to the upstream sequence of the donor *p44-1/18* locus and the downstream sequence of the *p44E* and was performed with DNA isolated from peripheral blood leukocytes from the SCID mouse. The primer pairs used were primers p1265450 (5′-GCTATGGGAGATTACTATTC-3′) and p1292839 and primers p1265376 (5′-CATTTTCTTTAAAAGGCAGAC-3′) and p1292327 (Fig. 1A).

PCR with *Pfu* DNA polymerase was performed in a 50-μl reaction mixture containing 5 μl of the DNA product, 10 pmol of each primer, a 0.2 mM concentration of each deoxynucleoside triphosphate, and 5 U of *Pfu* Ultra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA). PCR was performed with 2 min of denaturation at 94°C, followed by 35 cycles of 20 s of denaturation at 94°C, 20 s of annealing at 58°C, and 5 min of extension at 70°C. PCR products were purified from a gel and cloned into the pCR-Blunt vector (Invitrogen, Carlsbad, CA). DNA clones were randomly selected from the transformants and sequenced on an ABI 373XL Stretch DNA sequencer by using the ABI Prism BigDye terminator cycle sequencing reaction kit (ABI, Foster City, CA).

To examine the possibility of PCR artifacts formed during amplification, *p44-1/18* and *p44E* were cloned into the TA cloning vector pCRII (Invitrogen), and 20 ng of plasmid mixture was spiked in genomic DNA from an uninfected SCID mouse and used as the PCR template. The PCR was performed with the primer pairs p1265450-p1292839 and p1265376-p1292327. The first PCR product was 1:100 diluted, and 5 μl was used for the second PCR. To investigate possibility of the reciprocal crossing over between the upstream sequence of the *p44E* and the downstream sequence of donor *p44-1/18* locus, nested PCR was performed by using the same SCID mouse DNA sample. For this reaction, the two forward primers were p1290155 (5′-CGT TATTTGTTCTAGAGAAAG-3′) and p1290226 (5′-ATTGGACTTTTGAGCT-GTCTT-3′), and the reverse primers were p1263125 (5′-CACCACGCAGGAATA TCGATCT-3′) and p1263083 (5′-GCTTTTGCCACTAGAGACAGG-3′) (Fig. 1A).

The *p44-18* donor locus, *p44-1/18*, was PCR amplified by using the primer pair p1265450 and p1263125 from the SCID mice DNA and sequenced. The PCR conditions used were as described above.

Construction of a plasmid encoding *p44E* expression and *p44* donor loci and analysis of their recombination. Plasmid pEKD30, a double-origin (ColE1 and p15A) plasmid of 9.3 kb in length (see Fig. 3Ai) was constructed by using the plasmid pKEN33 (generously provided by Ichizo Kobayashi [38]) as the backbone. A recipient locus and a donor locus derived from *A. phagocytophilum* were inserted into the plasmid pKEN33. The recipient locus consisted of the 213 bp upstream of *p44E* (*p44E IR* in Fig. 3Ai), a kanamycin-resistant gene *km*, an *E. coli* ribosome binding site (TAAGGAG), and *p44-18E* and its 446 bp downstream sequence (*p44E DS* in Fig. 3Ai). The *km* gene was PCR amplified from plasmid pCR-XL-TOPO (Invitrogen). The 213-bp intergenic upstream sequence, *p44-18E* and its 466-bp downstream sequence were PCR amplified from *A. phagocytophilum* genomic DNA. The intergenic region was amplified by the primer pair p1291699 (5′-GGTCGACGGGCTAAGGGCTCCCCTTTT-3′) and p1292129 (5′-ATCTAGAGCAATAGACCCAGTAG-3′) to have XbaI and SalI sites at each end, respectively. The *p44-18E* and the downstream sequence was

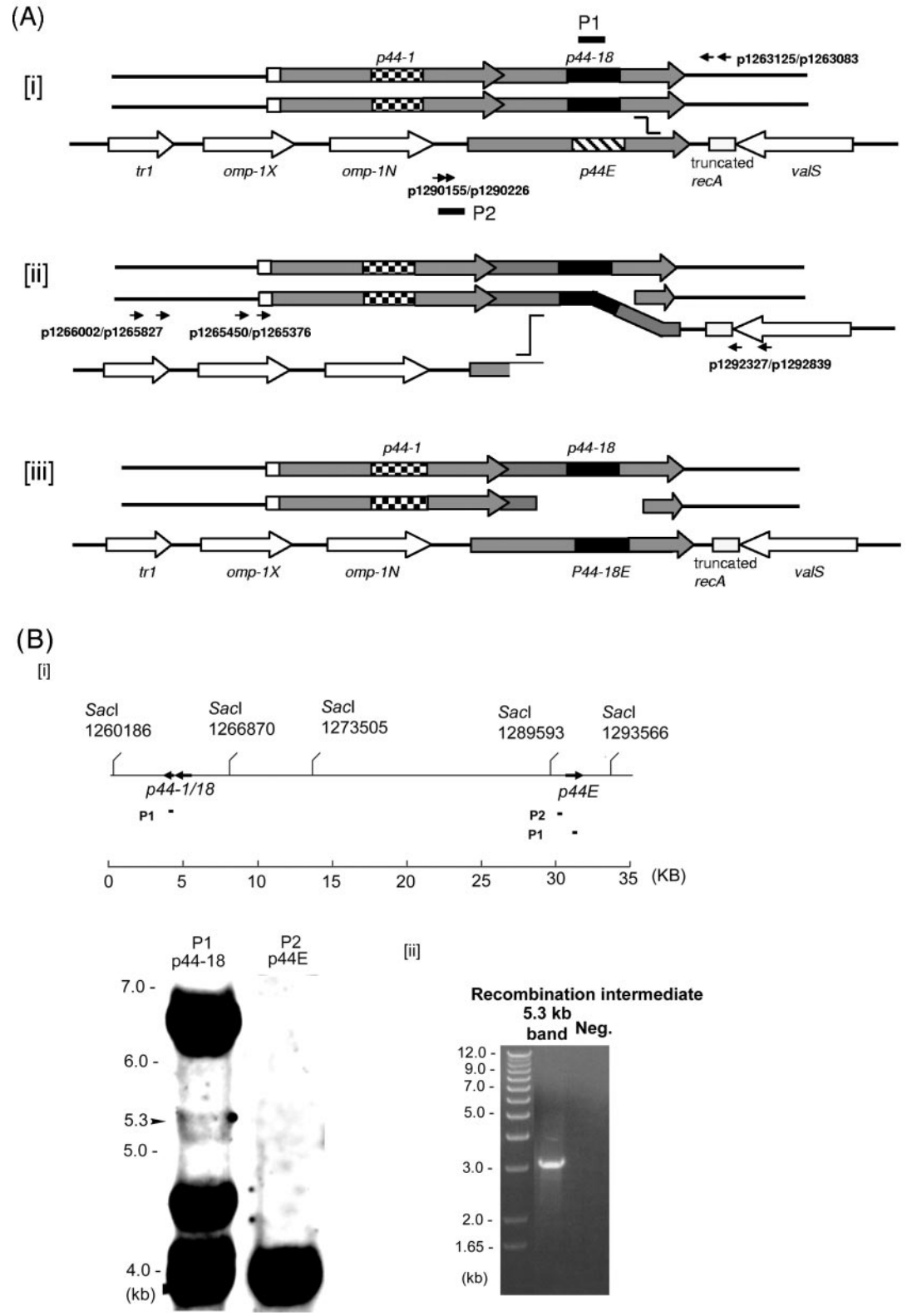

FIG. 1. Analysis of the *p44* recombination intermediate in *A. phagocytophilum*. (A) Successive half-crossover model for *p44-18* conversion to the *p44E* and the experimental design. [i] The donor locus was expected to be a part of replicated chromosome; the *p44-1/18* locus in a donor chromosome (*p44-1/18D*) synapses with the 3′ conserved region of *p44E* in the recipient chromosome. [ii] A half crossover between *p44E* and *p44-1/18D* generates one recombination duplex and two ends. The upstream of the synapsed region of *p44E* is presumably degraded to generate a single-stranded tail at the 5′ conserved region by an exonuclease. [iii] The putative final products of the successive crossover are one duplex with two double strands exchanged in the recipient *p44* expression locus and two ends in the donor locus. The gray boxes are *p44* conserved regions,

amplified by primer pair p1290170 (5′-GGGCATGCATGCGACGTCAGAAA GATGTGCGTAAGAGGTAA-3′) and p1291698 (5′-CCTGCAGCCCTCTTT AGATAAGCAAGCTTA-3′) to have AatII and PstI sites at each end, respectively. *km* was amplified by using the primer pair pkmF (5′-GCTGCAGTAAG GAGGTTTCGC ATGATTGAACAAGATG-3′) and pkmR (5′-CGTCGACTC AGAAGAACTCGTCAAGAAGG-3′), including a ribosomal binding site sequence (5′-TAAGGAG-3′) immediately upstream of the *km* gene and SalI and PstI site at each end, respectively. Restriction enzyme sites are underlined. These three fragments were digested by restriction enzymes AatII, PstI, SalI, and XbaI and were then ligated in direct orientation into pKEN33 that had been digested by XbaI and AatII to give a plasmid pKEN33-EKD.

As a donor *p44* sequence, *p44-30*, which is a truncated *p44*, 897 bp in length (as a SalI and NdeI fragment), was selected. In order to insert an *E. coli recA* promoter (5′-CAAAACACTTGATA$_{-35}$CTGTATGAGCATACAGTATAAT$_{-10}$T GCTTCA-3′) (28) into the *p44-30* hypervariable region, two primer pairs were designed to amplify the 5′ and 3′ fragments of *p44-30* that were overlapped at the RecA promoter sequence: p1416475 (5′-GGGTCGACGTCGAC GTATACCA AAAGCCTATGCAATAA-3′)-p1416768 (5′-TGAAGCAATTATACTGTATG CTCATACAGTATCAAGTGTTTTGGTATTCCGTTACGCTTCCTCC-3′) and p1416771 (5′-CAAAACACTTGATACTGTATGAGCATACAGTATAAT TGCTTCAGGCAGAGCCGGATGAAAACAC-3′)-p1417466 (5′-GGCATAT GCATATGAGAATTAAAGTAGAAAAGGGGAG-3′). Then, using these two PCR fragments as a template, the *p44-30* with the *E. coli recA* gene promoter in the hypervariable region (*p44-30PrecA*) was PCR amplified by primers p1416475 and p1417466. The PCR product was digested by restriction enzymes SalI and NdeI and was ligated into pKEN33-EKD that had been digested by SalI and NdeI (this also removed the original *km* gene in pKEN33) to give pEKD30. All inserted fragments were sequenced and the plasmid pEKD30 was kept in a Δ*recA* strain, INVαF′, with minimal generation numbers.

Detection and analysis of recombination. The isogenic *E. coli* K-12 strains JC7623 (*recB21 recC22 sbcB15 sbcC201*) (24, 29), JC8111 (*recB21 recC22 sbcB15 sbcC201 recF143*), and JC8679 (*recB21 recC22 sbcA23*) were from *E. coli* genetic stock center (Yale University, New Haven, CT). JC12190 (*recB21 recC22 sbcB15 sbcC201 recJ153*) (17, 30) was obtained from Ichizo Kobayashi (University of Tokyo, Tokyo, Japan). Competent cells were prepared as described previously (35). Approximately 200 ng of the intact plasmid DNA was delivered into 10^{10} bacterial cells at a field strength of 12.5 kV/cm for 5.2 to 5.5 ms. Even when recombination generates two plasmids, they may be lost during growth in the absence of selection; therefore, we only let the bacteria recover at 37°C, with rotation at 220 rpm for 1 h after electroporation. The bacteria were then applied to SOB plates containing different combinations of chloramphenicol (Cm), kanamycin (Km), and amphotericin B (Am), each at 50 μg/ml, and colony numbers on plates were determined to assess the recombination frequency. Km-resistant colonies were randomly selected and analyzed by site-specific PCR. The sites for binding of primers p1416475 and pkmR (sequence shown above) were located upstream of the donor *p44-30* and in the 3′ end of the *km* gene, respectively. The binding sites for the other primer pair, p1290226-pkmR, were located upstream of *p44E* and 3′ end of the *km* gene, respectively. The plasmid was extracted from the Km-resistant colonies and digested by XbaI or by XbaI and SalI to confirm the fragment sizes. The fragments were cloned and sequenced.

RESULTS

Analysis of the *p44* half-crossover structure in *A. phagocytophilum*-infected HL-60 cells by Southern blot analysis followed by sequencing. The majority (80 to 90%) of noncloned *A. phagocytophilum* HZ isolated after many passages in cell culture (>100 passages, with each passage after 3 to 4 days of culture) has *p44-18* at its *p44* expression locus (*p44-18E*) (39, 40, 44). Lower percentages are sometimes observed due to presently undefined reasons, but during subsequent passages the percentage eventually returns to the original 80 to 90% of *p44-18E* in the population. Therefore, we hypothesized that the recombination intermediate structure linking the donor *p44-18* sequence to the recipient *p44* expression locus is detected in *A. phagocytophilum* in culture. The donor *p44-1/18* locus consists of tandem *p44-1* (1,242-bp full-length *p44* gene) and *p44-18* (762-bp pseudogene) (Fig. 1A). We isolated genomic DNA from this high-passage *A. phagocytophilum* HZ to detect the recombination intermediate by Southern blot analysis. For Southern blot analysis we chose restriction enzyme SacI. As shown in our previous sequencing data (26) and the map of SacI restriction sites (Fig. 1Bi), there is no SacI site within the *p44* expression locus and the donor *p44-1/18* locus, and the *p44* expression locus is expected to be in an ~4.0-kb fragment and the *p44-1/18* locus is expected be in an ~6.6-kb fragment. The recombination intermediate structure is predicted to be approximately 5.3 kb (Fig. 1Aii and Bi).

Probes for Southern blotting were designed as shown in Fig. 1A and Bi. The P1 probe was designed to be specific to *p44-18* hypervariable region, and the P2 probe was designed to be specific to a region upstream of *p44E* in the expression locus. By using P1 probe, the ~6.6-kb band corresponding to the *p44-1/18* locus, and the ~4.0-kb band corresponding *p44-18E* in the expression locus were detected (Fig. 1Bi). In addition, a new and weak band of ~5.3 kb was consistently detected by using P1 probe (Fig. 1Bi). An ~4.7-kb band hybridized with P1 probe. *p44-18* was not detected in this band region; however, the sequence which has 134/171 bp (78%) identity with P1 probe was detected in the SacI fragment of *A. phagocytophilum* genomic DNA of this size.

The P2 probe was expected to hybridize to the expression locus 4.0-kb band. As shown Fig. 1Bi, the P2 probe hybridized to the 4.0-kb and did not hybridize to the 5.3-kb band, suggesting that if this band is the recombination intermediate, this is a nonreciprocal event (Fig. 1Bi). The 5.3 kb-band region which did not include 6.6- and 4.0-kb bands (therefore, there is no chance of strand jumping between these two fragments) was recovered from the agarose gel and used as a template for PCR using the primer pairs p1266002-p1292839 and p1265827-p1292327 (Fig. 1Aii). The PCR generated the 3.2-kb band (Fig. 1Bii). The sequencing result showed a half-crossover structure between the *p44-1/18* and the *p44* expression loci as shown in Fig. 1Aii and Fig. 2B. The GenBank accession number for the

and the large checkerboard, black, and wide downward diagonal are hypervariable regions of *p44-1*, *p44-18*, and the recipient *p44E*, respectively. The arrows of boxes indicate transcriptional orientation. Horizontal arrows with numbers indicate primers used to isolate the intermediate structure by PCR and primers used to demonstrate the absence of a reciprocal recombination product. Black short bars indicate the locations of two probes (P1 and P2) used for Southern blot analysis in Fig. 1B. The "stairstep" symbol indicates the potential pair of the recombination sites. (B) Southern blot analysis of the half-crossover intermediate structure of *A. phagocytophilum* cultured in HL-60 cells. [i] The probe positions and SacI digestion sites within the 35-kb region containing *p44-1/18* and *p44E* are indicated. The numbers under the SacI sites indicate the positions of the cleavage sites in the genome. The genomic DNA was purified from *A. phagocytophilum* cultivated in HL-60 cells. Southern blot analysis of genomic DNA digested with the restriction enzyme SacI with two probes (P1 and P2) showed the ~6.6-kb band containing the donor *p44-1/18* locus and a 4-kb band containing the recipient *p44E*. An ~5.3-kb band contained duplicated *p44-1/18*. [ii] PCR was performed using the primer pairs p1265450-p1292839 and p1265376-p1292327 (Fig. 1Aii) and the DNA isolated from the 5.3-kb region of the gel as a template. The numbers on the left indicate the molecular sizes. A band of approximately 3.2 kb was amplified and sequenced GenBank accession no. DQ011270. "Neg." refers to a negative control with water as a template.

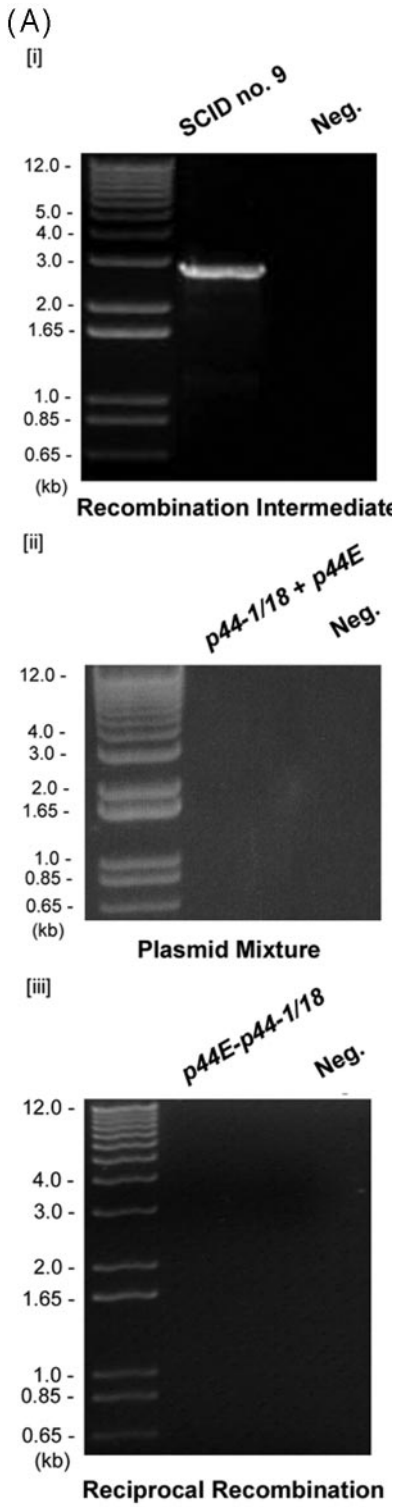

FIG. 2. Analysis of the *p44* recombination intermediate in *A. phagocytophilum* from an infected SCID mouse. (A) The half-crossover recombination intermediate was detected by PCR of peripheral blood leukocytes from an *A. phagocytophilum*-infected SCID mouse. [i] The ~2.9-kb band was amplified by the primer pairs p1265450-p1292839 and p1265376-p1292327 (Fig. 1Aii) using *Pfu* DNA polymerase. [ii] No PCR product was detected with the same primer pairs from uninfected SCID mouse DNA spiked with plasmids containing *p44-1/18* and *p44E* as a template. [iii] No PCR product was detected in peripheral blood leukocytes of infected mice with forward primers located in the intergenic region of *p44E* and *omp-1N* and with reverse primers located downstream of donor *p44-1/18* locus (Fig. 1Ai). "Neg." refers to a negative control with water as a template. (B) The nucleotide sequence of the half-crossover recombination duplex between *p44E* and *p44-1/18D*. The second set of primers used for the nested PCR are indicated by horizontal arrows and labeled with primer ID. *p44-1/18DE INT* is the intermediate structure containing donor site *p44-1/18* and the recipient site *p44E*. The sequence is identical to that expected for a half-crossover recombination intermediate as illustrated in Fig. 1Aii. The light-shaded areas indicate sequence identity between the crossover structure and the putative donor *p44-1/18* locus, and the dark shaded areas indicate identical sequence between the hybrid structure and the recipient *p44E*, or among all three structures. The boxed nucleotides are start codons for *p44-1* and for *p44-18*. The stop codons are underlined. Dashes indicate sequence gaps that helped to determine the origins of conserved sequences in the recombination intermediate.

intermediate sequence of the cell culture is DQ011270. Southern blot analyses were independently repeated by two investigators more than four times using different batch of *A. phagocytophilum* cultures and probe preparations of different sizes, as well as restriction enzymes purchased from different sources. The results with the P1 and P2 probes were reproducible. These results suggest that *p44E* recombination occurs via the RecF pathway successive half-crossover mechanism in the cell culture system.

Analysis of *p44* recombination intermediate structure from *A. phagocytophilum* infecting a SCID mouse. To confirm that the RecF-pathway recombination intermediate structure in *A. phagocytophilum* in infected animals, we analyzed peripheral blood leukocytes from a *A. phagocytophilum*-infected severe combined immunodeficiency (SCID) mouse 50 days after inoculation with *A. phagocytophilum*. This time point was used for analysis because populations of *p44-18E* appeared to increase at this time point in a previous study (27) and should provide a high probability of detecting the recombination intermediate structure. However, we could not obtain *A. phagocytophilum* DNA from infected SCID mice that were sufficiently enriched for Southern blot analysis due to the low infection rate of granulocytes and small amount of blood specimens. Therefore, we developed a nested PCR method as shown in Fig. 1A. The forward primers p1265450 and p1265376 are complementary to regions starting 136 and 60 bp upstream of *p44-1* of the *p44-1/18* locus, respectively; the reverse primers p1292839 and p1292327 are complementary to regions starting 619 and 107 bp from the 3′ end of the *valS* in the *p44* expression locus (Fig. 1A). Three elements of our PCR procedures were designed to prevent artifactual, recombination-like events that might occur during PCR. *Pfu* DNA polymerase was used for PCR, since it has been demonstrated that *Pfu* DNA polymerase does not have discernible recombination activity, in contrast to *Taq* and Vent DNA polymerases, due to in part high proofreading activity of *Pfu* DNA polymerase (37). We used prolonged polymerase extension times (4 min) in each cycle, since an incompletely extended segment of DNA and the partially amplified sequence act as a primer during subsequent amplification. In addition, rapid cooling between the denaturation step and the annealing step (20 s) was used to discourage annealing of incomplete products over the more abundant primer.

(B)

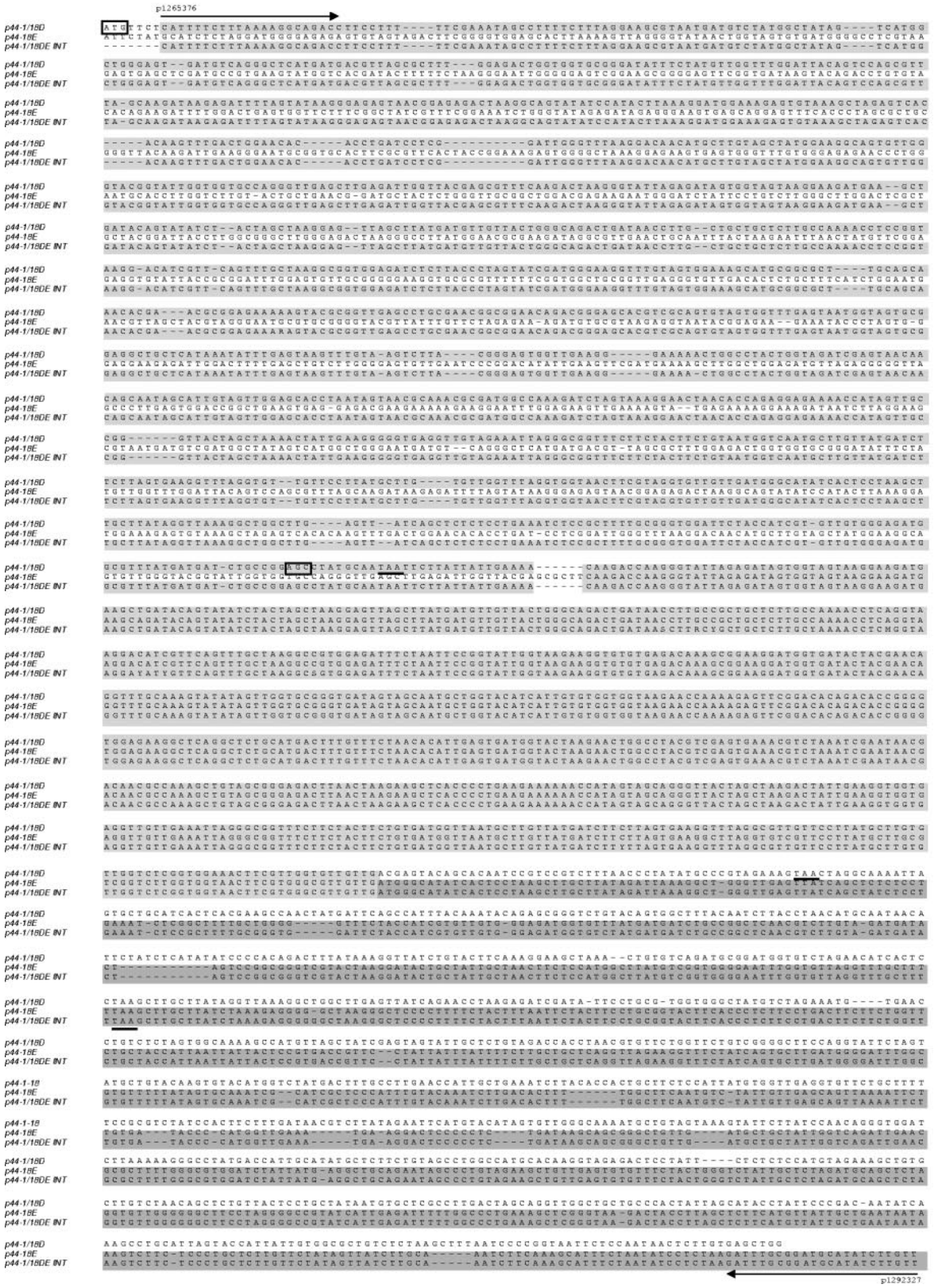

FIG. 2—*Continued.*

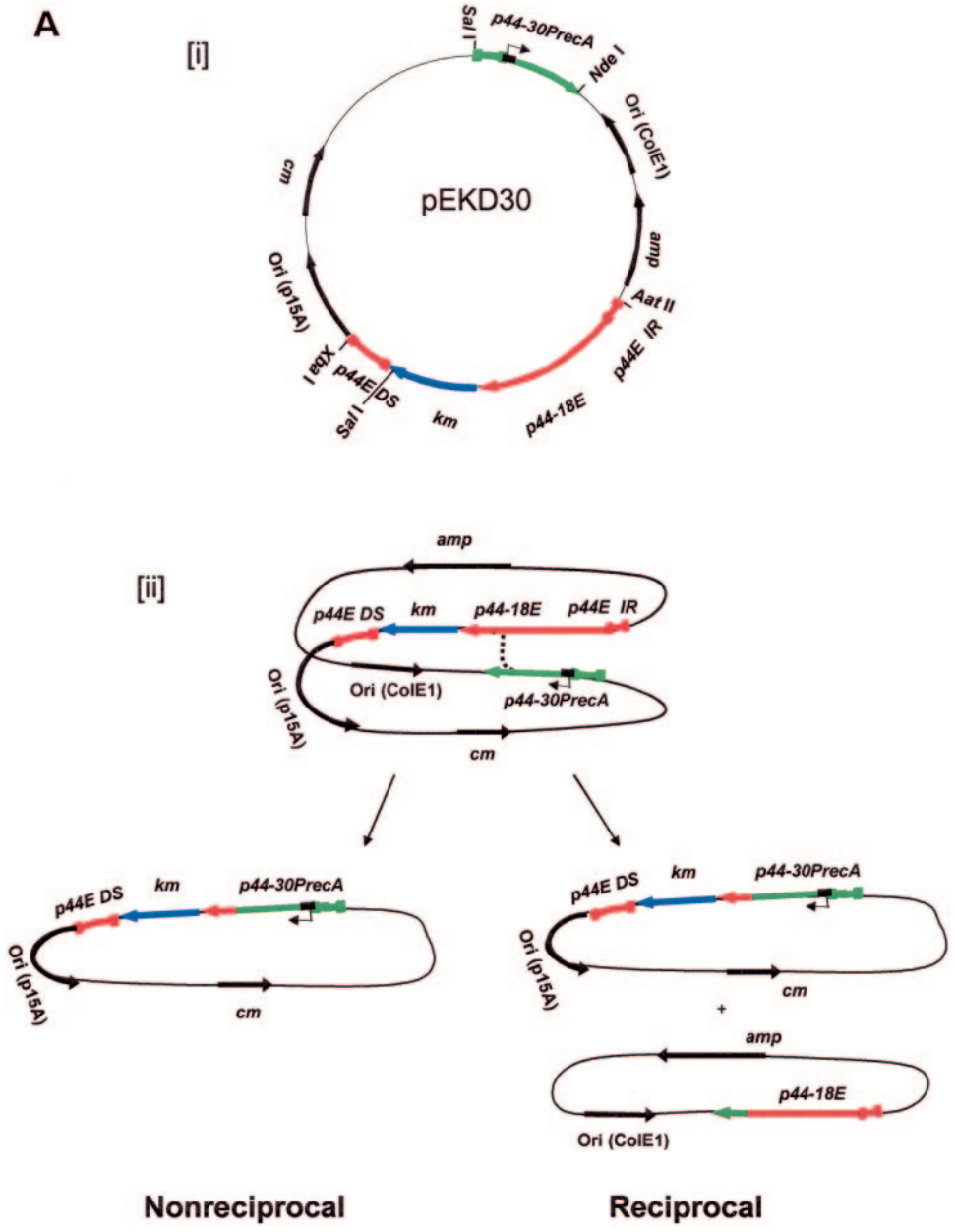

FIG. 3. Analysis of a *p44* recombination intermediate in *E. coli* using plasmid encoding *p44* expression and donor loci. (A) Double-origin plasmid pEKD30 and *E. coli* system design. [i] Plasmid pEKD30 carries the recipient site of the *p44* expression locus *p44E* upstream region (*p44E IR*)- *p44-18E-km–p44E* downstream sequence (*p44E DS*) and the donor site *p44-30PrecA* in a direct orientation. The donor *p44-30PrecA* has an *E. coli recA* gene promoter in the hypervariable region (bent arrow). The plasmid carries two more antibiotic-resistant genes (*cm* and *amp*) and two compatible replication origins (P15A and ColE). The restriction enzyme cleavage sites are shown. If *p44-30PrecA* recombines to *p44-18E*, *km* is transcribed from the recA promoter, allowing isolation of the recombination intermediates in the presence of Km. [ii] Experimental design. If nonreciprocal recombination occurs between *p44-30PrecA* and *p44-18E*, only one type of plasmid (5.7 kb) that carries *km* with upstream *p44-30PrecA* is expected to be isolated in the presence of Km. If reciprocal recombination occurs between the donor and the recipient sequences, two plasmids should be generated: one identical to the plasmid generated by the nonreciprocal recombination (5.7 kb) and another that carries *p44-18E* and *amp* (3.6 kb). Thus, in nonreciprocal recombination, Km^r *E. coli* strains are Am^s, while in reciprocal recombination Km^r *E. coli* strains are Am^r. [iii] Sequence alignment of 5′- and 3′-end conserved regions of donor *p44-30D* and recipient *p44-18E* in the plasmid pEKD30. The different nucleotides between *p44-30D* and *p44-18E* conserved regions are shaded in light gray. (B) Analysis of the Km^r Am^s plasmids isolated from *E. coli* strain JC7623 with an active RecF pathway. [i] PCR amplification using the primer pair located upstream of *p44-30PrecA* and downstream of *km*, respectively, showed a band of the expected size for the recombined *p44-30PrecA-km* structure in Km^r Am^s clones 11 and 19. The remaining three Km^r Am^s clones had the original *p44-18-km* structure. [ii] The two PCR positive plasmids from clones 11 and 19 were analyzed by digestion with XbaI to determine the size of the recombined plasmid. As predicted from the restriction sites shown in Fig. 3Ai, XbaI digestion generated a single 5.7-kb band from the recombined *p44-30-km* plasmid. [iii] The sequence between the two SalI sites in clones 11 and 19, which confirmed the recombination between *p44-30PrecA* and *p44-18E*.

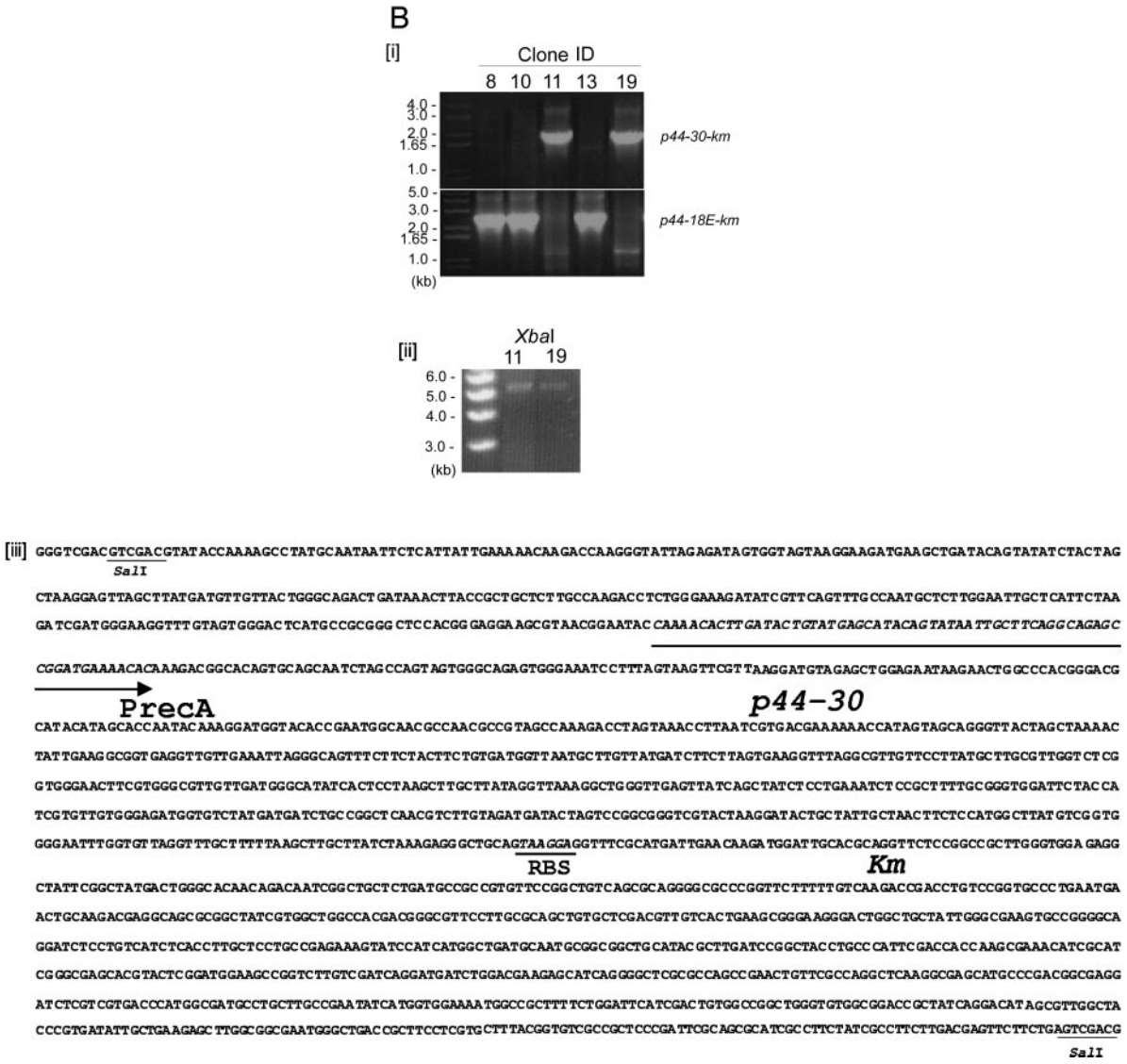

FIG. 3—*Continued.*

The PCR of DNA from peripheral blood leukocytes from the infected mouse amplified a band of ~2.9 kb (Fig. 2Ai). The size was smaller than the 3.2-kb band observed in the cell culture specimen since the 5′ primers used here anneal to a region further downstream of the donor locus than those used to analyze the cell culture samples (Fig. 1A). DNA sequencing (Fig. 2B) revealed that the 2.9-kb band had a sequence expected for the half-crossover structure consisting of the *p44-1*/*18* sequence upstream and a *p44E* sequence downstream as illustrated in Fig. 1Aii and almost identical to the sequence DQ011270 detected in cell culture. The next half-crossover event in the 5′ *p44* conserved region of *p44-18* of the intermediate is expected to create the structure illustrated in Fig. 1Aiii, which was previously demonstrated by PCR and sequencing (27).

As a control to rule out the possibility of a PCR shuffling between the *p44* expression locus and donor *p44-1*/*18*, we performed the same PCR using as a template a mixture of two *p44* plasmid clones each carrying donor *p44-1*/*18* and *p44E* sequences of the *A. phagocytophilum* HZ strain. Each of the DNAs at 20 ng was spiked into genomic DNA from an uninfected SCID mouse. The concentration of each *p44* in the plasmid present in this PCR was estimated to be >4,000-fold the concentration of total *p44* genes present in the mouse blood. No bands between 2 and 3 kb were amplified in this PCR control (Fig. 2Aii). As another control, the nested PCR was performed with forward primers complementary to the intergenic region of *p44E* and *omp-1N* and reverse primers that would anneal downstream of the *p44-1*/*18* locus (Fig. 1A). This PCR did not generate a band (Fig. 2Aiii). This result also confirmed the absence of reciprocal recombination between the recipient *p44* expression locus and the donor *p44-1*/*18* locus (Fig. 1 and 2Aiii). To confirm absence of crossover at the donor locus, we sequenced the *p44-1*/*18* locus by using DNA sample extracted from the blood of the same mouse. The sequences of randomly selected 10 clones of the PCR product were identical and did not show any sequence variation (data

not shown). These results support that the first half-crossover structure in the successive half-crossover model in the RecF pathway recombination is also present in *A. phagocytophilum* in the SCID mouse (22).

Construction of a plasmid encoding *p44E* expression and *p44* donor loci and their recombination through the RecF pathway. Kobayashi (22) could isolate half-crossover intermediates using a double-origin plasmid in the *E. coli* RecF active strain (22, 38, 41). A stable plasmid expression system or gene knockout system is not currently available for any obligate intracellular bacteria. It may be, however, possible to demonstrate the *p44* recombination intermediate by constructing an *E. coli* plasmid and by using *E. coli* mutants that are deficient in one or more of the recombination pathways. Therefore, in order to analyze the *p44* recombination mechanism, we developed an *E. coli* plasmid system. We constructed the pEKD30 plasmid from the double-origin plasmid pKEN33 (38) by replacing the two original Km resistance genes (*km*) with the recipient *p44* expression locus sequence and a donor *p44* sequence, respectively, in direct orientation (Fig. 3Ai). The double-origin plasmid was used, since intraplasmid recombination causes segregation of the plasmid into two plasmids and each origin allows recovery of two plasmids (Fig. 3Aii) under permissive conditions. The recipient *p44E* consists of *p44-18E* hypervariable region flanked by 5′ and 3′ conserved sequences (Fig. 3Aiii). A *km* gene was inserted downstream of *p44E* to create an operon fusion with an *E. coli* ribosome-binding site sequence (5′-TAAGGAG-3′) upstream of the ATG start codon of *km* gene. The *p44E-km* fragment contained a 213-bp sequence upstream of *p44-18E* (*p44E IR*) and a 446 bp downstream sequence (*p44E DS*). The donor was derived from the *p44-30* pseudogene (897 bp). An *E. coli recA* promoter (5′-C AAAACAC $\underline{\text{TTGATA}}_{-35}$ CTGTATGAGCATACAG $\underline{\text{TATA AT}}_{-10}$ TGCTTCA-3′) was inserted into the hypervariable region of the *p44-30* (Fig. 3Ai) to create *p44-30PrecA*. When the donor *p44-30PrecA* recombines to the *p44-18E*, *km* will be expressed from the RecA promoter in the *p44-30PrecA* and bacteria become resistant to Km; therefore, we could recover the recombined clones in the presence of Km. Various *E. coli* strains were cultured at 37°C for 1 h after introduction of the plasmid. The bacteria were then applied to SOB plates containing different combinations of Cm, Km, and Am. If recombination is nonreciprocal, the cells become Am sensitive, since nonreciprocal recombination causes loss of the Am resistance gene (*amp*) due to breakage in *p44E* (Fig. 3Aii). However, if recombination is reciprocal as catalyzed by the RecE pathway, the *E. coli* clone remains Am resistant, since *p44E* receives *p44-30* and the segregated plasmid with *amp* is ligated and thus survives (Fig. 3Aii).

In the RecBCD-deficient, RecF-active *E. coli* strain JC7623 (*recB21 recC22 sbcB15 sbcC201*), nonreciprocal recombination occurred as the Km-resistant, Cm-resistant, and Am-sensitive (Km^r Cm^r Am^s) transformants were detected. The ratio of Km^r/Am^r transformants was approximately 0.01 as determined by colony counting in the presence of Km and Am, respectively. DNA extracted from transformant clones were examined by two types of PCR: one specific for the expected product of recombination, using primers located downstream of the *km* gene and upstream of the donor *p44-30* (Fig. 3A), and another specific for the original *p44-18-km* locus using primers located downstream of the *km* gene and upstream of the donor *p44-18*. Two transformants of five Km^r clones examined had plasmids with the recombined *p44-30-km* locus and had lost the original *p44-18-km* locus (Fig. 3Bi). The isolated plasmids from the two recombined Km^r transformants were analyzed by restriction enzyme digestion with XbaI (restriction sites in the pEKD30 plasmid are shown in Fig. 3Ai). By using the restriction enzyme XbaI, a linear plasmid of ~5.7 kb was detected in the two Km^r transformants (Fig. 3Bii) rather than the original pEKD30 plasmid (9.3 kb). Sequencing of the amplicons (1,836 bp) obtained by using primers located upstream of the donor *p44-30* and in the 3′ end of the *km* gene, respectively, from the two clones demonstrated that these were indeed the half-crossover structure expected from the intraplasmid recombination (Fig. 3Biii). Thus, the *p44* gene recombination can occur in the nonreciprocal RecF pathway of *E. coli*.

recF and *recJ* are essential genes involved in RecF pathway homologous recombination (22, 23). In the *recF*-deficient strain JC8111 (*recB21 recC22 sbcB15 sbcC201 recF143*), no transformants were recovered from the plate with Km. Using this strain, the transformants on Am plate were 4×10^4 to 6×10^4. In the *recJ*-deficient strain JC12190 (*recB21 recC22 sbcB15 sbcC201 recJ153*), no transformants were recovered from the Km plate. The transformants in Am plate were 4×10^3 to 5×10^3. These results confirmed that the *p44* gene conversion in the pEKD30 plasmid was RecF dependent and that *recF* and *recJ* were essential for *p44* gene conversion in *E. coli*.

A. phagocytophilum lacks genes in the RecE pathway (26, 27). However, to test whether *p44* recombination could occur through the RecE pathway, we examined the recombination in the RecE-active strain JC8679 (*recB21recC22sbcA23*). The ratio of Km^r/Am^r transformants was less than 0.001, suggesting that RecE-dependent recombination (crossover) can also occur, albeit at a lower frequency than that observed through the RecF pathway.

DISCUSSION

The present experimental data generated using cell culture and infected animal specimens and the *E. coli* plasmid construct are consistent with our prediction based on the genome sequence data analysis (26, 27) and the characteristics of *p44* gene conversion (4, 11, 26, 27) that the *p44* gene conversion in *A. phagocytophilum* occurs through the RecF pathway. Isolation of recombination intermediates has been extremely difficult and possible thus far in limited systems, such as the yeast (16), *E. coli* (22, 41), and *N. gonorrhoeae* (18, 19). The recombined half-crossover-like structure in *A. phagocytophilum* in HL-60 cell culture and in the blood of an infected SCID mouse was very similar to those previously described in RecF-dependent intraplasmid recombination in an *E. coli* strain (22, 41). The weak 5.3-kb band in Southern blot analysis suggests that this structure is unstable or exists at very low frequency in *A. phagocytophilum* in cell culture under the conditions of the present study. Although a large amount of bacterial DNA can be isolated, *p44E* recombination rates appear to be much lower in cell culture than in animals (27). On the other hand, in infected animals a very small amount of *A. phagocytophilum* DNA is present in the overwhelming background of host cell DNA. These facts make the Southern blot detection extremely difficult. Further experiments and analysis would help find con-

ditions or factors that enhance the *p44* recombination rates to facilitate the detection of recombination intermediates in Southern blot analyses. The intermediate structure observed by sequencing is probably not a PCR shuffling artifact between a donor *p44* and a recipient *p44* expression locus DNA for reasons. (i) The intermediate structure sequence was obtained from the 5.3 kb-band region that did not include donor and recipient sequences. (ii) The existence of this structure was demonstrated by PCR with a high-fidelity *Pfu* DNA polymerase with no discernible recombination activity (37). (iii) Extremely low concentrations of *p44* DNAs in the total DNA from infected blood specimens, since most of it is host cell derived in our assay, prevents the PCR shuffling artifact. (iv) Even when the PCR was performed with a high concentration of two plasmids, one carrying *p44E* and another the *p44* donor sequence, a recombined sequence was not generated. (v) The PCR detected only a single unidirectional half-crossover structure in which 3′ end of donor *p44* recombined with the recipient *p44E* but not the structure in which the 5′ end of *p44E* recombined with the *p44* donor. (vi) Similar recombination intermediates using a double-origin plasmid carrying the *p44* expression locus and a donor *p44* locus were recovered in an *E. coli* strain with an active RecF recombination pathway.

A stable gene knockout method has not been developed for obligate intracellular organisms, but the *E. coli* system may provide an alternative strategy for characterization of factors involved in the *p44* recombination in *A. phagocytophilum*. We inserted a donor *p44* and the recipient *p44E* in the double-origin plasmid. Using this plasmid, pEKD30, we isolated the half-crossover intermediate in *E. coli* strain JC7623 with an active RecF-pathway (*recBCsbcBC*). The demonstrated fidelity of the *E. coli* system for *p44* recombination should encourage the use of *E. coli* enzymes and mutant systems for the analysis of antigenic variation in obligatory intracellular bacteria. Using a BLAST search, genes involved in the RecF recombination pathway were found in the *A. phagocytophilum* genome sequence database (26, 27). RecJ is a single-strand specific 5′ to 3′ exonuclease (23); the homolog in *A. phagocytophilum* may digest the 5′ tail end of *p44*. RecFOR and RecA homologs were also found that may help the 3′-tailed single strand invade the donor *p44* duplex. In addition, the *A. phagocytophilum* genome contains the Holliday junction DNA helicase genes, *ruvA* and *ruvB*, and the crossover junction endodeoxyribonuclease gene, *ruvC*. RuvA and RuvB denature and renature duplex DNA at the cruciform structure to promote its migration, while the RuvC protein acts to resolve the Holliday junction structure. Genes of the *recBCD*-dependent and *recE*-dependent pathways were absent from our bacterium, although homologs of AddAB have been found in *Bacillus* (8) and *Rhizobium etli* (45). AddAB was detected in the genome of *A. phagocytophilum* (APH0258 and APH0849 with E-values of 6e-79 and 9e-07, respectively) by BLAST search using AddAB of *R. etli*. This new class of enzymes have both helicase and nuclease activity and function like the RecBCD enzyme (8). In the future, we may be able to express these genes in our pEKD30 and *E. coli* system and define their roles in *p44* recombination.

Further experiments and analysis would help optimize the present *E. coli* system for molecular analysis of *p44* recombination. An experiment should be performed to determine how much noncoding sequence upstream and downstream of *p44E* is required for recombination. Three of five Km^r clones did not have the recombined *p44-30-km* locus but retained the original *p44-18-km* locus. These transformants were not investigated in detail. It is possible that this background transformation may be due to the insertion of the whole intergenic region between *omp-1N* and *p44E* into the plasmid. This region contains the *A. phagocytophilum* promoter sequence upstream of *p44E* (26) that may have been recognized by *E. coli* transcription and translation system. Thus, the downstream *km* gene could have been expressed without recombination. By removing or changing this promoter region, we may be able to reduce the Km^r background colonies. However, our speculation may not be correct, since we did not obtain any Km^r colonies when the *recF* mutant strain JC8111 or the *recJ* mutant strain JC12190 was used. An experiment that compares the lengths and sequences of donor *p44*s for their efficiency of recombination will be also informative. The functional pseudogene *p44-30* used for pEKD30 plasmid has approximately 200 bp of the 5′conserved region and 410 bp of the 3′ conserved region, and thus far all *p44*s found to recombine to *p44E* have at least 50 bp from each of these regions (26, 27, 40). According to Fujitani et al. (12), 50 bp is the minimum length required for the efficient homologous recombination. By systematically modifying the pEKD30 construct, we may be able to define the sequence that influences efficiency of *p44* recombination.

In the bovine intraerythrocytic agent, *Anaplasma marginale*, the major surface protein 2 (*msp2*) multigene family, homologous to the *p44* gene family, also shows variation in gene expression in a similar gene expression locus (3, 6, 7, 13). Recently, Futse et al. proposed an "anchoring" model of segmental gene conversion for annealing donor and recipient *msp*2 sequences (13). however, a recombination intermediate structure or a recombination mechanism has not been described. The RecF pathway recombination may also involve gene conversion of the *msp2* pseudogene to the *msp2* expression locus, because *A. marginale* also lacks the RecBCD and RecE pathway genes (31) and because *msp2*s have 5′ and 3′ conserved regions and a central hypervariable region similar to the *p44* locus suitable for homologous recombination.

In summary, the present study supports that the sequential half-crossover model accounts for the gene conversion in the *p44* expression locus. The present study opened the possibility of many mechanistic experiments to determine the molecular mechanisms of *p44* recombination and thus P44 antigenic variation.

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