Retron for the 67-base multicopy single-stranded DNA from *Escherichia coli*: A potential transposable element encoding both reverse transcriptase and Dam methylase functions

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ABSTRACT The region (retron-Ec67) required for the biosynthesis of a branched-RNA-linked multicopy singlestranded DNA (msDNA-Ec67) from a clinical isolate of Escherichia coli was mapped at a position equivalent to 19 min on the K-12 chromosome. The element containing the retron consisted of a unique 34-kilobase sequence that was flanked by direct repeats of a 26-base-pair sequence found in the K-12 chromosomal DNA. This suggests that the 34-kilobase element was probably integrated into the E. coli genome by a mechanism related to transposition or phage integration. In the 34-kilobase sequence an open reading frame of 285 residues was found, which displays 44% sequence identity with the E. coli Dam methylase. Interestingly, there are three GATC sequences, the site of Dam methylation, in the promoter region of the gene for reverse transcriptase.

msDNA-Ec67 is a 67-base multicopy single-stranded DNA from Escherichia coli that is linked at its 5' end to the 2' OH group of the 15th guanosine residue of a 58-base RNA molecule by a 2',5'-phosphodiester linkage (1). This peculiar RNA·DNA complex was found in a clinical isolate of E. coli, strain Cl-1, and reverse transcriptase (RT) has been shown to be required for msDNA synthesis (for a review, see ref. 2). It has been proposed that a long RNA transcript forms a unique secondary structure, which then serves as primer as well as template for msDNA synthesis by RT (3, 4). The gene for RT has been identified and shown to be closely associated with the region encoding both DNA and RNA molecules of msDNA (1, 5, 6). On the basis of sequence similarities of bacterial RTs to retroviral RTs, their evolutionary homology has been proposed (5). It is possible that the msDNAsynthesizing system may be evolutionarily related to retrotransposons and retroviruses, and Temin (7) proposed designating the system a "retron."

In this report, we first attempted to isolate and sequence DNA fragments* from the chromosome of E. coli strain Cl-1 that contain junction sites between the element containing the retron-Ec67 (retron responsible for the synthesis of msDNA-Ec67) and the E. coli genome. We found that a 34-kilobase (kb) foreign DNA fragment containing retron-Ec67 was integrated at a site equivalent to 19 min on the E. coli K-12 chromosome. This fragment did not hybridize with the K-12 chromosomal DNA and was found to be flanked by direct repeats of a 26-base-pair (bp) sequence found in the K-12 chromosomal DNA. This result raises a possibility that the 34-kb fragment was integrated into the E. coli genome by mechanism related to transposition or phage integration. A possibility that the 34-kb element might have been transposed as a retrotransposon will be discussed.

MATERIALS AND METHODS

Bacterial Strains and Plasmid. pUC9 (8) was used to clone chromosomal DNA and to subclone various DNA fragments. *E. coli* JM83 (8) was used for transformation, and *E. coli* GM33 dam⁻ (9) was used to assay Dam methylase activity. Those *E. coli* cells harboring plasmids were grown in L broth (10) containing ampicillin (50 μ g/ml). The λ library of the *E. coli* K-12 genome (11) was obtained from A. Ishihama (National Institute of Genetics, Mishima, Shijuoka-ken, Japan). The λ phage DNA filters were prepared by spotting 0.8 μ l of λ phage lysates on a nitrocellulose filter paper according to Kohara *et al.* (11).

DNA Sequencing and Chromosome Walking. DNA manipulations were performed as described by Maniatis *et al.* (12). To determine DNA sequence, large DNA fragments from chromosomal DNA were digested with various restriction enzymes and subcloned. DNA sequences were determined by the chain-termination method (13) using double-stranded plasmids and synthetic oligonucleotides as primers. To find the junction sites, DNA walking using the pCl-1E DNA as a probe was performed. Two neighboring DNA fragments from restriction enzyme digests of the Cl-1 chromosomal DNA were identified by Southern blot hybridization (14) with use of the left- and righthand end fragments from pCl-1E as probes. DNA walking was continued until DNA fragments from strain Cl-1 hybridized with the K-12 DNA.

RESULTS

Isolation of Junction Fragments. The circular gene map of the entire genome of E. coli K-12 (15) and the restriction map of its chromosomal DNA (11) have been established. Furthermore, a λ genomic library encompassing the entire genome of E. coli K-12 is also available (11). Therefore, we used E. coli K-12 as a reference strain to determine the relative map position of retron-Ec67 on the Cl-1 chromosome and the size of the DNA fragment containing retron-Ec67. Since the original clone of retron-Ec67, pCl-1E containing the 11.6-kb EcoRI fragment [E(a)-E(b) in Fig. 1A] from strain Cl-1 (1) did not hybridize with the K-12 DNA, DNA walking to the left on the Cl-1 chromosomal DNA was first carried out until a DNA fragment hybridizable with the K-12 DNA was identified. By using the lefthandmost 1-kb EcoRI [E(a)]-Sal I [S] fragment, a 6-kb Pst I fragment [P(a)-P(b)] was cloned from the Cl-1 DNA (see Fig. 1A). This Pst I fragment was found to hybridize with a 4.3-kb EcoRI fragment of the K-12 DNA by Southern blot hybridization (data not shown). The Pst I fragment was then used to identify λ clones from the K-12 DNA library (11). Out of 476 λ clones, two λ phage DNAs clearly hybridized with the probe. These two λ phages, 3H12

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Abbreviations: RT, reverse transcriptase; ORF, open reading frame; msDNA, multicopy single-stranded DNA.

^{*}The sequence reported in this paper has been deposited in the GenBank data base (accession no. M55249).

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FIG. 1. Restriction map of the 34-kb DNA containing retron-Ec67 (A) and the 4.3-kb EcoRI fragment from E. coli K-12 containing the integration site for retron-Ec67 (B). (A) The location and the orientation of msDNA and RNA linked to msDNA (msdRNA) are indicated by a small arrow and an open arrow, respectively. ORFs (see Fig. 3) are indicated by dashed arrows. A large solid arrow and a stippled arrow represent the genes for RT and the Dam methylase, respectively, and their orientation. An open bar represents the 34-kb retron Ec67, and solid bars represent DNA sequences that are common to K-12 DNA. A detailed restriction map is shown only between E(a) and E(b). A substantial part (≈ 15 kb) of the retron is omitted from the map. B, Bal I; E, EcoRI; EV, EcoRV; H, HindIII; P, Pst I; S, Sal I. DNA sequence from E(a) to B and junction regions between retron-Ec67 and the E. coli chromosomal DNA were determined. DNA sequence from B to E(b) was reported by Lampson et al. (1). (B) The location of the integration site of retron-Ec67 is shown by an open triangle. The DNA fragment was isolated from a λ phage containing a DNA fragment from 19 min on the E. coli K-12 chromosome. E, EcoRI; H2, HincII; R, Rsa I. Only two Rsa I sites used to determine the integration site are shown.

and 5F4, have been shown to contain DNA fragments from 19 min on the K-12 chromosome (11), indicating that retron-Ec67 is integrated into the Cl-1 genome at the position equivalent to 19 min on the K-12 chromosome. From the λ DNA identified above, a 4.3-kb *Eco*RI fragment was cloned, which hybridized with the 6-kb *Pst* I [P(a)–P(b); see Fig. 1A] fragment of the Cl-1 DNA.

This EcoRI fragment is expected to contain the retron-Ec67 integration site and was mapped as shown in Fig. 1B. It was found that the 0.55-kb E(A)-H2(A), the 0.60-kb H2(A)-H2(B), and the 2.55-kb H2(B)-H2(C) fragments were able to hybridize with the 6-kb P(a)-P(b) fragment (Fig. 1A) from the Cl-1 DNA, while the 0.6-kb H2(C)-E(B) did not (data not shown). By cloning the 2.55-kb HincII [H2(B)-H2(C)] fragment, further mapping of this fragment with Rsa I was carried out, and it was found that the righthand end 0.6-kb R(B)-H2(C) fragment could not hybridize with the 6-kb P(a)-P(b)fragment, but the 0.55-kb R(A)-R(B) fragment did (data not shown). DNA sequencing at the junction regions revealed that the 0.55-kb R(A)-R(B) fragment was directly connected to the 0.6-kb R(B)-H2(C) fragment as shown in Fig. 1B. These results indicate that the integration site of retron-Ec67 was within the R(A)-R(B) fragment of the K-12 DNA.

To isolate a DNA fragment that contains the junction between the righthand end of retron-Ec67 and the host chromosomal DNA, the 1.9-kb EV(b)-E(b) fragment (Fig. 1A) was used as a probe. As a result a 6.6-kb *Eco*RV fragment [EV(b)-EV(c) in Fig. 1A] was cloned. This *Eco*RV fragment hybridized to the 11.6-kb *Eco*RI fragment from E(a) to E(b) as well as to another 22-kb *Eco*RI fragment. We were unsuccessful in cloning this 22-kb *Eco*RI fragment from the Cl-1 chromosomal DNA. Therefore, to isolate a junction fragment from the Cl-1 DNA containing the righthand junction region, the 0.6-kb R(B)-H2(C) fragment from the K-12 DNA (Fig. 1B) was used as probe. Subsequently, a 2.1-kb *Pst* I-*Eco*RI fragment [P(k)-E(c) in Fig. 1A] was cloned. This 2.1-kb *Pst* I-*Eco*RI fragment hybridized to the 22-kb *Eco*RI fragment in Southern blot hybridization as did the 6.6-kb EcoRV fragment (data not shown). Since the 6.6-kb EcoRV fragment contained only one EcoRI site, it was concluded that the 22-kb EcoRI fragment was the adjacent fragment [E(b)–E(c)] to the 11.6-kb EcoRI fragment [E(a)–E(b)]. Thus the restriction map around the retron-Ec67 integration site within the 39-kb P(a)–E(c) region was determined as shown in Fig. 1A.

From the results described above, we conclude that the 10.8-kb *Pst* I fragment from the Cl-1 DNA [P(k)-P(1)] contained the junction site between the righthand end of retron-Ec67 and the host chromosomal DNA. To determine the junction site, the *Pst* I fragment was digested with *Eco*RI, which generated two fragments, the 2.1-kb P(k)-E(c) fragment and the remaining 8.7-kb fragment (see Fig. 1A). The P(k)-E(c) fragment from the K-12 DNA (Fig. 1B), indicating that the junction site exists in the P(k)-E(c) fragment. It was found that the P(k)-E(c) fragment hybridized to the same λ clones that hybridized to the P(a)-P(b) probe (see Fig. 1A). This indicates that the host chromosomal sequences at the left- and the righthand junctions are located in a close proximity in the K-12 chromosomal DNA.

DNA Sequences of the Junction Sites. To determine the junction sequences at the left and right ends of retron-Ec67, we first determined the DNA sequence of the 0.55-kb R(A)–R(B) fragment from the K-12 chromosomal DNA (Fig. 1B), which is considered to contain the retron-Ec67 integration site. Subsequently, the junction sequences from the Cl-1 DNA were searched using the DNA sequence of the R(A)–R(B) fragment determined above as a reference. At the lefthand end, ≈ 2.3 kb leftward from the P(b) site (Fig. 1A), the sequence found in the K-12 fragment appeared as shown in Fig. 2. For the righthand end, the K-12 sequence also appeared ≈ 0.3 kb rightward from the P(k) site (Fig. 1A) (Fig. 2). The same 26-bp sequence found in the K-12 DNA (boxed in Fig. 2) is repeated at both ends (Cl-1L and Cl-1R) of the

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K-12 ATGGTTGAGAGGGTTGCAGGGTAGTAGATAAGTTTTAGATAACAAAAAACCCATCAACCTTGAACCGAAATGGCGGGGGTT
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CI-1L ATGGTTGAGAGGGTTGCAGGGTAGTAGATAAATTTTAGGCAACAAAAAACCCActtatctaaatgggttaataaaaacaa
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CI-1R gcttctaacatcatgattttaaaaggqATAAATTTTAGACAACAAAAAAACCCACCAACCTTGAACCGAAATGGCGGGGGTT

FIG. 2. DNA sequence of the integration site of retron-Ec67 in K-12 DNA and regions from strain Cl-1. DNA sequences from K-12 or Cl-1 are shown with uppercase letters and the retron-Ec67 are shown with lowercase letters. Cl-1L and Cl-1R represent left and right junction regions in Fig. 1A, respectively. Twenty-six bases shared among the three DNA sequences are boxed. Asterisks represent base substitutions between the sequences.

GAATTCATTT TTAAATCCTT TTACTATATC AATTTGAAAC TATTGTACTT CTTATACAAG CAACCACTTC AATATCAGAA 80 ANTIGENEATT CHARGETATT CTCTGTAGTT GTAATTTTAN TCATACCTTT TGGAAGCTTT GTGATTTTTC GAATGGAATA 160 GETTECETES ATGTTGATTA ACCAGTGTEC ATCAAGTACT TEAGAAAATT TITGATCACA AATGTATGTG ACAAGECCAT 240 CONGRATARC ANTIGOTORY GARGAGETTIC CROCRAGERS TORTORATIC RETRIETED ARCATOTIC ARTCRTTTIC 320 400 CETGEGGTAA GGEGAAACTT GGGAAGETET TETGTGGETA ETGTEAGEGA GETTTGETTA AGTEETTGEE EGGTGGTTAA CCATTTCAGT GAGGTTCCTG TTTCAAGAGC ACATTGGATT ATCCAGTCTG CAGGAAACGT ATCCCGCAAA TACCTGTTCG 480 CONGAGTGOT TTTTGATACT TOCANGTGAT CAGOTAAAGC CTGTCGTGTC GTAAAGCCAT AAGCTTCGAC TAATCGCTCA 560 640 ATCGCAGCCT TACCACCTTT ATTGGGATTT ATTTTGATCT CACTTGGGTA CTTTGATGTT GACATATCTC TTTTGCGATC CTAGTATCAG TTTTGTCCCC ATTTGGGTAC TTGTCACGAT TACTACAGGC TCACCACAAG CCAATAGGAG ATGTTGCATC 720 ATGACCCCTA ACATTTCAAT AACTCTGAAT ACGCCACATG TCACAATTGA GCGTTATAGC GAACTTACTG GTCTTTCAAT 800 CGACACAATT AACGATATGC TGGCTGACGG TCGCATCCCT CGGCATCGCC TTCGGAAAGA CAAGAAAAGA GAAAAGGTGA 880 TGATCAACCT TECTECTCTT ACCEPTEATE CACTTACTER TTECAATETT STATTCAACT ASTTCCATTT TEEGATACAT 960 CAGGGGGTGTC GACCATGTTT GATTACCAAG TTTCCAAACA TCCACATTTT GATGAAGCCT GTCGTGCATT CGCATTGCGC 1040 CACAACCTGG TGCAACTGGC AGAACGTGCT GGCATGAATG TGCAGATTCT GCGGAACAAG CTGAACCCAT CTCAACCTCA 1120 TTTATTAACC GCACCAGAAA TCTGGCTGCT TACCGATCTG ACTGAAGATT CAACGCTGGT AGATGGTTTT CTGGCACAGA 1200 TTCACTGCCT GCCATGTGTA CCGATTAATG AGGTTGCAAA AGAGAAACTG CCGCATTACG TCATGAGCGC AACTGCAGAG 1280 ATCGGACGTG TTGCAGCAGG TGCGGTATCT GGTGATGTAA AAACTAGTGC CGGTCGTCGT GATGCTATCA GCAGCATTAA 1360 CTCTGTAACA CGACTGATGG CGCTGGCTGC TGTTTCATTG CAGGCCCGTT TACAGGCTAA TCCTGCGATG GCGAGTGCAG 1440 TTGATACCET GACTEGECTC GETECTTCAT TEGETTTECT STEAGETECT TATECTEACE AAAGAACCAT CATTTECATC 1520 GCTGCTGGTA ANACANAGCC CGGCAATGCA CTACGGTCAC GGCTGGATCA TGGGTGAGGA TGGTAAACGC TGGCATCCGT 1600 GCCGTTCACA AGATGAATTG CTGGCAGAAC TATCTACGAA AAAACGGGGG AACAAATGGC TATTGAAGGC GCTGCGGCGA 1680 TEGTTCCATT AAGCCCCCGGT GAACGCCTGA ATGGACTTAA TCACATTGCG GAATTAAGGG CGAAAGTATT TGGCCTGAAT 1760 ATTGAGTCAG AGCTTGAGCG GTTTATTAAA GATATGCGTG ATCCACGGGA TATCAATAAT GAACAAAATA AACGAGCACT 1840 GCTGCCATA TTCTTTATGG CAAAAATTCC AGCTGAACGT CATAGCATCA GCATTAATGA GCTGACCACT GACGAAAAGC 1920 GGGAGTTGAT TAAAGCAATG AATCATTTTC GTGCAGTGGT GAGCTTATTT CCCAGACGGC TAACCATGCC GAATTAACCA 2000 ACTAATGAAA TTAATGGCGT AAACCCGCCG GGTATCCCTT TATCTAAATT CAGGAGAATT GATTATGCGT AATATTGAAA 2080 CTCTCACGAC TAAAACCCGGA CCGGATGATG CAGGGCTTAA TATTTTACTG ACAGAGGCTC GTCTGGAAGA ACGCCGGGCA 2160 AGGGCTGAAG CAATGGCAGC TCGCCTTGAT AGCCTGGCGT GTCATATCAC ATCCCGCCAG CTAAACCACG TCGAAGCAGC 2240 AGAACTGCTG CGTGTAACTG CTGAAGCAAT CCAGAACGAA GCGCAGGAGA TCCACTAATG GCTGATGCAA TGGATCTCGT 2320 ACAGCAGCGC GTTGAAGAAG AACGCCAGCG CCATATCCGT GCTGCCCGTG CCAAAACGCC GGGCGTGTCC CGCGTGCTTT 2400 GCATTGAGTG TGAAGCGCCA ATTCCGCCAG CACGCCGCCG TGCCATTCCG GGTGTGCAGC TTTGCATTAC CTGTCAGGAA 2480 ATCECAGAGC TEAAAGECAA ACATTACAAC GEAGETECTE TATEACAAGE GEAGTECETA TECATCAATT AAAAATTECA 2560 CCTAAGTATT TCAACGCTGT GGTTGCAGGT CAAAAGACGG CTGAACTTCG TAAAGACGAT CGTGGCTATA AAGTTGGTGA 2640 TGTTCTTTCT CTTTGCGAAT GGAAGCATGG CGTATTTACG GGTAGGGAAT GGGCCGCTGT TATCTCTCAT GTGCTTCCGG 2720 TTAATGACGT CATGGCAGTT TCAGAACAAT GGGTGATGCT ATCAATTCGC CCATTAACCC CATTAGAAGC TTTAGGATAT 2800 GTTATTGCAG GAGGTGCTGT ATGAGCACCA TCCTGAAATG GGCGGGGAAAT AAAACCGCCA TTATGTCCGA ACTGAAAAAA 2880 ORF 1 M S T I L K W A G N K T A I N S E L K K CACCTTCCTG CTGGCCCGGG ACTGGTTGAA CCTTTCGCGG GTTCCTGTGC TGTGATGATG GCGACGGATT ACCCCAGCTA 2960 H L P A G P R L V E P F A G S C A V M M A T D Y P S Y TCTGGTTGCG GATATTAATC CTGATTTAAT CAACCTCTAT AAAAAGATTG CCGCTGATTG CGAGGCGTTT ATATCTCGCG L V A D I N P D L I N L Y K K I A A D C E A F I S R 3040 CCAGAGTTTT ATTTGAGATC GCAAACAGGG AGGTGGCTTA TTACAACATA AGGCAGGAGT TTAATTACTC AACTGAAATT A R V L F E I A N R E V A Y Y N I R Q E F N Y S T E I 3120 ACTGATTTCA TGAAAGCGGT ATATTTCCTG TATCTCAATC GTCATGGTTA CCGTGGGTTA TGTCGCTATA ACAAGAGCGG T D F M K A V Y F L Y L N R H G Y R G L C R Y N K S G 3200 GCATTTCAAC ATTCCCTACG GGAATTATAA AAATCCGTAT TTCCCTGAAA AAGAAATTCG CAAATTTGCA GAAAAAGCCC H F N I P Y G N Y K N P Y F P E K E I R K F A E K A 3280 AGCGAGCAAC GTTTATCTGC GCCAGCTTTG ATGAAACGCT GGCGATGCTG CAGGTGGGGG ATGTGGTGTA TTGCGATCCG Q R A T F I C A S F D E T L A M L Q V G D V V Y C D P 3360 CCTTATGACG GTACGTTTTC CGGCTATCAC ACTGACGGCT TCACTGAAGA TGACCAGTAT CACCTGGCAT CTGTTCTTGA P Y D G T F S G Y H T D G F T E D D Q Y H L A S V L E 3440 ATATCGATCA TCAGAAGGTC ATCCAGTCAT TGTTTCTAAC AGTGACACAT CCCTGATCCG TTCGCTGTAT CGCAATTTCA Y R S S E G H P V I V S N S D T S L I R S L Y R N F 3520 3600 CTCACCACTA CATCAAGGCA AAACGCAGCA TCGGCGTGTC GGCTGGCGAG AGTAAATCTG CAACAGAAAT CATTGCTGTT T H H Y I K A K R S I G V S A G E S K S A T E I I A V TCCGGGGGGC GCTGCTGGGATTGAT CCTTCGCGTG GCGTGGATAG TTCTGCTGTG TACGAGGTGC GTGTATGAGT 3680 S G A R C W V G F D P S R G V D S S A V Y E V R V categoegata teagegacte tageggettt aacgaggeeg etgeageatt teatggaac ggeeggaaaa aggeertaaa ORF 2 m e r p e k g h k 3760 CCCTTATCTG GACCCGGCGG AAGTTGCGCC GTTTCTGCGC TTTCAAACCT GATCACTCTG TATGCTGCGC ATAACGAGCA P L S G P G G S C A V S A L S N L I T L Y A A D N E Q 3840 3920 GGAACAGCTG CGCCGCGAGG AATTGAGTGA ACAGGTCTGG GAGCGTTATT TCTTTAATGA ATCCCGTGAT CCTGTCCAGC E Q L R R E E L S E Q V W E R Y F F N E S R D P V Q GCGAAATGGA GCAGGATAAG CTCATTAGTC GGGCAAAGCT GGGGCATGAG CAGGAGCGTT TTAACCCGGA CATGGTCATT R E M E Q D K L I S R A K L A H E Q Q R F N P D M V I 4000 CTGGCGGACG TCAGCGCCCA GCCCACCCAT ATCAGCAAGC CGCTGATGCA ACGTATCGAA TACTTCAGCA GCCTGGGCAG L A D V S A Q P T H I S K P L M Q R I E Y F S S L G R 4080 GCCAAAGGCT TATTCCCGCT ATTTGCGTGA GACGATTAAG CCATGTCTGG AGCGACTGGA TTGTGTACGT GACAGTCAGC P K A Y S R Y L R E T I K P C L E R L D C V R D S O 4160 TATCTGCTIC TITCCGTTTT ATGGCAAGCC ATCAAGGGCT GGAGGGCCTG CTGATCCTGC CTGAAATGAG TCAGGATCAG L S A S F R F M A S H Q G L E G L L I L P E M S Q D Q 4240 GTGAAACGCC TGTCCACCCT GGTAGCAGCG CATATGAGCA TGTGTCTTGA GGCCGCTTGT GGTGATTTGT ATGCCACCGA 4320 V K R L S T L V A A H M S M C L E A A C G D L Y A T D

TGACGTTAAG CCAGAAGAAA TCCGCAAGAC ATGGGAAAAG GTGGCAGGGG AAACCCTGCG TCTGGATGTT ATCCCGCCTG D V K P E E I R K T W E K V A A E T L R L D V I P P 4400 4480 CGTTTGAGAA ACTTCGTCGG AAAAGAAACC GCCGCAAACC CGTGCCCTAT GAACTCATAC CGGGTTCGCT GGCACGTATG A F E K L R R K R N R R K P V P Y E L I P G S L A R M 4560 CTITIGCGCCG ACTGGTGGTA TCGGAAATTG TGGAAGAGG GTGGGGAAGAG CAGTTGCGTG CIGTCTGTCT L C A D N W Y R K L N K M R C E W R E E Q L R A V C L GGTCAGCAAA AAAGCATCTC CCTATGTCAG CTATGAAGCA GTGACACATA AACGTGAGCA GCGCCGCAAG TCGCTGGAGT V S K K A S P Y V S Y E A V T H K R E Q R R K S L E 4640 TTTTCCGTTC TCATGAACTG GTGAATGAAG ACGGAGACAC GCTGGATATG GAGGATGTGG TAAACGCCAG CAGCAGCAAT 4720 F F R S H E L V N E D G D T L D M E D V V N A S S S N CCGGCGCATC GCCGCAATGA GATGATGGCC TGTGTTAAAG GTCTGGAGCT TATCGCAGAA ATGCGCGGGT ACTGCGCCGT 4800 P A H R R N E M M A C V K G L E L I A E M R G D C A V TTTCTACACT ATCACCTGTC CGTCACGTTT CCATTCCACG CTCAACAACG GCAGACCCAA CCCGACCTGG ACAAATGCGA FYT T T C P S R F H S T L N N G R P N P T W T N A CGGTAAGACA AAGCAGCGAT TATCTGGTCG GCATGTTTGC TGCATTTCGT AAGCCTATGC AACCAAAGCC GGGTTGCGCT T V R Q S S D Y L V G M F A A F R K A M Q P K P G C A 4960 GGTATGGGGT GGGGGGGGGT GAGCCGCACC ATGACGGTAC TGTGCACTGG CATCTCATGT GTTTCATGGG CAAAAAAGAC G M A C G W L S R T M T V L C T G I S C V S C A K K T M C F M R K K D MF3 M C F M R K K D 5040 decergence thatecatt streatard titecates strangages transmarks observed a signal transmark of the set of the 5120 CTGATAA ACCCGCGCAA AGGAACGCCG ACAAGCTACA TCGCGAAATA CATCAGTAAG AACATTGACG 5200 N P R K G T P T S Y I A K Y I S K N I D GACGTGGTCT GGCTGGCGAG ATCAGCAAGG AAACGGGTAA ATCCCTGCGT GATAACGCGG AATACGTGAA TGCCTGG G R G L A G E I S K E T G K S L R D N A E Y V N A W TCTCTACATC GTGTTCAGCA GTTCCGCTTC TTTGGTATTC CGGGGGCGTCA GGGGTACCGT GAACTTCGCT TGCTGGCTGG S L H R V Q Q F R F F G I P G R Q A Y R E L R L L A G 5360 TCAGGCGGCA AGGCAACAGG GTGACAAAAA AGCAAGTACG CCGATACTGG ATGACCCGGC CCTTGATGCC ATCCTGGCTG Q A A R Q Q G D K K A S T P I L D D P R L D A I L A 5440 CCGCTGATGC TGGCTGTTTT GCCACCTACA TCATGAAGCA GGGCGGCGTAC TGGTTCCCCG CAAATATCAC CTTATCAGAA A A D A G C F A T Y I M K Q G A Y W F P A N I T L S E 5520 TTATGA AATTAACGAA GAGCCGACCG CTTATGGCGA TCACGGTATT CGTATTTATG GCATCGGTC ACCCATTGTA L M K L T K S R P L M A I T V F V F M A S G H P L Y 5600 CAGGGCAAGA TCTGCACTCA TGCAATGAAA TGGAAAANTGG TTCGTAAGGC CGTTGACGTT CAGGAGGCGG CAGCCGACCA 5680 GGCGCTTGC GCCCCTTGGA CTCGTGGCAA TAACTGTCCC CTTGCTGAAA ATTTGAACCA ACAGGAGAAA GATAAATCAG 5760 CTGATGGGGA CACCAGAACG GACATTACCC GCATGGATGA CAAGGAGTTG CACGATCACC TGCACAGTAT GAGCAAAAAA 5840 GAGCGCCGGG AACTGGCTGC AAGGTTACGC CTGGTTAAAC CGATACGGCG TAAAGACTAC AAACAGCGAA TTACAGATCA 5920 TCAGCGACAG CAGCTCGTTT ATGAGCTGAA GTCCAGAGGA TTTGATGGCA GCGAGAAAGA GGTCGATTTA CTCCTTCGCG 6000 GCGGCAGTAT TCCGTCAGGA GCAGGCCTGC GTATCTTCTA TCGGAACCAG CGTCTGCAGG AGGATGATAA ATGGCGGAAC 6080 CTGTATTAAT TACGCTGGTT AACAATTCGT GCTCTTAATA ATACCAGGCA TATCAGGCTG ATGAGCGTAA AAAAACGTTT 6160 TACATCAGTA AGATTATTAT ATACTGTARA TATARACAGT GGTTATGTGT ACAGTATTGC TTGTGGTGTC ATAGGAGGAA 6240 AAATGCAGGA CTATTTTTTG GAGTCTTTGA AGCTCCAGCG CATTGATTTT TTTCTTAAGC TTGTAGCGGC TAGTGAGTGT 6320 AGTGATGAAG AGAAGGGGGCT GGCCTGCAGT GGGTTTCTGA ACTGACAGAT GAACTCATGG CAAAAATCAG AACCCACGAA 6400 TACAACCGCT CAATGGATGT CATCAGTTGA GGTGACTTTT ATGCGCATTG AAATAATGAT CGATAAAGAG CAGAAGATTA 6480 GCCAGTCTAC CCTGGACGCC CTTGAATCCG AGCTTTACCG CAATCTGCGC CCCCTGTATC CCAAAACGGT AATTCGTATC 6560 CGTAAAGGTA GCTCTAACGG TGTGGAACTG ACCGGACTGC AACTGGATGA AGAAAGAAAA CAAGTGATGA AAATTATGCA 6640 GAAGGTGTGG GAAGACGACA GCTGGCTGCA TTAAGAAACG TTGCCCCCCAG GAGGATTCAT TCTGATGGGG GCTAGTTTGG 6720 GCAACGAGTG AAACGAGGCG TAAGGTGGGT GGGCATTTTG ATAAGTGATC GTCCGCTTTG TGTCAGAAGC AGAAGTGGGA 6800 GTGTCTGAGA CTCTCTAAAA GTTGATGATT CACTTACAAA ACCATTTTCC TGATGAATGC TTACACTTAC GAATGATCAT 6880 CTGTTTAGT CTTCATGAGA ANATCCCGAN TCTTTGCCAN GTTTGANTTT TCGGGGATTG ANTCCATGTA CGGATTCACG 6960 TCAAACACT AATTGCTCAG ATTCAACGAA AGCCTGCCAC TCTACTGGGT CTAGCTCAAA TCCCATTGCT GTCATATCAC 7040 TTTCATCTTC CCCCTCGATC CAGTGGACTA AATAAAACAA TTCATCATCA GGAGAATCAT CCTCACCATC AACAATATCA 7120 ACATCGGTTA CAGTGATACG TAGGGTGGGA TCGATTTTTG AGACATAAAT TCCTAACTGT GGTATAGGGG GAATATTGTT CATGGAAACT CCTTTTATTG GTGTTTGCTA TTTGAATAAA AATACGCCAA ACAGAGTGTT AACTGTGAAT ATGGAAAGAA 7280 TCGTTGTAAG TACCCGCTTA GGTGTCCCAT TCTATTTTAG AGGTTCTCGT GCATATGGAT TATGTTATCT GAAAGCTAAC 7360 TTCCGCTTCC CGTTCACAGC GAACATTCAT CTTTGTAAAC CCGTATGATC CGTTTTTCAA GTGGCCATTC AGATACGGAT 7440 TTTCACTTCC TTGACAGTGC ATGACTATGC TGCATGAAAT CGCATGATGG ATTGAGGATC GTCTTTGCTC AGATCCGCCA 7520 GAACTGGCGG GCTTTTGCTC ATGTCATGCA TGTGCATGAA AACCACTGCA TAAAGCGGGGC AGGCGTGGCG, GGGATACGAG 7600 CECCECECTAT CACCEANAAT AGCCANAATA CTTCTEGAAA ACAGAAAGTT GAAGTGATAT GTTCATAAAC ACCATETAG GCGCCGCATA GTGGCTTTTA TCGGTTTTAT GAAGACCTTT TGTCTTTCAA CTTCACTATA CAAGTATTTG TGCGTACATC 7680 GENERATTEST TEGETETERAN TEGECANCERE TEGECETTANT GECKEGAGEA ATCECCTECE TANANCETT GATTERARGE CETETANACA ACCANCACTT AGCETTEGETE ACCEGENATTA CCGTECTECET TAGEGENEGE ATTTINGENA CTANGTETEG 7760 II CONTRACTOR A CO GACAAAAACA TCTAAACTTG ACGCACTTAG GGCTGCTACT TCACGTGAAA ACTTGGCTAA AATTTTAAGAT ATTAAGTTGG 7920 T K T S K L D A L R A A T S R E D L A K I L D I K L 8000 TATTTTTAAC TAACGTTCTA TATAGAATCG GCTCGGATAA TCAATACACT CAATTTACAA TACCGAAGAA AGGAAAAGGG V F L T N V L Y R I G S D N O Y T O F T I P K K G K G GTAAGGACTA TTTCTGCACC TACAGACCAGG TTGAAGGACA TCCAACGAAG AATATGTGAC TTACTTTCTG ATTGTAGAGA 8080 V R T I S A P T D R L K D I Q R R I C D L L S D C R D TGAGATCTTT GCTATAAGGA AAATTAGTAA CAACTATTCC TTTGGTTTTG AGAGGGGAAA ATCAATAATC CTAAATGCTT 8160 E I F A I R K I S N N Y S F G F E R G K S I I L N A ATANGCATAG AGGCAAACAA ATAATAATAA ATATAGATCT TAAGGATTTT TTTGAGGCT TY K H R G K Q I I L N I D L K D F F E S

FIG. 3. DNA sequence of the 8.2-kb E(a)-H(e) fragment from retron-Ec67. The amino acid sequences of ORF1, -2, and -3 and the sequence of the amino-terminal region of ORF4 are shown by single-letter code. The DNA sequence from residue 7422 to residue 8221 has been determined (1). The restriction enzyme sites used to detect Dam methylase activity are shown. P, *Pst* I site at P(g); H, *Hin*dIII site at H(e) in Fig. 1A. The three *Cla* I sites between P(g) and H(e) are also indicated by C. Three GATC sequences upstream of *msr* are boxed. The sequences of RNA linked to msDNA and msDNA are boxed, and their orientations are indicated by open arrows. The branched G residue at position 7684 is circled. The inverted repeat sequences required for biosynthesis of msDNA-Ec67 are shown by arrows with a1 and a2 (1).

retron DNA, except that there were three and two mismatches between K-12 and Cl-1L and between K-12 and Cl-1R, respectively (indicated by asterisks). It should be noted that there is only one mismatch between Cl-1L and Cl-1R. The DNA sequence of the 292-bp sequence upstream of Cl-1L was determined and was found to have only four single-base mismatches when compared with the corresponding region of the K-12 DNA. Similarly there were two mismatches in the 220-bp sequence downstream of Cl-1R. On the contrary, there was little homology between the downstream sequence of Cl-1L and the corresponding region of the K-12 DNA and between the upstream sequence of Cl-1R and the corresponding sequence of the K-12 DNA. These results clearly demonstrate that a 34-kb foreign DNA fragment (retron Ec67) was integrated into the Cl-1 genome and is flanked by a duplicated 26-bp host sequence.

Dam Methylase Linked to Retron-Ec67. The DNA sequence of 7.4 kb from E(a) to B (see Fig. 1A) was determined. The DNA sequence 4.2 kb downstream of site B, which contains msr, msd, and the RT gene, has been determined. Fig. 3 shows the 8221-kb DNA sequence from residue 1 [E(a)] to residue 8221 [H(e)] of which the sequence from residue 7422 (B site) to 8221 was determined (1). In the region from E(a) to E(b), there are five large open reading frames (ORFs; >200 amino acid residues) including the ORF for RT. All of the ORFs are in the same orientation except for ORF5, with the first (ORF1) from residue 2821 to residue 3675, the second (ORF2) from residue 3734 to residue 5134, the third (ORF3) from residue 5017 to 5625, the fourth (ORF4; RT-coding ORF) from residue 7839 to residue 9596 (1), and the fifth (ORF5) from residue 10,667 to residue 9690 (data not shown; available from GenBank) (see Fig. 1A). Although the amino acid sequences of ORF2, ORF3, and ORF5 show no significant similarities to any known protein sequences, ORF1 shows significantly high sequence similarity to E. coli DNA adenine methylase (Dam methylase; ref. 16), as shown in Fig. 4. Out of 285 residues, there are 125 identical residues (44% identity) between the two Dam methylases.

To examine if ORF1 is able to produce a functional Dam methylase, we transformed Dam⁻ E. coli strain GM33 with pCl-1E harboring the 11.6-kb E(a)-E(b) fragment, which contains all the ORFs described above (see Fig. 1A). After overnight culture, the plasmid DNA was isolated and digested with Pst I and HindIII. Subsequently, the 1.8-kb P(g)-H(e) fragment (Fig. 1A) was purified and examined by digestion with Cla I. As a control, strain GM33 was also transformed with pCl-1EP5 containing the 5-kb P(g)-E(b) fragment, which does not contain ORF1 (see Fig. 1A). The 1.8-kb P(g)-H(e) fragment was also isolated from PCl-1EP5 and digested with Cla I. If a guanosine residue preceded a Cla I site (ATCGAT), a Dam methylase recognition site (GATC) is created. All three Cla I sites within the 1.8-kb fragment do form the Dam methylation sites. Therefore, in the presence of Dam methylase, all the Cla I sites are methylated to become resistant to Cla I digestion. As shown in Fig. 5, lane 6, the fragment from pCl-1EP5 was completely digested by Cla I, yielding four bands at 0.73, 0.69, 0.34, and 0.12 kb, as expected. On the contrary, the 1.8-kb fragment from pCl-1E was hardly digested with Cla I (lane 4). It should be noted that a few faint bands under the undigested band are observed. The band densities did not change after prolonged digestion or digestion with more enzyme, indicating that the Cla I sites in the fragment were partially unmethylated. The Dam methylase of retron-Ec67 may be less active than the E. coli chromosomal Dam methylase.

There are a total of nine additional ORFs of >100 amino acid residues in the regions where no ORFs are assigned in Fig. 3, ORFa (bases 616–939; 108 amino acid residues), ORFb (bases 975–1481; 169 residues), ORFc (bases 1656– 1994; 113 residues), ORFd (bases 2522–2821; 100 residues), ORFe (bases 5625–6086; 154 residues), ORFf (bases 6369–



FIG. 5. Cla I digestion patterns of the 1.8-kb P(g)-H(e) fragment isolated from pCl-1E and pCl-1EP5. Plasmids were isolated from GM33 (dam⁻) (9) and digested by Pst I and HindIII. The 1.8-kb P(g)-H(e) fragments were isolated by polyacrylamide gel electrophoresis and subjected to Cla I digestion followed by polyacrylamide gel electrophoresis. The bands were visualized by staining with ethidium bromide. Lanes: 1, 0.3 μ g of λ DNA digested with HindIII and EcoRI; 2, pBR322 digested with HaeIII as molecular weight markers; 3 and 4, 1.8-kb fragment isolated from pCl-1E; 5 and 6, the 1.8-kb fragment isolated from pCl-1EP5; 3 and 5, without Cla I digestion; 4 and 6, with Cla I digestion. Numbers to the left are sizes of DNA in kb.

6671; 101 residues), ORFg (bases 7203-6889; 105 residues), ORFh (bases 1110-799; 104 residues), and ORFi (bases 625-32; 198 residues). There is one overlapping ORF (ORFj) with ORF1 (bases 3164-3493; 110 residues). Interestingly, when these ORFs were analyzed by the Genetics Computer Group Sequence Program (26) and the TFastA Program (27), it was found that ORFi has 30% identity to the cI gene product and ORFb 70% to the cp76 gene product of bacteriophage 186 (28, 29). In addition, these two genes are situated in a similar manner as found in the DNA sequence shown in Fig. 3. This finding raises an intriguing possibility that the 33-kb foreign DNA fragment might be a prophage related to phage 186, a member of the temperate P2 family of *E. coli* phages.

DISCUSSION

From the hybridization analysis between the *E. coli* K-12 DNA and strain Cl-1 DNA, the junction sites between the DNA fragment containing retron-Ec67 and the host chromosomal DNA were identified. The size of this fragment was determined to be 34 kb and to have little homology to K-12 DNA. The 34-kb fragment was flanked by 26-bp direct repeats, the sequence of which was found at 19 min on the

EC EC67	Dam Dam	MKKNRAFLKWAGGKTPLLDDIKRHLPKGECLVEPFVGAGSVFLNTDFSRYILADINSDLISLYNIVKMRTDEYVQAAR MSTILKWAGNKTAIMSELKKHLPAGPRLVEPFAGSCAVMMATDYPSYLVADINPDLINLYKKIAADCEAFISRAR • • • • • • • • • • • • • • • • • • •	78 75	
Ec	Dam	ELVFPETNCAEVYYQFREEFNKSQDPFRRAVLFLYLNRYGYNGLCRYNLRGEFNVPFGRYKKPYFPEAELYHFAEK	154	F
EC67	Dam	VL-FEIANREVAYYNIRQEFNYSTEITDFMKAVYFLYLNRHGYRGLCRYNKSGHFNIPYGNYKNPYFPEKEIRKFAEK • • • • • • • • • • • • • • • • • • •	152	quer retro with
Ec	Dam	AQNAFFYCESYADSMARADDASVVYCDPPYAPLSATANFTAYHTNSFTLEQQAHLAEIAEGLV-ERHIPVLISNHDTM	231	Ami
EC67	Dam	AQRATFICASFDETLAMLQVGDVVYCDPPYDGTFSGYHTDGFTEDDQYHLASVLEYRSSEGH-PVIVSNSDTS	224	aligr quer 0. Ic
Ec	Dam	LTREWYQRAKLHVVKVRRSISSNGGTRKKVDELLALYKPGVVSPAKK	278	
Ec67	Dam	LIRSLYRNFTHHYIKAKRSIGVSAGESKSATEIIAVSGARCWVGFDPSRGVDSSAVYEVRV	285	rach

FIG. 4. Amino acid sequence alignment of the putative retron Ec-67 Dam methylase with *E. coli* dam methylase. Amino acid sequences are aligned using IBI Pustell sequence analysis programs. ● and O, Identical and functionally homologous amino acids, respectively. K-12 chromosome. These results suggest that the 34-kb DNA fragment containing retron-Ec67 was integrated into the E. coli genome like a transposon-like element; a staggered cut was made at the 26-bp target sequence, and the retron-Ec67 containing DNA was then joined at the protruding ends of the chromosomal DNA. Subsequently, the gap at the target sequence was filled in by a repair reaction or DNA replication resulting in the duplication of the 26-bp sequence at both ends of the DNA fragment. Various transposons are considered to be integrated at their individual target sites, as described above (for reviews, see refs. 17 and 18). Alternatively, the 34-kb DNA fragment was integrated like a prophage at the 26-bp sequence on the E. coli genome by site-specific recombination. Such site-specific recombination has been shown for bacteriophages (for a review, see ref. 19) as well as for some plasmids (for example, see ref. 20). We have found (J. Sun, M.I., and S.I., unpublished results) another clinical E. coli strain that contains another retron. In this case the retron-containing fragment is integrated at 82 min on the E. coli chromosome and is flanked by 29-bp direct repeats that did not show any homology to 26-base repeats in Fig. 3.

How and when the retron-Ec67 containing DNA fragment was integrated into the genome of strain Cl-1 remains unanswered. However, comparative analysis of various enzymes of strain Cl-1 with other well-established *E. coli* strains [*E. coli* strains (*ECOR*); ref. 21] revealed that strain Cl-1 is closely related to ECOR35 strain among other 72 strains (22). Importantly, we found that ECOR35 also contained msDNA, which is highly similar to msDNA-Ec67. Furthermore, the chromosomal DNA isolated from ECOR35 contained a DNA fragment that hybridized with the RT gene from retron-Ec67. In the phylogenetic tree established for the ECOR collection, ECOR35 and ECOR36 are most related. Surprisingly, ECOR36 neither contained msDNA nor contained the RT gene. These results suggest that retron-Ec67 was recently acquired by the genome of strain Cl-1.

A most intriguing question is whether the RT function is required for transposition of the retron-Ec67 containing fragment. If the DNA fragment is a retrotransposon, the entire 34-kb DNA sequence had to be first transcribed. The resulting RNA transcript then had to be converted to cDNA by RT. The single-stranded cDNA was also converted to doublestrand DNA by RT and is then assumed to have integrated into an E. coli genome. Although we have demonstrated that RT from retron-Ec67 is able to synthesize cDNA as well as double-stranded DNA in vitro by using synthetic primers and templates (4), it remains to be seen if retron-Ec67 is able to synthesize cDNA in vivo. It is interesting to point out that a complementary sequence to the 3' end of msDNA-Ec67, 5'---TccTGCG-3', is found at the junctions between the 34-kb DNA fragment and the righthand direct repeat sequence, Cl-1R (see Fig. 2). In this sequence, 5'-GGCAtaA-3', the first three bases (GGC) are from retron Ec67 and the remaining four bases are from the direct repeat (AtaA), where lowercase letters represent mismatches. It is possible that the 3' end of msDNA may serve as a primer for DNA synthesis from the very end of retron Ec67, which may play a role in transposition.

The present finding of the *dam* gene in the element containing retron-Ec67 raises the intriguing possibility that the retron Dam methylase may play a regulatory role in the functions of the retron. Dam methylases have been shown to

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play positive and negative roles in expression of several important genes in *E. coli*, which contain GATC sequences in their promoters (for reviews, see refs. 23 and 24). Furthermore, transposition of Tn10 has been shown to be regulated by Dam methylase (25). It is interesting to note that there are three GATC sequences clustered within the 30-bp sequence (residue 7486 to residue 7515 in Fig. 3; the GATC sequences are boxed) 154 bp upstream of the *msr* gene. Methylation of these sequences may be involved in the regulation of the transcription of the *msr-msd*-RT region.

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