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Distal regulatory functions for the *uvrC* gene of *E. coli*

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ABSTRACT

We find that the uvrC gene is preceded by three promoters (P1, P2 and P3), identified by heparin-resistant RNA polymerase-DNA complex formation. P2 and P3 promoters are located proximal to the 5' end of the uvrC gene, while the P1 promoter is separated from the uvrC structural gene by an interposed DNA region of more than 1 kb. We have reported that P2 and P3 are not sufficient to promote uvrC complementation. However, plasmids containing the direct fusion of the P1 promoter to the uvrC gene complements the uvrC defect. Insertion of IS1 downstream from the P1 promoter leads to efficient synthesis of the uvrC protein as measured in maxicells. Fusion of the lac promoter to the uvrC structural gene can substitute for in vivo regulatory functions. We conclude that uvrC protein synthesis is controlled in a complex manner and that a distal promoter, P1, is required.

INTRODUCTION

The the uvrA, uvrB and uvrC genes control nucleotide excision repair of DNA damage (1,2). Recently, the uvrA, uvrB and uvrC genes have been cloned and characterized (3-9). The regulation of expression of these genes appears to be complex. For example, the uvrA and uvrB genes have been shown to be regulated by the recA-lexA control system, and thus their gene products can be induced in exponentially growing cells via "SOS" response (5,10,11). This is in contrast to the previously held notion that these genes were constitutively expressed. Such "SOS"-controlled regulation has not yet been defined for the uvrC gene.

We showed that the uvrC gene is located on a 1.9 kb fragment and codes for a protein of 66,000 Mr (7). The 1.9 kb fragment does not appear to contain the uvrC gene promoter. To identify the control regions for the uvrC gene, we subcloned the uvrC structural gene together with 5'-flanking DNA. We concluded (12) that (a) the 1.9 kb fragment when fused at the 5' end with a heterologous promoter complemented the uvrC defect, (b) an adjacent region of at least 1 kb 5' to the structural gene did not contain an active in vivo uvrC promoter, and (c) a distal regulatory region(s) was required for optimal in

vivo complementation of the uvrC defect. Interestingly, a plasmid (pUV7) containing the 1.9 Kb structural region and 2.4 Kb 5' to the gene, failed to promote synthesis of detectable amounts of the uvrC protein under non-induced conditions, although this plasmid confers normal UV resistance to uvrC$^-$ cells and codes for a 27,000-Mr protein in addition to uvrC (12).

We have carried out fine mapping for the E. coli RNA polymerase binding sites on the 2.4 kb upstream DNA sequences, and constructed deletion and insertion mutations to identify the presence of putative promoter regions. Our results suggest that the uvrC gene is preceded by three promoters. In addition, we have utilized the lac promoter to bypass the distal promoter-mediated regulation effect and to overproduce the uvrC protein. We conclude that a distal regulatory region is involved in in vivo regulation of the uvrC gene.

MATERIALS AND METHODS

E. coli strains AB1157 (1), AB1184 uvrC$^-$ (1), SR57 uvrC$^-$, recA$^-$ (6), CSR603 uvrA$^-$, recA1, phr$^-$ (13), and AD10 recA$^-$, pgsA (14) , were used as host strains. General procedures for construction and cloning of plasmids, bacterial transformation, purification of plasmid DNA and restriction enzyme analysis were as described (7,12). Maxi-cell preparations and ^{35}S-methionine labeling of plasmid encoded proteins were done as reported (15). UV-survival of AB1884 (uvrC$^-$) transformed with plasmids was measured using cell dilutions at different UV-doses (12).

Plasmids

Plasmid TP88, the source of the lac promoter fragment, was a gift from Dr. Anthony R. Poteete of the University of Massachusetts Medical School. Plasmids pUV201 and pUV301 have been previously described (7,12).

Construction of plasmid pUV7-8 required ligation of Hind III linkers. The preparation of "blunt ends" for linker ligation was done by filling in protruding ends generated by Bgl II. The digested DNA (5 µg) was incubated with Klenow fragment of DNA polymerase I (15 units) for 15 minutes at 14°C in a reaction mixture (100 µl) containing 60 mM Tris-HCl, pH 7.6, 8 mM $MgCl_2$, 10 mM DTT, 400 µM ATP, and 200 µM dNTPs. This reaction was immediately followed by addition of ^{32}P-labeled Hind III linkers and by treatment with T4 phage DNA ligase (40 units). Ligation was performed by incubating the reaction mixture at 14°C for 2 hours, and then continuing the incubation at 4°C overnight. The ligation mixture was precipitated and digested with excess Hind III. Required fragments were first identified by autoradiography and isolated for further

use. For construction of plasmid pUV7-3, blunt end ligation was carried out as described above.

RNA Polymerase Binding Assay

Reaction conditions for RNA polymerase-DNA interactions were as described (12). In brief, isolated DNA fragments were digested with restriction enzymes to generate smaller fragments. DNA fragments were incubated with RNA polymerase (4:1 RNA polymerase:DNA ratio) for 30 minutes at 37°C Weak RNA polymerase-DNA complexes were further competed out with heparin (200 µg). The protein-DNA complexes were retained on nitrocellulose filters, eluted and separated on 7.5% acrylamide gel.

Selection of the lac Promoter-Containing Transformants

Transformants containing hybrid plasmids which contain lac promoters were selected on ampicillin-agar plates containing the indicator 5-chloro-4-bromo-3-indolyl-β-D galactoside (XG) (16). The lac operator present on the lac promoter fragment can titrate the cellular repressor allowing synthesis of β-galactosidase (17). The synthesis of β-galactosidase in transformants gives blue colonies on XG indicator plates. We purified single colonies on fresh XG plates and selected dark blue colonies. XG solution (20 mg/ml) was prepared in N,N-dimethyl formamide (16), and 50 µl of this solution was spread on agar plates.

Materials

Growth media were from Difco. Ampicillin, tetracycline, chloramphenicol, and D-cycloserine, were purchased from Sigma. Restriction endonucleases and T4 ligase were obtained from Bethesda Research Laboratories, New England Biolabs, and Boehringer Biochemicals.

RESULTS

Multiple Promoters of the uvrC Gene

Our previous studies have shown that a plasmid, pUV301, containing the uvrC structural gene and the contiguous 0.9 kb 5' region does not complement the uvrC defect, in contrast to plasmid pUV7 which carries a 1.5 kb additional upstream region with the 0.9 kb fragment (12). This suggested the presence of uvrC control regions in the distal 1.5 kb fragment. To identify putative regulatory region(s) on the 1.5 kb and 0.9 kb fragments (Fig. 1A), we previously used the approach of selective binding of RNA polymerase holoenzyme to promoter-operator regions (12). Heparin resistant RNA polymerase-promoter complexes can be retained on nitrocellulose filters and characterized by gel electrophoresis (18). The 1.5 kb and the 0.9 kb fragment on plasmid pUV7

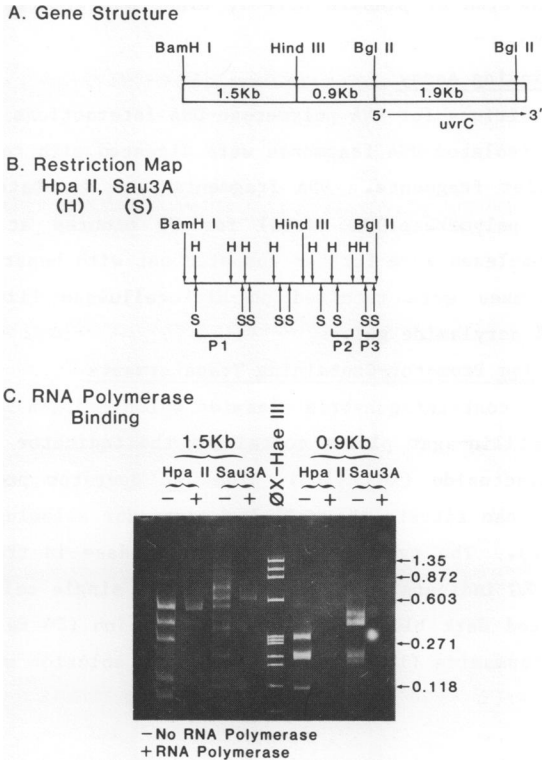

Figure 1. RNA polymerase binding sites in the uvrC gene unit. (A) A physical map is shown for the uvrC structural gene and its upstream region. The 5'-end of the structural gene is located downstream from the Bgl II site. (B) The Bam HI-Bgl II (2.4 Kb) fragment encompassing the uvrC upstream region was isolated from the parental plasmid pUV7 after labeling the Bam HI site in the presence of γ-^{32}P ATP and polynucleotide kinase. The end-labeled fragment was subjected to partial digestion with Sau 3A and Hpa II independently (0.5, 1, 3, 10, 30, 60 min), and the resulting fragments were separated on a 7.5% acrylamide gel. The approximate sites for Sau 3A and Hpa II were determined with respect to the distance from the labeled end. (C) Plasmid pUV7 was digested with Bam HI, Bgl II and Hind III, and the 1.5 Kb and 0.9 Kb fragments were separated on a 1% agarose gel and purified by electro-elution. The purified fragments (2 µg) were digested with Sau 3A and Hpa II respectively. The resulting fragments were mixed with RNA polymerase (4:1), passed through nitrocellulose filters and then eluted from the filters as described in Materials and Methods. The eluted DNA fragments were run on 7.5% agarose gel and visualized by ethidium bromide staining. Lanes: a, 1.5 Kb fragment + Hpa II; b, lane a products + RNA polymerase; c, 1.5 Kb fragment + Sau 3A; d, lane c products + RNA polymerase; e, ϕX174 DNA Hae III markers; f, 0.9 Kb fragment + Hpa II; g, lane f products + RNA polymerase; h, 0.9 Kb fragment + Sau 3A; i, lane h products + RNA polymerase.

(Fig. 1A) exhibited stronger binding of RNA polymerase than the 0.9 kb fragment. Our plasmid constructions (7,12) indicated that the promoter on the 1.5 kb fragment is required, whereas the promoter(s) on the 0.9 kb fragment are not alone sufficient. In the same experiments, the 2.2 kb fragment containing the uvrC structural gene did not show any binding to RNA polymerase and served as an internal control. These results are consistent with the observation that plasmid pUV301, lacking the distal 5' region, fails to complement the uvrC defect.

To obtain a more accurate map of the RNA polymerase-promoter interaction sites on the uvrC upstream region, we first established a map of the 1.5 kb and the 0.9 kb fragments with respect to restriction enzymes Sau 3A and Hpa II by using the partial digestion method of Smith and Bernsteil (19). These two enzymes generate fragments of various sizes with random cuts in a non-overlapping fashion as shown in Fig. 1B. Both of the fragments were independently digested with Sau 3A or Hpa II and the resulting fragments were assayed for heparin-resistant complexes with a 4:1 ratio of RNA polymerase to DNA, a ratio giving clear binding to promoter sequences (12). Fig. 1C shows the restriction fragment(s) which exhibited selective binding to RNA polymerase. From Hpa II and Sau 3A digests (lane a and c) of the 1.5 kb fragment, two fragments of sizes 0.45 kb (Hpa II, lane b) and 0.6 kb (Sau 3A, lane d), showed strong binding to RNA polymerase. These two fragments overlap each other and represent the P1 promoter region (Fig. 1B). In the case of the 0.9 kb fragment for the Hpa II digest (lane f), two non-overlapping fragments (0.3 kb and 0.2 kb, lane g) exhibited weak binding to RNA polymerase and represent the P2 and P3 promoters. For the Sau 3A digest (lane h) of the 0.9 kb fragment, we did not observe any detectable binding. These results indicate that the uvrC gene is preceded by one distal (P1) and two proximal (P2 and P3) promoters.

Deletions and Insertions in the uvrC Regulation Region

Construction of internal deletions. To delineate the physiological role of the P1 promoter, we have generated deletions involving promoters P2 and P3. If promoter P1 is indeed an *in vivo* requisite for regulation of the uvrC gene, the fusion product of this region with the structural gene might be proficient in complementing the uvrC defect. We found this is the case. Fig. 2 shows the underlying strategy and results of such experiments. Plasmid pUV7 was first digested with Bgl II and the Bgl II ends were converted into Hind III sites by ligating Hind III linkers. Hind III-digestion of the resulting plasmid generated fragments of 5.5 kb, 2.2 kb and 0.9 kb, respectively. The

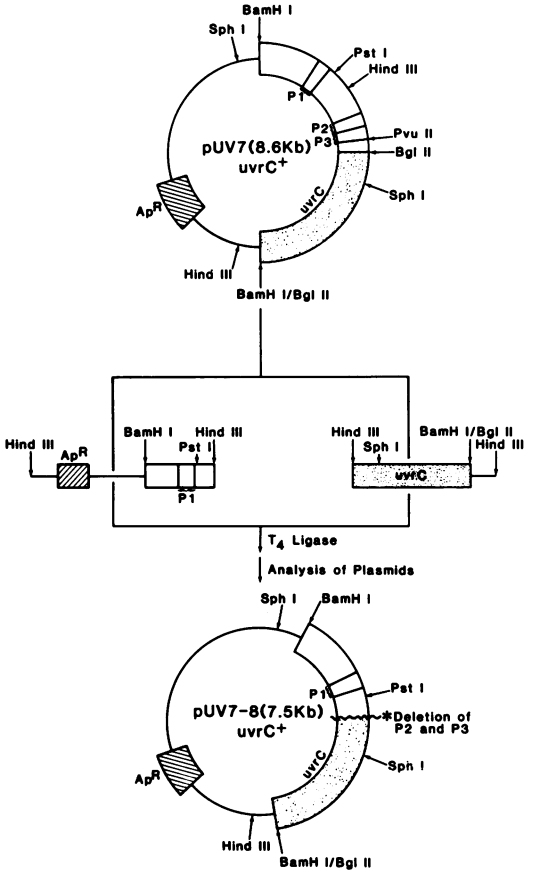

Figure 2. Strategy for deletion of P2 and P3 promoters. Relevant positions for P1, P2 and P3 promoters on plasmid pUV7 are indicated. pUV7 (15 µg) was linearized with Bgl II. The Bgl II site was converted into a Hind III site by Hind III linker ligation as described in Materials and Methods. The resulting plasmid was digested with excess Hind III and the fragments representing the uvrC structural gene (2.2 Kb) and P1 promoter (5.4 Kb), respectively, were isolated, ligated and used to transform the uvrC⁻ strain. Transformants were selected on ampicillin/L-broth agar plates and streak-tested for UV^R phenotype. The UV^R colonies were analyzed for plasmid size and structure. One of the UV^R plasmids, pUV7-8, is shown depicting deletion of the 0.9 Kb fragment containing the P2 and P3 promoters.

5.5 kb fragment contained pBR322 sequences and the P1 region, while the 2.2 kb fragment represented the structural gene without P2 and P3 regions. These two fragments were isolated, ligated with T4 ligase, and used to transform AB1884 (uvrC⁻). Ampicillin-resistant colonies were tested for UV^R phenotype. Plasmids isolated from UV^R colonies were analyzed for the presence of appropriate

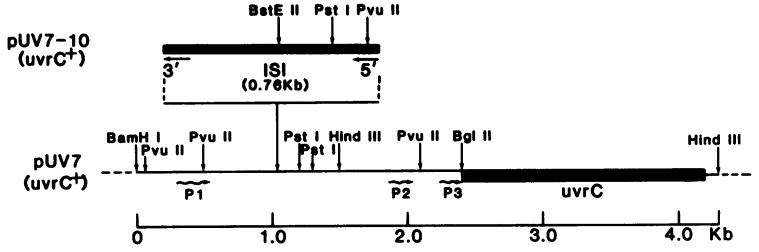

Figure 3. Structure of plasmid pUV7-10. The pUV7-10 DNA is represented linearized with respect to plasmid pUV7. pBR322 DNA sequences are represented by broken lines, the uvrC structural gene by the heavy line, and the approximate locations of P1, P2 and P3 promoters by wavy lines. The IS1 element is represented by heavy lines on top of the pUV7 sequences. The orientation was determined by cleavage at the unique Pst I and Pvu II sites in the IS1 element and in pUV7.

P1-uvrC gene fusions. Two such plasmids, pUV7-8 and pUV7-16, were further characterized and found to have the same structure, as shown in Fig. 2. These results confirm the requirement for a distal uvrC control region. The P1 promoter region lies at least 1.3 kb away from the 5' end of the uvrC gene on the basis of results presented in Fig. 1B. Our results indicate that P2 and P3 are not needed for uvrC gene function in the plasmid.

Spontaneous insertions in the P1 promoter regions. Transposons and insertion elements have been used to inactivate or activate genes. We have used an E. coli strain (AD10) which potentiates the insertion of foreign DNA fragments in the transfected plasmid DNA at high frequency (14). AD10 was transfected with plasmid pUV7, then plasmid DNA was isolated from individual colonies and analyzed for major restriction fragments. Plasmid pUV7 digested with Bam HI, Hind III and Bgl II produces four fragments: 1.5 kb (Bam HI-Hind III), 0.9 kb (Hind III-Bgl II), and 2.2 kb (Bgl II-Hind III) all chromosomal; pBR322, 4.05 kb (see Fig. 1A). Digestion of a plasmid isolated from an AD10 transformant (pUV7-10) with Bam HI, Hind III and Bgl II gives rise to a similar pattern except that the 1.5 kb fragment comigrates with the 2.2 kb fragment (data not shown), suggesting an insertion of 0.7-0.8 kb in the 1.5 kb fragment. According to the size and unique restriction sites, the insert is a IS1 insertion element.

To confirm and map the IS1 insertion in the 1.5 kb fragment, we have made use of unique Pst I and Pvu II sites in the IS1 element (Fig. 3). IS1 is inserted in "opposite" orientation downstream from the P1 promoter.

Fig. 4 shows quantitation of UV survival of AB1884 when transformed with

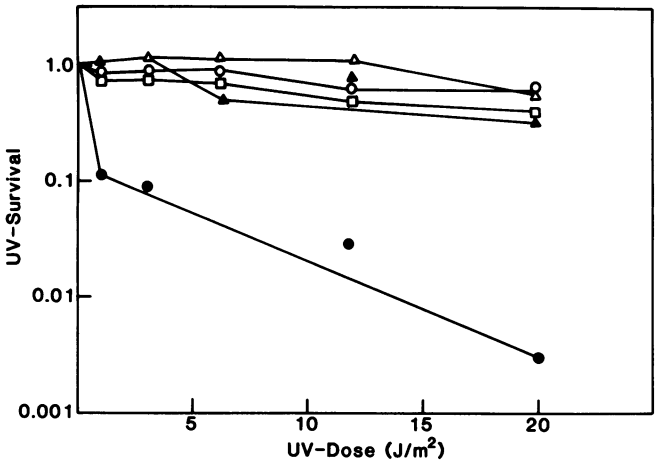

Figure 4. Effect of deletion and insertion on uvrC gene complementation. Wild-type and uvrC⁻ cells were grown to $8x10^8$ cells/ml in M9 medium, centrifuged and washed with M9 buffer. For quantitative UV survival, cells were resuspended in M9 buffer and subjected to various UV doses as described in Materials and Methods. The symbols represent: Δ, AB1157 wild-type/pBR322; ○, AB1884 uvrC⁻/pUV7; □, AB1884/pUV7-8; ▲, AB1884/pUV7-10; ●, AB1884/pBR322.

either insertion or deletion plasmids. Plasmid pUV7, pUV7-8, and pUV7-10 do not differ much in their proficiency for complementing the uvrC defect.

Effect of deletions and insertions on uvrC protein expression. We have previously reported that plasmid pUV7 does not promote synthesis of the uvrC protein to detectable levels in maxi-cells (12), although other uvrC plasmids do. Perhaps in the case of pUV7, regulatory factors might be responsible for low apparent synthesis of the uvrC protein. We tested uvrC protein synthesis directed by plasmids pUV7-8 and pUV7-10.

Fig. 5 shows the autoradiogram of proteins expressed by various uvrC⁺ plasmids under "maxi-cell" conditions. Our previous results have demonstrated that plasmid pUV201 (containing the uvrC structural gene fused to the tet promoter) codes for the uvrC protein (Mr ≃ 66,000, lane b). Plasmid pUV7 (lane c) failed to show any synthesis of the uvrC protein. Plasmid pUV7-8 behaved similarly to pUV7 in its apparent low synthesis of the uvrC protein. However, plasmid pUV7-10 (containing IS1) promoted efficient synthesis of the uvrC protein.

By-Pass of the uvrC Control by Insertion of the lac promoter

To increase synthesis of the uvrC protein and to further analyze the regulation of the uvrC gene, we have used the approach of inserting a strong

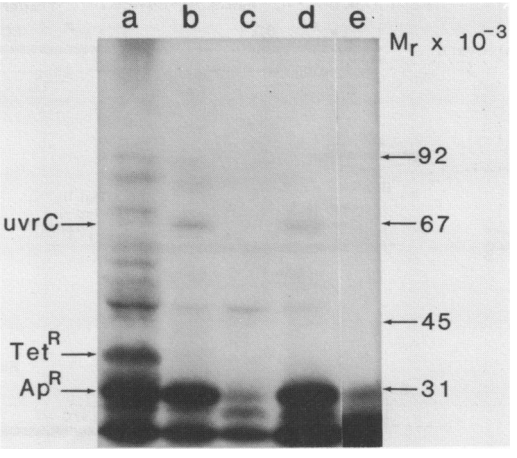

Figure 5. Expression of the uvrC protein by $uvrC^+$ plasmids. Host strain CSR603 transformed with various plasmids was processed for protein synthesis in maxi-cells as described (12). Proteins were labeled with ^{35}S-methionine (10 µCi/ml) and separated on 10% polyacrylamide/0.1% SDS gel. The labeled proteins were detected by fluorography. Lanes: a, CSR603/pBR322; b, CSR603/pUV201; c, CSR603/pUV7; d, CSR603/pUV7-10; e, CSR603/pUV7-8. Mr of standard protein markers: phosphorylase b, 92,000; bovine serum albumin, 67,000; ovalbumin, 45,000; carbonic anhydrase, 31,000.

promoter in front of the gene. We have made use of the lac promoter for the construction of lacP-uvrC fusions. A 285 bp DNA fragment which is flanked by Eco RI sites and harbors two 95 bp lac promoter fragments ligated through a 95 bp non-promoter fragment (A.R. Poteete, personal communication) was inserted at the 5' end of the uvrC gene. Both of the lac promoters in the 285 bp fragment direct transcription in the same direction (Fig. 6A). Each promoter fragment contains a Shine-Dalgarno (SD) sequence, a site for ribosome binding, but no initiator codon for the lacZ gene (20). Each promoter fragment contains the UV5 mutation which relieves the promoter of CAP dependence.

Fig. 6 shows the construction of the lacP-uvrC fusions. We have described a $uvrC^+$ plasmid, pUV201, that contains the smallest (1.9 kb) chromosomal fragment which we found to code for uvrC function, with synthesis of the uvrC protein under control of the tet promoter of the vector (7). We inserted the lac promoters into plasmids pUV201 and pUV7. Two derivative plasmids, pUV201-9 and pUV7-3, were further processed for uvrC protein overproduction.

Fig. 7 shows the autoradiogram of an SDS-polyacrylamide gel representing proteins encoded by lac promoter-uvrC hybrid plasmids. The uvrC protein is efficiently expressed by plasmid pUV201-9 and pUV7-3, as expected in the

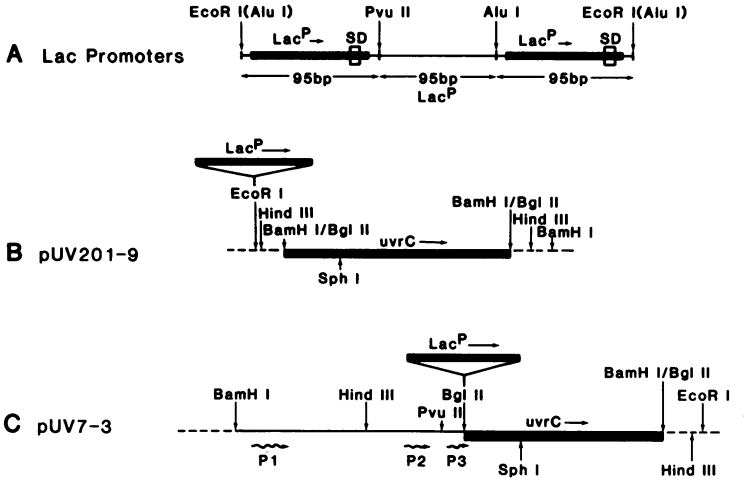

Figure 6. Structure of plasmids pUV201-9 and pUV7-3. (A) Schematic representation of the DNA fragment (285 bp) bearing two promoters. Each lac promoter fragment (96 bp), represented by the thick line, is a UV5 allele and contains an SD sequence but no initiation codon of the lacZ gene. The thin line represents a heterologous fragment (96 bp). (B) The Eco RI fragment (285 bp) was inserted into the Eco RI site of plasmid pUV201, which resulted in construction of plasmid pUV201-9. Thick and broken lines represent uvrC structural gene and pBR322 sequences respectively. (C) Plasmid pUV7-3 was constructed by blunt end ligation of the Eco RI fragment (285 bp) at the Bgl II site of plasmid pUV7. Relevant locations for P1, P2 and P3 promoters and the uvrC structural gene are indicated. The orientation of the Eco RI fragment in plasmids pVU201-9 and pUV7-3 was determined by size of fragments generated by Sph I and Pvu II.

favorable lac promoter-uvrC fusions. In the case of overproducers, there appear to be two bands for uvrC protein on 10% polyacrylamide gels. The lac-uvrC fusions complement the UV sensitivity of uvrC strains fully. The overproducing plasmids, pUV201-9 and pUV7-3, do not differ significantly from pUV7 in quantitative complementation. This argues that increased levels of uvrC protein are not needed for normal repair. Previously (7), we have used a low-copy number plasmid, pSC101, containing the uvrC gene for complementation, with the same results.

Maxi-cell analysis was done with 12.5% gels for pUV201 and pUV201-9. The intensities of the densitometer tracings of the resulting autoradiograms were standardized to the β-lactamase band. We estimate that the uvrC gene product is overproduced 100-fold over cellular background. We see a single band of protein at 66 kd on 12.5% gels (data not shown).

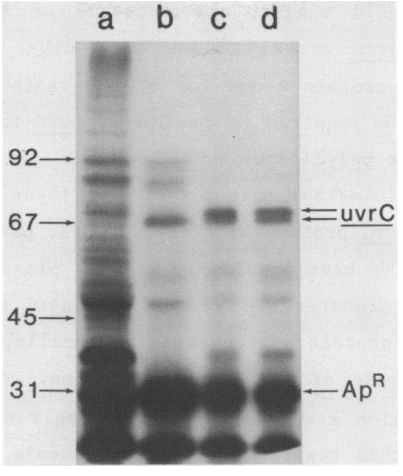

Figure 7. The ^{35}S-methionine labeled proteins encoded by plasmids containing the lac promoter-uvrC fusion. CSR603 was transformed with plasmids, and maxi-cells labeled with ^{35}S-methionine (10 μCi/ml) were prepared as described (12). Fifty μg of protein in cell extracts was analyzed on 10% polyacrylamide/0.1% SDS gel. The lanes represent: a, pBR322; b, pUV201; c, pUV201-9; d, pUV7-3. The standard protein markers are the same as described in the legend to Fig. 5. The arrows represent two bands seen in uvrC-complementing plasmids.

DISCUSSION

Although no quantitative analysis is available for the regulatory region(s) of the uvrC gene, our results indicate that the uvrC gene is preceded by three promoters. We have probed the putative promoter sites by studying the heparin-resistant RNA polymerase interaction sites on a 2.4 kb uvrC upstream region and have identified promoter regions P1, P2 and P3. These results have been compared with the biological characteristics of plasmids carrying fusions of the uvrC structural gene and fragments containing either P1, P2 or P3 promoters. We have already shown that a plasmid containing a fragment encompassing P2-P3 and the structural gene fails to express and complement the uvrC gene (12). However, plasmid pUV7-8, containing only the P1 promoter and the structural gene restores UV^R phenotype of uvrC$^-$ cells, as does a plasmid with all three promoters, pUV7. It is, therefore, probable that the in vivo control promoter for the uvrC gene is located in the P1 promoter region. This conclusion is also supported by preferential strong RNA polymerase binding to the P1 promoter region as compared to the P2 and P3 regions. If such in vitro RNA polymerase interactions with the P1 promoter and initiation of transcription are indicative of in vivo uvrC transcription

events, our results would suggest the presence of long transcripts which should code for the uvrC protein along with other protein(s). We have identified a 27,000-Mr protein coded for by the region 5' to the uvrC gene, which does not seem to be required to complement uvrC function (12). However, there is no evidence for polycistronic uvrC RNA.

To deduce the physiological role for each of these promoters, we examined the expression of the uvrC protein by plasmids pUV7, pUV7-16 and pUV7-10 under maxi-cell conditions. We have previously shown a plasmid containing the uvrC gene fused to the tet promoter can express the uvrC protein. Plasmid pUV7 failed to promote uvrC protein synthesis in maxi-cells, although this plasmid exhibited wild-type levels of uvrC complementation. The possibility exists that plasmid pUV7 contains a site(s) that negatively controls the regulation of the uvrC gene. In this regard, efficient synthesis of the uvrC protein by plasmid pUV7-10 is surprising. Plasmids pUV7-10 and pUV7 are structurally similar except that plasmid pUV7-10 has a 0.75 kb IS1 insertion downstream from the P1 promoter region.

IS1 insertion in gene operons have been shown to mainly inactivate genes by virtue of a polarity affect. Our results indicate that IS1 insertion can also activate genes. This could be explained by one of two models: (1) IS1 insertion could provide a stronger promoter region; (2) IS1 insertion could inactivate a negative control site, thus promoting transcription. The IS1 element has been shown to have one promoter region in normal orientation; however, transcription from this promoter is terminated within the IS1 element (21). In plasmid pUV7-10, the IS1 sequences are inserted in opposite orientation with respect to uvrC gene transcription. Thus, it appears unlikely that the IS1 promoter is responsible for promoting efficient synthesis of the uvrC protein in plasmid pUV7-10. In the second model, one could propose that there is a signal downstream from the P1 promoter region that is deleterious for uvrC transcription, and IS1 insertion disrupts this signal. Such an explanation has been given for activation of the cryptic bgl operon of E. coli by IS1 and IS5 insertions (22). We can not rule out the possibility that insertion of IS1 downstream from P1 provides an opportunity for either the P2 or P3 promoter to become activated.

We have also taken advantage of foreign promoter insertion to study regulation and overproduction of the uvrC protein. Insertion of a strong promoter at the 5'-end of the uvrC gene should help by-pass the normal regulation. Our results are consistent with this hypothesis as shown by the uvrC protein expression by plasmid pUV7-3, even in the presence of uvrC

upstream sequences, in contrast with plasmid pUV7.

Taken together, our data suggest that the uvrC gene has a complex regulatory structure. The uvrB gene has been shown to have three promoters, clustered at the 5'-end of the gene. At least two uvrB promoters are controlled by the recA-lexA system (23). The uvrA gene has been shown to be preceded by only one promoter, which is also controlled by the lexA-recA system.

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