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Molecular Analysis of the *Escherichia coli ruvC* Gene, Which Encodes a Holliday Junction-Specific Endonuclease

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The *Escherichia coli ruvC* gene is involved in DNA repair and recombination and encodes an endonuclease that resolves Holliday structure in vitro. The 2.8-kb chromosomal DNA fragment that encompasses the *ruvC* gene and its flanking regions was cloned and sequenced. Four open reading frames were identified in the order *orf17-orf26-ruvC-orf23* immediately upstream of the *ruvAB* operon, and their orientations are the same as the *ruvAB* operon, except for *orf23*. Proteins encoded by *orf17*, *orf26*, and *ruvC* (*orf19*) were identified by the maxicell method, and their sizes agreed with those predicted from the DNA sequences. Among the open reading frames in this region, only *ruvC* is involved in the repair of UV-damaged DNA. *ruvC* appeared to be regulated by at least two promoters, but, in contrast to the *ruvAB* operon, *ruvC* is not regulated by the SOS system as demonstrated by operon fusions.

Escherichia coli strains carrying mutations in the *ruv* locus were isolated initially as mutants which were sensitive to mitomycin C (19) or which showed reduced ability of recombination between duplicated *gal* genes (32). These mutants are also sensitive to various DNA-damaging agents such as UV, ionizing radiation, and some chemical mutagens, and they form multinucleated cells after treatment with low doses of such agents (18–20, 32). Although *ruv* mutations have little effect on conjugal recombination in otherwise wild-type strains, they reduce the ability of recombination in *recBC sbcA* and *recBC sbcBC* strains (11–13). This evidence suggests that the *ruv* locus may be involved in recombinational repair by the RecE and the RecF pathways.

The *ruv* locus contains two genes, *ruvA* and *ruvB*, that constitute an operon regulated by the SOS system (3, 27, 29). Recently these genes were also shown to be involved in mutagenesis induced by UV and X irradiation and by some chemicals (9, 24). The purified RuvB protein possesses weak ATPase activity (7), which is stimulated by the RuvA protein in the presence of DNA (26). The RuvA-RuvB complex in the presence of ATP renatures cruciform structure in supercoiled DNA with a palindromic sequence, indicating that it may promote strand exchange reactions in homologous recombination (26).

While we were cloning the chromosomal DNA fragment that complemented the repair defect in the *ruv* mutants, we noticed that the phenotype of one of the *ruv* mutants we analyzed, CS85 (*ruv-53*), was not complemented by the plasmid carrying the *ruvAB* operon, but was complemented by the chromosomal DNA region upstream of the *ruvAB* operon. Sharples et al. (25) have identified the approximate location of this mutation on the physical map of this region and designated it *ruvC*. The *ruvC* mutants, as with the other *ruv* mutants, are defective in DNA repair and recombination of the RecE and the RecF pathways (1, 12, 19, 25, 30). In this study we analyzed the nucleotide sequence of the *ruvC* gene and its flanking regions and identified the proteins encoded by the *ruvC* region.

RuvC protein has been highly purified recently and has an endonuclease activity that specifically cleaves Holliday junctions (33).

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and media. *E. coli* CS85 (*ruv-53*) (30) was used as the host strain for cloning and subcloning of the *ruvC* gene. CS40a (11) was used as a *ruv*$^+$ control strain. The Ruv phenotype was examined by the sensitivity of the mutant to UV irradiation. CSR603 (*uvrA6 recA1 phr-1*) was used to identify plasmid-encoded proteins by the maxicell method (22). DM2558 (Δ*lac-pro*) (5) was used as a host for the plasmids carrying the *lacZ* operon fusions. JM103 (16) was used as a host for derivatives of M13 phage.

pUC19 (35), pSCH18 (8), and pSCH19, which is similar to pSCH18 but carries the 2.3-kb *Hae*II fragment of pUC19 that contains multiple cloning sites, were used as cloning vectors. pRS415 (31) was used to construct operon fusions with *lacZ*. Phage M13mp18 (35) was used for subcloning and sequencing of the *ruvC* region.

Bacteria were grown routinely in LB medium. M9 medium supplemented with appropriate amino acids was used for labeling the cells by the maxicell procedure. YT and 2YT medium were used for M13 phage multiplication. All the media mentioned above were described by Miller (17).

Manipulation of recombinant DNA. Plasmids and phage M13 DNA were prepared and manipulated in vitro by standard procedures as described previously (14).

Sequencing of the cloned DNA. A DNA fragment containing the upstream region of the *ruvAB* operon was subcloned from pHS102 (27) into pUC19 or M13mp18. A series of phage clones that contained successive deletions from one end of the cloned DNA fragment were prepared as reported previously (6). Nucleotide sequence was analyzed by the dideoxy-chain termination method (23) with these M13 phage derivatives as templates. Ambiguous sequences were also analyzed by the method of Maxam and Gilbert (15).

Identification of proteins encoded by recombinant plasmids. Proteins encoded by recombinant plasmids were identified by the maxicell method (22). UV-irradiated CSR603 cells which harbored plasmids were labeled with [^{35}S]methionine.

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The labeled proteins were separated by electrophoresis in a 14.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) by the method of Laemmli (10) and visualized by autoradiography.

UV sensitivity test. Overnight cultures suspended in M9 buffer (2×10^8 to 4×10^8 cells per ml) in a petri dish were irradiated with a germicidal UV lamp, diluted appropriately, and plated on LB agar plates. The UV dose at 254 nm was measured by using the Topcon radiometer UVR-254 (Tokyo Kogaku Kikai, Tokyo, Japan). Surviving colonies were counted after incubation for 24 h at 37°C in the dark.

β-Galactosidase assay. Overnight cultures of DM2558 (Δ*lac-pro*) carrying the *lacZ* operon fusions were diluted 100-fold with fresh medium, and the cells were grown at 37°C with shaking. At a density of 3×10^8 to 4×10^8 cells per ml, the β-galactosidase activity of the culture was measured and the specific activity of the enzyme was expressed in Miller units (17).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence data bases under the accession number D90392.

RESULTS

Nucleotide sequence of the *ruvC* region and identification of the *ruvC* gene. CS85 was originally isolated as a *ruv* mutant by Shurvinton et al., and the *ruv-53* mutation was mapped to the *ruv* locus (30). Introduction of pHS501, a low-copy-number plasmid carrying the 2.7-kb *Pvu*II fragment containing the *ruvA* and *ruvB* genes, did not complement the *ruv-53* mutation in CS85. However, introduction of pHS530, which carries the 2.8-kb *Pst*I-*Pvu*II fragment upstream of the *ruvAB* operon (27) on the low-copy-number vector pSCH18 (8), into the same mutant restored UV resistance to the wild-type level. Therefore, we concluded that the *ruv-53* mutation is distinct from the *ruvA* and *ruvB* mutations; this is in agreement with the report by Sharples et al. (25), who showed that the *ruv-51* and *ruv-53* mutations are complemented by the plasmids carrying the chromosomal fragments upstream of the *ruvAB* operon. They designated these mutations *ruvC*, because their phenotypes are very similar to those of *ruvB* and *ruvAB* mutants; i.e., *ruvC* mutants are sensitive to DNA-damaging agents and are defective in recombination when they carry additional mutations in *recBC sbcA* or *recBC sbcBC* (11, 12).

To analyze the structure of the *ruvC* region, we isolated the *Pst*I-*Pvu*II fragment of pHS102, converted the *Pst*I site to blunt ends, and inserted it into the *Sma*I site of M13mp18. We prepared a series of nested deletion clones from the recombinant phage and analyzed the sequence of the *ruvC* region (Fig. 1). Three open reading frames (ORFs), *orf17*, *orf26*, and *orf19*, which could encode proteins of 17, 26, and 19 kDa, respectively, were found in this region (Fig. 1 and 2). Among them, alternative initiation codons could be assigned for *orf17*. We think that GTG at nucleotides 511 to 513 or 523 to 525 is the initiation codon for this frame, since they are preceded by well-conserved sequences for ribosome-binding sites (28) located at appropriate positions.

To identify which ORF of the three is *ruvC*, we recloned the inserts from some of the nested deletion clones into pSCH18, giving plasmids pHS532 to pHS540 (Fig. 2). To subclone the *Hinc*II-*Nde*I fragment (Fig. 2), we converted the *Nde*I-digested end to a blunt end and inserted the fragment into the *Sma*I site of pSCH18, giving pHS544. Complementation of the UV sensitivity of CS85 (*ruv-53*) was examined by introduction of these recombinant plasmids (Fig. 2). Introduction of pHS530 and pHS538 restored the UV resistance in the *ruv-53* mutant to the wild-type level. Introduction of pHS540 partially restored the UV resistance. pHS540 carries the chromosomal fragment of nucleotides 1677 to 2807 (Fig. 1) that includes the entire *orf19* and 90 nucleotides upstream of the putative ATG initiation codon. We suspected that *orf19* is the *ruvC* coding region and that the partial complementation of the UV sensitivity by pHS540 may be caused by insufficient expression of *ruvC* as a result of the complete or partial deletion of the *ruvC* promoter in pHS540. To test this possibility, we inserted the same DNA fragment into pSCH19 so that *orf19* would be placed downstream of the *lac* promoter on pSCH19. The resultant plasmid, pHS542, fully restored the UV resistance of the *ruvC* mutant (data not shown). Other plasmids shown in Fig. 2 had no effect on the UV sensitivity of the *ruv-53* mutant. From these results, we conclude that the *ruvC* coding region is *orf19*.

Proteins encoded by the *ruvC* region. To identify the products of the three ORFs in the *ruvC* region, the DNA fragments inserted into a low-copy-number vector, pSCH18, were recloned into a high-copy-number vector, pUC19 (Fig. 2). In these pUC19 derivative plasmids, the three ORFs were inserted into the vector just downstream of the *lac* promoter in the same orientation as the *lac* promoter, except for pHS238 and pHS240, so that the ORFs lacking a promoter can also be transcribed from the *lac* promoter. The proteins encoded by the plasmids were labeled with [^{35}S]methionine by the maxicell method and analyzed by SDS-PAGE (Fig. 3).

Plasmid pHS230 (Fig. 3, lane 3) encoded three proteins with apparent molecular masses of 33, 18, and 16 kDa, in addition to β-lactamase (29 kDa) and a 14.5-kDa protein, both of which were synthesized by the strain carrying the vector (lane 2). The 18-kDa protein was also produced by pHS238 (lane 5) and pHS240 (lane 6), but not by pHS244 (lane 4), pHS232 (lane 7), or pHS234 (lane 8). Only these plasmids which carried *orf19* produced the 18-kDa protein (Fig. 3) and complemented the repair defect of the *ruvC* mutant (Fig. 2). Therefore, we concluded that the product of *ruvC* (*orf19*) is the protein with an apparent molecular mass of 18 kDa, which agrees with the molecular mass deduced from the DNA sequence (19 kDa). The 33-kDa protein was produced by pHS230, pHS244, pHS238, and pHS232 (Fig. 3), all of which contained the *orf26* region (Fig. 2). Therefore, *orf26* probably encodes the protein with an apparent molecular mass of 33 kDa. Although the deduced size of the protein was smaller than the size estimated by SDS-PAGE, this is not a rare occurrence. The protein with an apparent molecular mass of 16 kDa was produced by pHS230, pHS244, pHS232, and pHS234 (Fig. 3), all of which carry *orf17* (Fig. 2); therefore, it should be the product of *orf17*.

The region flanking the *Pvu*II site (Fig. 2) that is located between *ruvC* and *ruvA* was also sequenced (Fig. 1). The sequence data confirmed that the 2.8-kb *Pst*I-*Pvu*II region is contiguous to the 2.7-kb *Pvu*II region containing the *ruvAB* operon, which had been previously sequenced (3, 27). From the DNA sequence and the maxicell analysis, we assigned the ORFs as shown in Fig. 1 and 2. The orientations of *orf17*, *orf26*, *ruvC*, and *ruvAB* are the same. An ORF whose orientation is opposite to that of *ruvC* and *ruvAB* was found between *ruvC* and *ruvA*. Although the ORF can be extended further to nucleotide 2991, we cannot find a reasonable sequence for a ribosome-binding site upstream of this GTG codon at an appropriate position (Fig. 1). Therefore, the

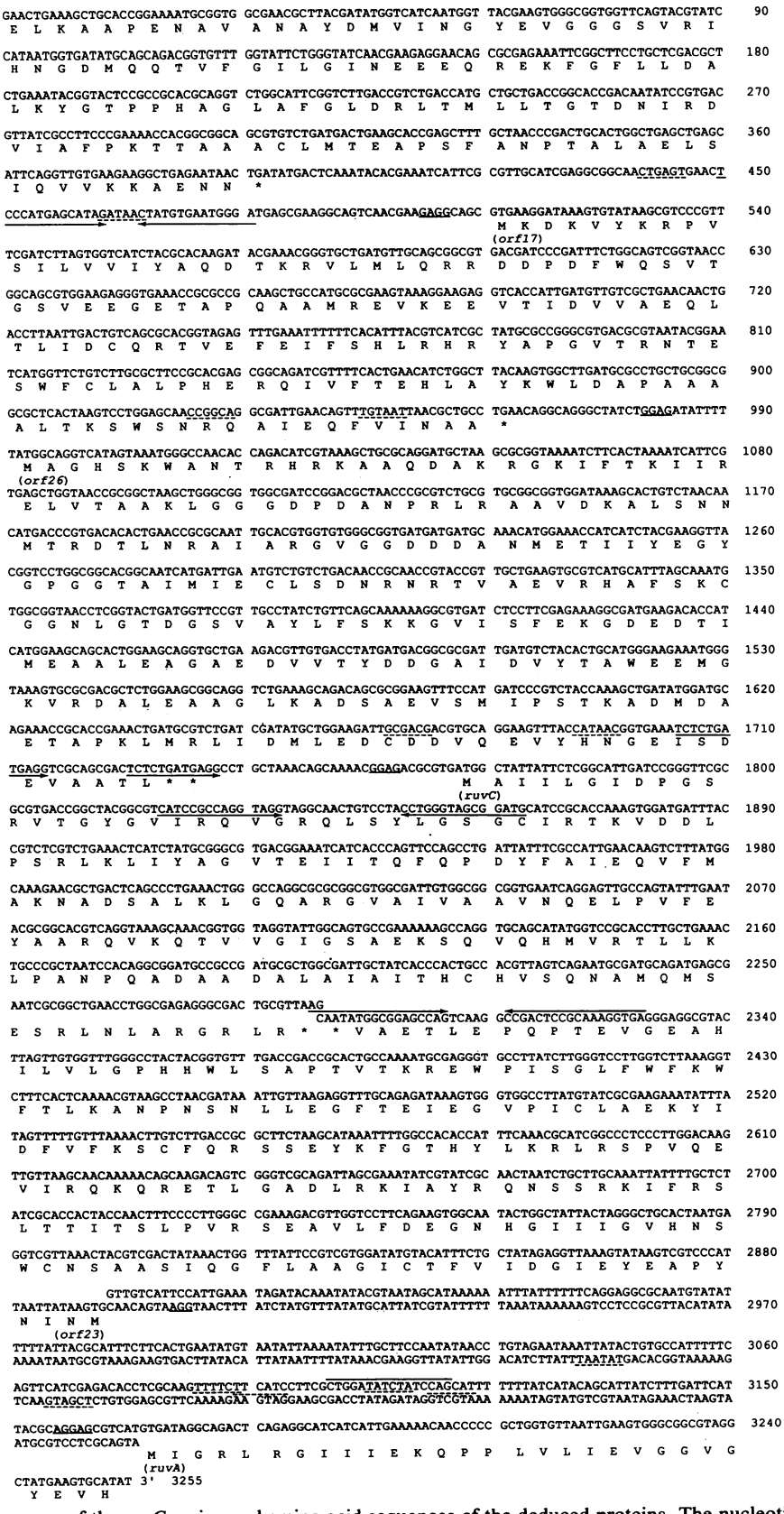
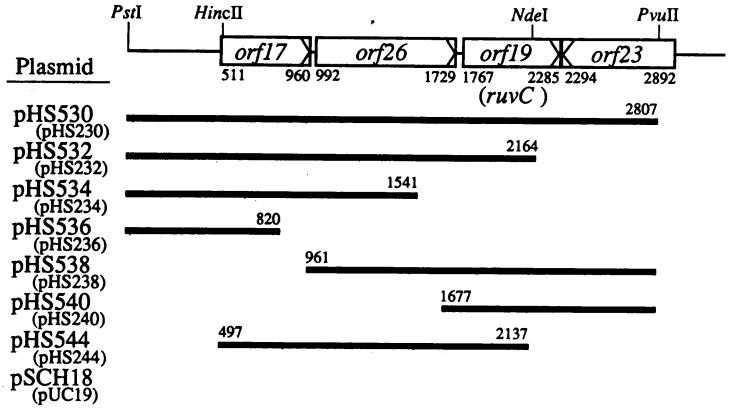


FIG. 1. Nucleotide sequence of the *ruvC* region and amino acid sequences of the deduced proteins. The nucleotides are numbered from the *Pst*I cleavage site. Putative ribosome-binding sites are underlined. For *orf17*, an alternative initiation codon, GTG at nucleotides 523 to 525, with a putative ribosome-binding site at nucleotides 513 to 517, is also plausible in addition to the one assigned in the figure. Putative −35 and −10 regions of promoters are indicated by dashed lines. The direct and inverted repeat sequences are indicated by horizontal arrows. The SOS box of the *ruvAB* operon is overlined. Sequences of both strands are shown for the region from nucleotides 2893 to 3166 to indicate the putative ribosome-binding sites, promoters, and SOS box for the two divergent genes, *orf23* and *ruvA*.



Plasmid	Surviving Fraction CS40a (*ruv*+)	CS85 (*ruv-53*)
pHS530 (pHS230)	0.74	0.55
pHS532 (pHS232)	0.68	0.026
pHS534 (pHS234)	0.63	0.033
pHS536 (pHS236)	0.59	0.042
pHS538 (pHS238)	0.65	0.45
pHS540 (pHS240)	0.61	0.20
pHS544 (pHS244)	0.58	0.036
pSCH18 (pUC19)	0.61	0.026

FIG. 2. Physical map of the *ruvC* regions cloned on plasmid vectors and complementation of UV sensitivity of the *ruv-53* mutant by the recombinant plasmids. The extent and direction of four ORFs predicted from the DNA sequence are illustrated by the arrows in the boxed ORFs at the top. The regions which are carried on the plasmids are shown by heavy lines, and the deletion endpoints are indicated by the nucleotide numbers counted from the *Pst*I site (Fig. 1). On the right, complementing activities of the pHS500 series plasmids are expressed by the surviving fractions of the cells irradiated by UV (30 J/m^2) carrying the indicated plasmids. For the pHS500 series plasmids, the chromosomal fragments are inserted into the low-copy-number vector pSCH18, and for the pHS200 series plasmids, the same fragments are inserted into the high-copy-number vector pUC19.

GTG codon at nucleotides 2890 to 2892, for which we could find a typical sequence for a ribosome-binding site at an appropriate position, is probably the initiation codon for this ORF. This ORF could code for a 23-kDa protein (*orf23*). The product of *orf23* could not be detected in the maxicells labeled with [^{35}S]methionine, but this might have been because *orf23* does not encode methionine except for the initiation codon. A strain with an *orf23'-'lacZ* gene fusion produced a hybrid β-galactosidase protein of the expected size (data not shown). This suggests that *orf23* is actually transcribed and translated in vivo.

Upstream regulatory elements of *ruvC*. To identify the minimal *ruvC* region that complements the DNA repair

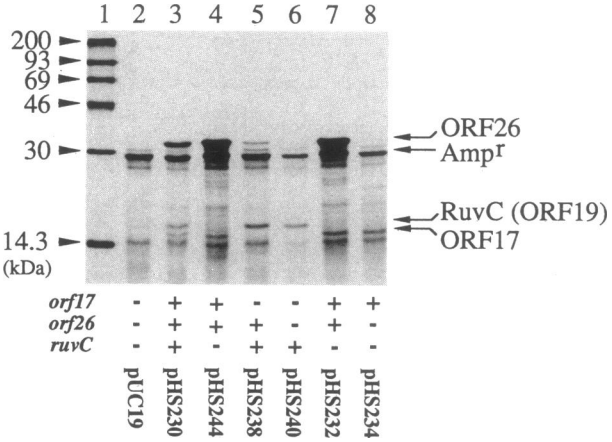


FIG. 3. Identification of proteins encoded by plasmids carrying various chromosomal fragments of the *ruvC* region. The proteins encoded by the plasmids were labeled with [^{35}S]methionine by the maxicell method and analyzed by electrophoresis on an SDS–14.5% polyacrylamide gel. At the bottom are shown the ORFs carried by each plasmid. ^{14}C-methylated protein mixture was run as size markers in lane 1. The numbers on the left show the size of each protein marker. The positions of the gene products identified are shown on the right. Amp^r indicates the position of β-lactamase (29 kDa) encoded by the *bla* gene on the vector.

defect of the *ruv-53* mutant, we recloned from pHS538 the DNA fragments carrying the *ruvC* coding region and various lengths of the upstream region into the low-copy-number vector, pSCH18. The UV sensitivity of the *ruv-53* strains carrying these plasmids was examined (Table 1). Plasmids pHS554 and pHS556, which carry the upstream region up to nucleotide 1653, fully complemented the mutation, whereas pHS552 partially complemented it and pHS550 did not complement it at all. The complete inability of pHS550 is probably due to the lack of the ribosome-binding site required for translational initiation of *ruvC*. We could identify putative promoter sequences (cATAAc for −10 and gcGACg for −35) in the region that is present in pHS554 and absent in pHS552 (Table 1; Fig. 1), and the lack of these sequences may account for the partial complementation of the mutation by pHS552.

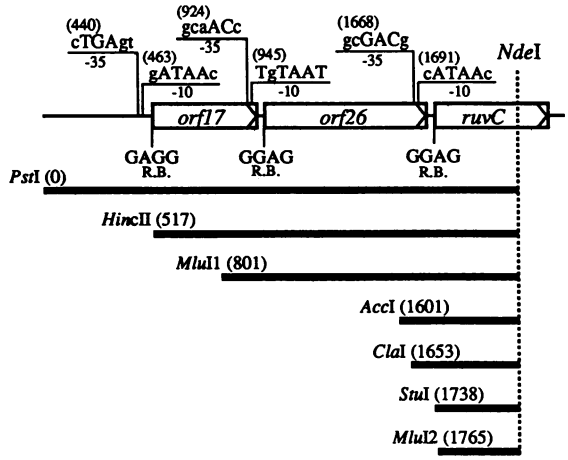
To examine the effects of the upstream regions on the transcription of *ruvC*, we constructed operon fusions of various *ruvC* upstream regions with *lacZ* on a promoter assay vector, pRS415, and assayed the promoter activity of the DNA fragments by measuring the β-galactosidase activity in the *lacZ* deletion strain (DM2558) carrying the plasmids with these operon fusions (Fig. 4). Plasmids of operon fusions with *lacZ* were constructed as follows. pHS230 (Fig.

TABLE 1. Effect of the upstream regions of the *ruvC* gene on the expression of the *ruvC* function

Plasmid	Region of inserted fragment[a]	Surviving fraction[b] CS40a (*ruv*+)	CS85 (*ruv-53*)
pHS550	1765–2807 (*Mlu*I2-*Pvu*II)	0.79	0.014
pHS552	1738–2807 (*Stu*I-*Pvu*II)	0.73	0.22
pHS554	1653–2807 (*Cla*I-*Pvu*II)	0.76	0.75
pHS556	1601–2807 (*Acc*I-*Pvu*II)	0.72	0.70
pSCH18		0.73	0.010
pHS538	961–2807	0.72	0.68

[a] The nucleotide numbers indicate the ends of the inserted chromosome fragments as defined in Fig. 1. See Fig. 4 for a comparison.

[b] Values relate to the fraction of cells surviving to form colonies after a UV dose of 60 J/m^2.



Plasmid	Specific activity of β-galactosidase (units/OD_{600})
pHS1250	256
pHS1252	228
pHS1254	216
pHS1262	117
pHS1264	125
pHS1266	13.4
pHS1268	14.7
pRS415	7.6

FIG. 4. Promoter activity of the upstream regions for *ruvC* expression as examined by operon fusions. The heavy lines indicate the chromosomal fragments inserted into the operon fusion vector. OD_{600}, optical density at 600 nm.

2) was double digested with *Nde*I and *Eco*RI, whose recognition sites lie in the *ruvC* coding region and the upstream flanking vector region, respectively. The staggered ends were converted to blunt ends with mung bean nuclease, and the smaller fragment was ligated into the *Sma*I site on pUC19, giving pHS270. pHS246, which carries the *Hin*cII-*Nde*I fragment with the blunted *Nde*I end in the *Sma*I site of pUC19 in the opposite orientation to pHS244 (Fig. 2), was digested by one of the restriction enzymes *Mlu*I, *Acc*I, *Cla*I, or *Stu*I. Each of the linearized DNAs was converted to blunt ends and ligated with an *Eco*RI linker (5′-GGAATTCC). From these derivative plasmids, the parental pHS246 plasmid, and pHS270, the inserted fragments, which contain various lengths of the *ruvC* upstream region, were excised by cutting with *Eco*RI and *Bam*HI, which recognize the flanking sites, and inserted into the *Eco*RI-*Bam*HI site on pRS415 vector to construct *lacZ* operon fusions; the resultant plasmids are shown in Fig. 4.

Little expression of β-galactosidase was observed in the plasmids pHS1266 and pHS1268, which lack the putative promoter of *ruvC* mentioned above. The cells with pHS1250, pHS1252, and pHS1254 were twice as active as those with pHS1262 and pHS1264. The cells with these two plasmids, pHS1262 and pHS1264, showed the same level of β-galactosidase activity, which was about 10-fold higher than those with pHS1266 and pHS1268. The activities of β-galactosidase encoded by these operon fusions did not increase after treatment with mitomycin C (data not shown), indicating that these promoters are not regulated by the LexA repressor. These results suggest that the *ruvC* expression is controlled by at least two promoters, one located between nucleotides 1653 and 1738 and the other located between nucleotides 801 and 1601. The sequences of the putative promoters in these regions are shown in Fig. 1 and 4. Promoterlike sequences are also found upstream of *orf17*; however, deleting this region in pHS1252 and pHS1254 did not reduce the expression of *ruvC* from the level of expression by pHS1250. However, this result does not eliminate the possibility that the promoterlike sequences upstream of *orf17* function as the promoter for *ruvC* under specific physiological conditions.

No ORFs in this region outside of *ruvC* are required for DNA repair. We wanted to know whether any ORF in the sequenced region outside of *ruvC* is required for repairing UV-damaged DNA. We constructed a strain (HRS1024) in which the *Mlu*I1-*Mlu*I2 chromosomal region (Fig. 4) was replaced with the gene coding for chloramphenicol acetyltransferase by homologous recombination by the method described by Winans et al. (34). HRS1024 should lack the region between nucleotides 797 and 1764, which includes part of *orf17*, the entire *orf26* region, and the putative ribosome-binding site of *ruvC*. HRS1024 was more UV sensitive than its *ruv*$^+$ parent strain AB1157 (2), and the UV sensitivity was fully restored to the wild-type level by pHS542 which carries *ruvC* only (data not shown). pHS542 carries the same chromosomal insert as pHS540 (Fig. 2), but in the opposite orientation downstream of the *lac* promoter on the vector, which enables full complementation of the *ruvC* mutation (see above). These results suggest that neither *orf17* nor *orf26* is required for DNA repair. Iwasaki et al. (8) showed that disruption of *orf23* at the *Pvu*II site in the chromosome did not make the cell UV sensitive, which suggests that *orf23* is also not required for DNA repair.

DISCUSSION

The new *ruv* gene (*ruvC*) was independently identified by Sharples et al. (25) and located between the *ruvAB* operon and *orf26* (*orf33*, according to them). Our data are consistent with theirs. Since the plasmids carrying *orf19* complement the *ruvC* mutant and encode an 18-kDa protein as shown by the maxicells, we conclude that *orf19* is *ruvC* and the protein with an apparent molecular mass of 18 kDa is the gene product.

Either the ATG codon as shown in Fig. 1 or the GTG codon immediately upstream of this codon could have been assigned as the initiation codon for *ruvC*. However, the GTG codon is too close to the well-conserved sequence for the ribosome-binding site, GGAG, while the ATG codon is located at an appropriate distance from the putative ribosome-binding site. Thus, we assigned the ATG codon as the initiation codon of *ruvC*.

orf17, *orf26*, and *ruvC* are arranged in this order in the same orientation with only small spaces between them and

they appear to constitute an operon. However, of these, only *ruvC* is required for repair of UV-damaged DNA. These three ORFs are separated from the unknown upstream *orf* by 120 nucleotides, and in this region is found a dyad symmetry which may function as a transcriptional terminator (21) or a binding site for regulatory protein. A putative terminator is also found downstream of *ruvC* in the noncoding region. *ruvC* is likely to be controlled by a promoter located between nucleotides 1653 and 1738 in *orf26* (Table 1; Fig. 4) and also by a promoter located further upstream in *orf17* or *orf26* (Fig. 4). There is an inverted-repeat sequence in the beginning of the *ruvC* coding region which may function as an attenuator. This may explain the lower expression of *ruvC* compared with *orf26* as examined by the maxicells (Fig. 3).

There are some unique DNA sequences in the sequenced region. Overlapping the promoterlike sequence upstream of *orf26*, two SOS box-like sequences were found: CTGgAgca AccggCAG (nucleotides 915 to 930) and CTGccTgaAcAgg CAG (nucleotides 956 to 971). According to a quantitative analysis described by Berg (4), the affinity of these SOS box-like sequences for LexA protein is estimated to be too low to have a functional interaction. In fact, *ruvC* was not induced by DNA-damaging agents, as demonstrated by *lacZ* operon fusions with the upstream regions of *ruvC*. Direct-repeat sequences, which are composed of 12 nucleotides (TCTCTGATGAGG) separated by 10 bp, are found around the termination codon of *orf26*, although we have no idea about their function, if any.

The *ruvC* gene product has been recently purified from an overproducing strain and has an endonuclease activity that resolves the Holliday structure, a putative intermediate of recombination, in vitro (33). We could not find any proteins whose sequences are substantially similar to the ORFs in the sequenced region by computer analysis with a protein primary structure data base, PRF (release 89.9), including T4 endonuclease VII and T7 endonuclease I, which also cleave Holliday junctions.

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