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Expression of the Cloned *uvrB* Gene of *Escherichia coli*: Mode of Transcription and Orientation

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The *Escherichia coli uvrB* gene, located on a 1.5-megadalton *Eco*RI fragment F, derived from transducing phage λ*b2att*^{2} [λ*b2cI857intam6Δ*(*bioAB*)*bioFCD*$^+$*uvrB*$^+$], has been cloned in the unique *Eco*RI site of several "relaxed" plasmids, i.e., pMB9, pBR322, and pBH20 (=pBR322, including the *lac* regulatory elements [K. Itakura, T. Hirose, R. Crea, A. D. Riggs, H. L. Heyneker, F. Bolivar, and H. W. Boyer, Science **198**:1056-1063, 1977]). Expression of the *uvrB* gene, both on pMB9 and on pBH20, occurs only when fragment F has one particular orientation. Cloning of this fragment on pBR322 in either orientation does not allow expression of the *uvrB* gene. Transcription of this gene on pNP5 (=pMB9 *uvrB*) is shown to be dependent on a pMB9 promotor that is located on a 0.22-megadalton *Eco*RI-*Hin*dIII fragment. Using plasmid pBH20 as a vector, we could demonstrate that expression of the *uvrB* gene is under control of the *lac* promotor-operator region. From deoxyribonucleic acid-deoxyribonucleic acid hybridization experiments with λp*gal8* deoxyribonucleic acid and restriction fragments of pNP5 deoxyribonucleic acid it could be shown that the *uvrB* gene is transcribed clockwise on the chromosome.

The *Escherichia coli uvrB* gene product is implicated in initial step(s) of the excision repair process which restores the integrity of DNA after irradiation with UV light. Mutations in this locus lead to UV sensitivity of the bacterium, probably due to the lack of an endonuclease specific for UV-irradiated DNA (6, 7, 25, 26). Similar phenotypic properties of bacteria are observed when mutations are created in two other separate loci, namely the *uvrA* and *uvrC* genes (17, 31). Recently, it has been demonstrated by in vitro complementation that reconstitution of the UvrA$^+$, UvrB$^+$, and UvrC$^+$ gene products yield an ATP-dependent endonuclease specific for UV-irradiated DNA (25). The actual function of the separate components in this apparent complex has not been resolved yet.

The *uvrB* gene product might participate in other DNA-metabolizing processes as well. It has been shown that UvrB PolA double mutants are not viable, whereas UvrA PolA double mutants are (20). These observations suggest that the *uvrB* gene product is involved also in another pathway common to the enzyme, DNA polymerase I. These considerations prompted us to investigate the *uvrB* region in more detail, to obtain more information about the genetic constitution of this locus. Furthermore, such details could enable us to elucidate the mode of synthesis of the *uvrB* gene product, including its genetic regulation.

Recombinant DNA technology has greatly facilitated the possibilities of studying particular genetic entities, both of procaryotic (9) and of eucaryotic origin (8). We have also employed this technique to clone the *uvrB* gene (23). For that purpose we have derived the *uvrB* gene from the transducing phage λ*b2att*^{2} (λ*b2cI857intam6* (*bioAB*)*bioFCD*$^+$*uvrB*$^+$). To study the genetic constitution of this region and its regulation, it is essential that this phage contains an intact *uvrB* gene, including its regulatory functions. This requirement is fulfilled, since the *uvrB* gene on the phage can be expressed under conditions that exclude interference of phage-regulatory elements (22, 23). In these studies we have also shown that the *uvrB* gene, when cloned in plasmids like pMB9 and introduced into UvrB strains, renders the bacteria Uv^r.

In this paper we report on the mode of expression of the cloned *uvrB* gene and we discuss the possibility that the *uvrB* gene is part of a more comprehensive genetic unit.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains that we employed are listed in Table 1. Other strains are described in the preceding paper (22). Strains containing the multicopy plasmids pMB9, pBR322, and pBH20 were obtained from H. L. Heyneker. The characteristics of these plasmids and of those constructed in this work are given in Table 2.

TABLE 1. *Bacterial strains of E. coli K-12*

Strain	Relevant genotype	Origin
RR1-322	r_K m_K containing plasmid pBR322	H. L. Heyneker
RR1-20	r_K m_K containing plasmid pBH20	H. L. Heyneker
GMI/XAC	F'*L8 lacI*^{q} Lac$^+$ Pro$^+$ Δ(*lac-pro*) *rif*^{r}	J. H. Miller via B. W. Glickman
HP3448	*sup-6* Δ*uvrB261* containing plasmid pNP6	This work and ref. 22
HP3460	*sup*$^+$ Δ*uvrB261* containing plasmid pNP7	This work
HP3461	HP3460 containing F'*L8 lacI*^{q} Lac$^+$ Pro$^+$	This work
HP3462	*sup-6* Δ*uvrB261* containing plasmid pNP8	This work
HP3463	*sup-6* Δ*uvrB261* containing plasmid pNP9	This work

TABLE 2. *Plasmids employed or constructed*

Plasmids	Characteristics	Origin
pMB9	Tc^r	Rodriguez et al. (24)
pBR322	Tc^r Ap^r	Bolivar et al. (5)
pBH20	pBR322, including *lacP*$^+$*O*$^+$	Itakura et al. (18)
pNP5	pMB9; *Eco*RI fragment F inserted containing *uvrB* gene	Our work (23)
pNP6	pMB9; fragment F inserted in opposite orientation, compared with pNP5	This work
pNP7	pBH20; *uvrB* on fragment F under control of *lacP*$^+$*O*$^+$	This work
pNP8	pBR322; inserted *Eco*RI-*Hin*dIII fragment (0.22 Mdal) of pMB9 and fragment F	This work
pNP9	pBR322; fragment F containing *uvrB* inserted	This work

Measurement of UV survival of bacteria. The determination of the survival of bacteria after irradiation with different doses of UV light is outlined in the preceding paper (22). A rapid analysis of the UV resistance of single colonies was done by streaking on L-broth agar plates (in some cases supplemented with either 50 μg of ampicillin or 20 μg of tetracycline per ml), followed by irradiation of a segment of these plates with a UV dose of 125 to 500 ergs/mm^2.

Preparation of plasmid DNA. Plasmid DNA was prepared by first amplifying L-broth-grown cultures by the addition of 150 μg of chloramphenicol per ml during logarithmic phase of growth and a further incubation of 4 to 6 h at 37°C (10). Extraction and purification of plasmid DNA was achieved by a cleared lysate technique described by Meagher et al. (19), followed by dye-buoyant density centrifugation in a cesium chloride-propidium diiodide gradient as outlined by Bolivar et al. (4). Cleared lysates are sufficiently pure for an analysis with restriction endonucleases; however, in those incubations RNase A was added to a final concentration of 20 μg/ml, to prevent sticking of proteins to RNA.

Digestion of DNA with restriction endonucleases. Incubations of restriction endonucleases with plasmid or phage DNA (50 μg/ml) were carried out for 1 h at 37°C with a two- to threefold excess of enzyme over DNA (2 to 3 units/μg of DNA). Various buffers were used with different restriction endonucleases, notably, for *Eco*RI: 0.1 M Tris-hydrochloride (pH 7.5)–0.01 M $MgCl_2$–0.05 M NaCl; for *Bam*HI: 0.1 M Tris-hydrochloride (pH 7.5)–0.01 M $MgCl_2$; for *Hin*dIII, *Bst*EII, *Hin*dII, and *Pst*I: 6 mM Tris-hydrochloride (pH 7.5)–6 mM $MgCl_2$–6 mM β-mercaptoethanol; for *Sal*I: 6 mM Tris-hydrochloride (pH 7.9)–6 mM $MgCl_2$–6 mM β-mercaptoethanol–0.1 M NaCl. Digestions were arrested by heating for 5 min at 65°C, followed by quenching in ice. Samples to be analyzed were made 3 to 5% (vol/vol) Ficoll and submitted directly to electrophoresis on 0.8% agarose (15) or on 5% polyacrylamide slab gels (4).

Ligation of restriction fragments and transformation of competent cells. DNA preparations digested with restriction endonucleases were extracted with phenol (saturated with 50 mM Tris-hydrochloride [pH 7.5]–1 mM EDTA). Excess of phenol in the aqueous layer was removed by two successive extractions with chloroform. Then chloroform was removed with a stream of dry nitrogen. Finally, the DNA preparation was dialyzed against 10 mM Tris-hydrochloride (pH 7.5)–0.1 mM EDTA. Ligation of fragments having "cohesive ends" was carried out for 16 h at 14°C in 0.05 M Tris-hydrochloride (pH 7.5), 0.01 M $MgCl_2$, 0.2 mM ATP, 0.01 M dithiothreitol, 0.1 mM EDTA, T4-induced DNA ligase (5 to 10 units/ml), and 7 to 10 nM *Eco*RI or *Hin*dIII termini of digested DNA. After ligation, the mixture was again extracted with phenol and chloroform as described above and finally dialyzed against 2 mM Tris-hydrochloride (pH 7.2)–0.1 mM EDTA. $CaCl_2$ was added to a final concentration of 30 mM.

Transformation of competent cells and selection of transformed bacteria with the desired phenotype were done as previously described (23).

DNA-DNA hybridizations. Plasmid pNP5 DNA was labeled in vivo with [^{3}H]thymidine as outlined in a previous paper (11). ^{3}H-labeled pNP5 DNA (1.2 μg, corresponding to 3.7×10^5 cpm) was digested with both *Eco*RI and *Bst*EII. The resulting fragments were separated by electrophoresis on a 1% agarose slab gel. *Bst*EII-*Eco*RI fragments of 0.68 megadaltons (Mdal) and 0.81 Mdal were extracted from the gel, according

to the hydroxyapatite technique of Tabak and Flavell (30).

A 60-μg quantity of each of the separated strands of λ*pgal8* or λ*cb2* DNA (a gift from W. F. Stevens) was loaded by filtration onto Schleicher and Schüll nitrocellulose membranes (0.45-μm; diameter, 40 mm). Single-stranded DNA was immobilized by baking the filters for 4 h at 80°C under vacuum. Small filters (diameter, 12 mm; loaded with approximately 5 μg of each strand) were punched out and used for hybridization. For that purpose isolated ^{3}H-labeled fragments of 0.68 and 0.81 Mdal were sonicated extensively and subsequently denatured in 0.25 N NaOH for 10 min at 0°C. The solutions were quickly neutralized with 0.5 N HCl-0.1 M Tris-hydrochloride (pH 7.6). Samples of denatured ^{3}H-labeled fragments (12.6 to 15.6 ng; 3,800 to 4,700 cpm), in 0.5 ml of 50% (vol/vol) formamide-2× SSC (SSC equals 0.15 M NaCl-0.015 M sodium citrate)-10 mM Tris-hydrochloride (pH 7.5)-1 mM EDTA, were added to the filters and hybridized for 80 h at 40°C. Filters were successively washed with 20 ml of hybridization buffer, 30 ml of 2× SSC, 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, and 20 ml of ethanol, and finally counted in a xylene-based scintillation liquid (Lumac).

Enzymes. Restriction endonucleases *Eco*RI, *Hin*dIII, and *Bam*HI were purchased from Miles Laboratories Inc. (Elkhart, Ind.). *Sal*I was from BioLabs (Beverly, Mass.). The enzymes *Pst*I, T4 DNA ligase (1,000 units/ml), and *Bst*EII were kindly provided by H. L. Heyneker (this laboratory). *Hin*dII was donated by F. van Mansveld (State University, Utrecht).

RESULTS

Orientation of the cloned *uvrB* gene on pMB9. We have previously reported on the construction of a recombinant plasmid, named pNP5 (molecular weight 5.1 Mdal), which consists of the plasmid pMB9 (molecular weight 3.6 Mdal) carrying a determinant coding for resistance against the antibiotic tetracycline (Tc^r) and an *Eco*RI fragment F of phage λ*b2att*^{2} (23). This restriction fragment, containing the *uvrB* gene, has been inserted in the unique *Eco*RI site of pMB9. Strains that have a chromosomal *uvrB* deletion become Uv^r Tc^r upon transformation with plasmid pNP5, provided that an amber suppressor is present (22, 23).

We have isolated plasmid DNA from eight independent Uv^r Tc^r transformants obtained from the original cloning experiment (23) and analyzed their composition after digestion with restriction enzymes. The enzymes *Hin*dIII and *Bst*EII are particularly suited for our purpose to determine the orientation of fragment F on these plasmids. Both pNP5 DNA and pMB9 DNA have only one *Hin*dIII site, i.e., within the promotor of the Tc^r determinant. *Bst*EII cleaves the *Eco*RI fragment F once, yielding fragments of 0.68 and 0.81 Mdal, whereas pMB9 is cleaved at a site 2.1 Mdal apart from the *Eco*RI site and 1.7 Mdal from the *Bam*I site (Fig. 1). Digestion of pNP5 DNA with both *Bst*EII and *Hin*dIII should result in fragments either of 2.26, 1.90, and 0.90 Mdal or of 2.13, 1.90, and 1.03 Mdal. Surprisingly, the restriction patterns of the plasmid DNA preparations of all different clones were identical. In all cases, a *Bst*EII-*Hin*dIII fragment of 0.90 Mdal was found (see lane 5 of Fig. 2); a fragment of 1.03 Mdal was not detected. These results suggest that for the *uvrB* gene to be expressed fragment F must be inserted in a specific orientation.

To verify this presumption we have inverted the orientation of fragment F, and thus of the *uvrB* gene, on vector pMB9, to determine whether UV resistance is obligatorily coupled to the orientation of the *uvrB* gene. To accomplish inversion of fragment F, pNP5 DNA was digested with *Eco*RI and subsequently religated. Competent cells of strain HP3435 *sup-6* Δ*uvrB* (22) were transformed with the ligation mixture, and transformants were selected on the basis of their Tc^r phenotype. Cleared lysates of 10 transformants were subjected to digestion with both *Bst*EII and *Hin*dIII. We found four transformants which contained plasmid DNA, denoted pNP6, consisting of pMB9 and fragment F, but this fragment had now been inserted in the opposite orientation as compared to pNP5 (Fig. 2). Digestion of either pNP5 or pNP6 with

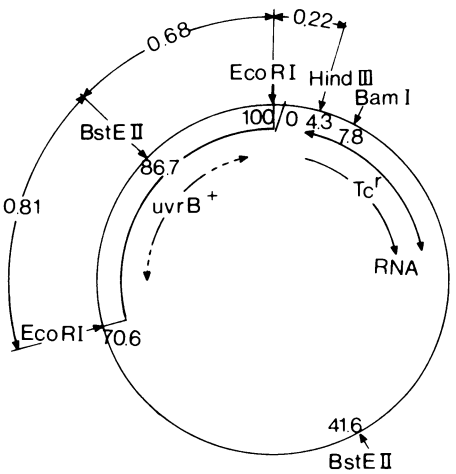

FIG. 1. *Diagrammatic representation of the structure of plasmid pNP5. pNP5 DNA contains unique restriction sites for the enzymes HindIII and BamHI. The enzymes EcoRI and BstEII have two restriction sites. These sites are given in percentages of the molecular weight of pNP5 DNA (5.1 Mdal). The outer circle represents the molecular weight (in Mdal) of some relevant fragments. An inner circle shows the direction of transcription of the Tc^r determinant.*

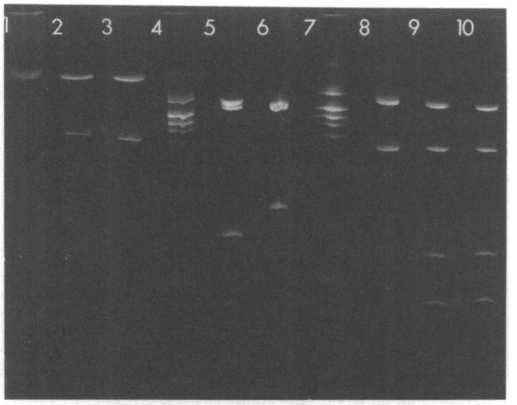

FIG. 2. *Restriction endonuclease analyses of pNP5 and pNP6 DNA. Molecular weight estimates are based on seven pBR322 fragments generated by, respectively, EcoRI (2.88 Mdal), HindII (2.15 Mdal, 0.73 Mdal), PstI and HindIII (2.36 Mdal, 0.52 Mdal), and PstI and SalI (1.96 Mdal, 0.93 Mdal), plus two pNP5 fragments generated by EcoRI (3.6 Mdal, 1.5 Mdal). A mixture of nonequimolar amounts of these fragments is visualized in lanes 4 and 7. Lane 1, pMB9 digested with EcoRI (3.6 Mdal). Lanes 2 and 3, respectively, pNP5 and pNP6 digested with EcoRI (3.6, 1.5 Mdal). Lane 5, pNP5 digested with BstEII and HindIII (2.26, 1.90, 0.90 Mdal). Lane 6, pNP6 digested with BstEII and HindIII (2.13, 1.90, 1.03 Mdal). Lane 8, pMB9 digested with EcoRI and BstEII (2.1, 1.5 Mdal). Lanes 9 and 10, respectively, pNP5 and pNP6 digested with EcoRI and BstEII (2.1, 1.5, 0.81, 0.68 Mdal). Electrophoresis on a 5% polyacrylamide slab gel was performed as described (4).*

*Eco*RI (Fig. 2, lanes 2 and 3) or with *Eco*RI and *Bst*EII (lanes 9 and 10) showed that these plasmids contain the same components. However, the restriction patterns of pNP5 and pNP6 markedly differ after an incubation with *Bst*EII and *Hin*dIII (lanes 5 and 6). pNP5 yields fragments of 2.26, 1.90, and 0.90 Mdal, whereas pNP6 yields fragments of 2.13, 1.90, and 1.03 Mdal. From these results we conclude that fragment F has been inverted on pNP6 as compared to the orientation on pNP5.

Strain HP3435 was transformed with pNP6 DNA to allow a comparison between the UV resistance of strain HP 3448 [= HP3435(pNP6)] and that of strain HP3442 [= HP3435(pNP5)]. The UV survival curves (Fig. 3) show that HP3442 is equally as resistant to irradiation with UV light as the isogenic untransformed UvrB$^+$ strain XA106. However, strain HP3448 carrying pNP6 is only slightly more UV resistant than the untransformed UvrB strain HP3435. Our data clearly demonstrate that UV resistance of UvrB strains transformed with pNP5-like plasmids is strictly coupled to the orientation of the *uvrB* gene on the vector pMB9. Moreover, our data suggest that expression of the cloned *uvrB* gene is a result of read-through transcription initiated at a promotor on pMB9. Alternatively, transcription started at a site on pMB9 could interfere with *uvrB* transcription started at its own promotor, especially when the direction of transcription is opposite to that of the pMB9-initiated RNA synthesis. To discriminate between these two options we have attempted to link the same restriction fragment F to a well-defined promotor, i.e., the *lac* promotor located on plasmid pBH20 (18). We expect that *uvrB* gene expression will be controlled by the *lac* regulatory elements. Furthermore, we have cloned fragment F within the unique *Eco*RI site of plasmid pBR322 (5). In this case no vector-promotor is available that could affect the expression of the cloned *uvrB* gene, since the region between the Ap^r and the Tc^r determinants, which contains the *Eco*RI site, does not harbor a promotor (29).

lac-promoted transcription of the cloned *uvrB* gene. Plasmid pBH20 has been developed to permit *lac*-promoted expression of a cloned eucaryotic gene (18). For that purpose a *Hae*III restriction fragment (203 base pairs) of λp*lac*5 DNA was employed which contains the *lac* pro-

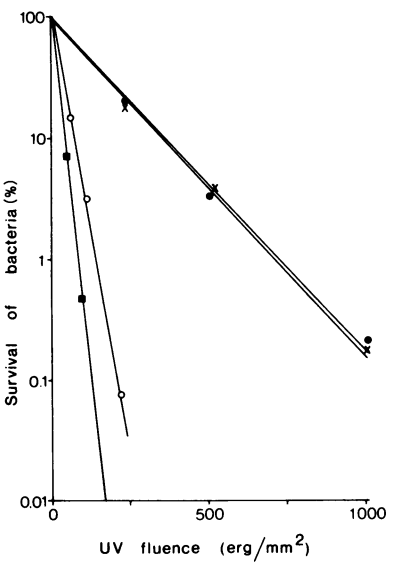

FIG. 3. *UV survival of UvrB strains transformed with pNP5 and pNP6. Strains HP3435 sup-6 ΔuvrB, HP3442 [=HP3435(pNP5)], HP3448 [=HP3435-(pNP6)], and XA106 (UvrB$^+$) were grown to the exponential phase, and the survival of bacteria after irradiation with UV light was as outlined before (23). Symbols:* (×) *XA106;* (●) *HP3442;* (○) *HP3448;* (■) *HP3435.*

motor-operator region and 24 base pairs coding for the first eight amino acid residues of β-galactosidase. This fragment was inserted in the *Eco*RI site of pBR322; hence, this DNA segment was flanked by *Eco*RI sites. Subsequently, the *Eco*RI site ahead of the *lac* promotor was removed while the other one remained as a cloning site for *Eco*RI fragments.

We have cloned the *uvrB*-containing fragment F in the *Eco*RI site of pBH20. To achieve this, both pBH20 and pNP5 DNA were digested with *Eco*RI and ligated, and the mixture was administered to competent cells of strain HP 3430 *sup*$^+$ Δ*uvrB*. After transformation we selected for Ap^r Tc^r Uv^r clones. Such clones were investigated in more detail to determine whether they contain recombinant plasmid DNA and whether the expression of the *uvrB* gene was entirely under control of the *lac* regulatory elements. *lac*-promoted expression should be abolished when sufficient *lac* repressor (*lacI* gene product) is available in the cell to prevent transcription initiated at the *lac* promotor. Therefore, the episome F′*lacI*^{q} Lac$^+$ Pro$^+$ from donor strain GMI/XAC, which produces about 10-fold more *lac* repressor than the Lac$^+$ wild-type strains, was crossed into strain HP3460 [HP3430(pBH20 *uvrB*)]. We used an overproducing *lacI* allele (*lacI*^{q}) to bind all the *lac* operator copies present in cells containing a derivative of the multicopy number plasmid pBH20.

The UV survival curves of the sexductant HP3461, the recipient HP3460, and the isogenic *uvrB* deletion strain HP3430 were determined (Fig. 4). Strain HP3460 is clearly more UV resistant than the untransformed strain HP3430. Furthermore, our data show that the presence of the *lac* repressor largely prevents the expression of the *uvrB* gene, since strain HP3461 containing F′*lacI*^{q} is about 10-fold more UV sensitive than the recipient strain HP3460. Repression can be relieved by growing strain HP3461 in a medium containing 1 mM isopropyl-β-D-thiogalactopyranoside, an inducer of the *lac* system (results not shown). We conclude that the expression of the apparently cloned *uvrB* gene on plasmid pBH20 is largely dependent on transcription initiated at the *lac* promotor.

To verify whether indeed a recombinant plasmid carrying the *uvrB* gene (designated pNP7) is present in strain HP3460, we analyzed a cleared lysate with restriction enzymes. Also, we determined the orientation of fragment F versus the *lac* promotor (Fig. 5). Again, we employed the enzyme *Bst*EII in combination with others, i.e. *Hin*dIII and *Bam*HI, to establish the composition of plasmid DNAs. As outlined before, fragment F is cleaved once by *Bst*EII, but plasmid pBH20 is not cut by this enzyme. pBH20

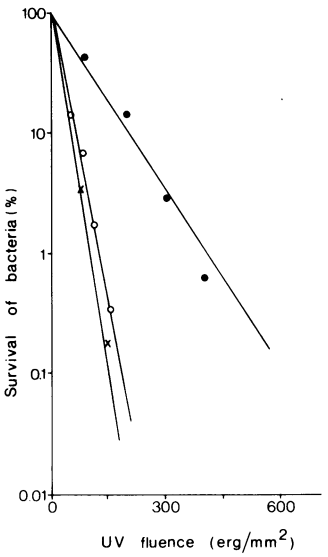

FIG. 4. *UV survival of UvrB strains transformed with pNP7 DNA. Strain HP3460 ΔuvrB Δ(lac-pro) rpsL(pNP7) was crossed with strain GMI/XAC F′L8 lacI^q Lac$^+$ Δ(lac-pro). Sexductants (HP3461) were selected on minimal medium agar plates, containing 100 μg of streptomycin per ml and without proline. Strains HP3430 ΔuvrB, HP3460 [=HP3430(pNP7)], and HP3461 [=HP3460 F′ L8 lacI^q Lac$^+$ Pro$^+$] were grown to the exponential phase, and the UV survival of the bacteria was determined as described before (23). Symbols:* (×) *HP3430;* (●) *HP3460;* (○) *HP3461.*

harbors unique restriction sites for *Hin*dIII and *Bam*HI within the Tc^r determinant (distance between these sites is 0.18 Mdal [13]). Plasmid DNA prepared from strain HP3460 (=pNP7) was digested either with *Bst*EII and *Bam*HI (Fig. 5, lane 2) or with *Bst*EII and *Hin*dIII (lane 3). The first incubation yields fragments of 3.4 and 1.00 Mdal (=0.81 + 0.18), whereas the latter gives fragments of 3.55 and 0.83 Mdal. Since the *Eco*RI and *Hin*dIII of pBH20 are very close (31 base pairs apart [14]), this segment can be disregarded in our calculations.

From these results it is obvious that the *Bst*EII-*Eco*RI fragment of 0.68 Mdal (see also Fig. 2) is adjacent to the *lac* region, whereas the larger *Bst*EII-*Eco*RI fragment of 0.81 Mdal is adjacent to the Tc^r determinant. Our data allow the conclusion that, when the *uvrB* gene occupies most of fragment F, the 0.68-Mdal fragment is transcribed before the 0.81-Mdal fragment. Furthermore, we can conclude that the orientation of fragment F relative to the Tc^r determinant on pNP7 is opposite to that on pNP5 (see Fig. 1 and Fig. 5B).

Another conclusion that we can draw from these experiments is that transcription of the *uvrB* gene proceeds via a "foreign" promotor.

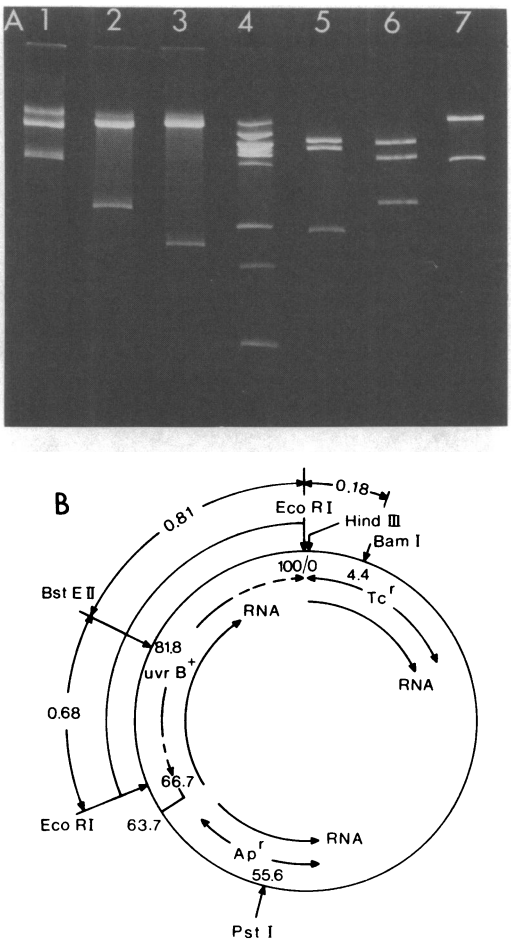

FIG. 5. *(A) Restriction endonuclease analysis of pNP7 DNA. Molecular weight calculations were based on the standards given in the legend of Fig. 2, namely 3.6, 2.36, 2.15, 1.96, 1.5, 0.93, 0.73, and 0.52 Mdal. Lane 1, pNP7 digested with EcoRI (3.0, 1.5 Mdal). Lane 2, pNP7 digested with BstEII and BamHI (3.4, 1.0 Mdal). Lane 3, pNP7 digested with BstEII and HindIII (3.55, 0.83 Mdal). Lane 4, marker DNA fragments. Lane 5, pNP5 digested with BstEII and HindIII (2.26, 1.90, 0.90 Mdal). Lane 6, pNP5 digested with BstEII and BamHI (2.26, 1.72, 1.08 Mdal). Lane 7, pNP5 digested with EcoRI (3.6, 1.5 Mdal). Electrophoresis on a 5% polyacrylamide slab gel was done as described (4). (B) Diagrammatic representation of the structure of plasmid pNP7. pNP7 contains unique restriction sites for the enzymes HindIII, BamI, PstI, and BstEII. EcoRI cleaves pNP7 twice. Restriction sites are given as percentages of the molecular weight of pNP7 DNA (4.5 Mdal). The outer circle represents the molecular weight of some relevant fragments. An inner circle shows the direction of transcription of the Tc^r and the Ap^r determinants and of the uvrB gene. The region between coordinates 63.7 and 66.7 consists of the lac promotor-operator. lac-promoted transcription proceeds clockwise on the map of pNP7.*

Consequently, we must reject the option made in the previous paragraph that, on pNP5 DNA, transcription initiated on a pMB9 promotor might interfere with autonomous *uvrB* mRNA synthesis. Instead, we propose that transcription of the *uvrB* gene on pNP5 is initiated on a pMB9 promotor, provided this gene is inserted in the correct orientation. In the next section we have localized the position of this unknown promotor.

Assignment of a promotor function to the 0.22-Mdal fragment. Rodriguez et al. (24) have shown that the region between the *Eco*RI and *Hind*III sites of the vectors pMB9 and pBR322 is entirely different. This segment of pMB9 extends about 350 base pairs, whereas on pBR322 only 31 base pairs separates these sites (29). As will be shown below, the stretch of 350 base pairs (molecular weight 0.22 Mdal) of plasmid pMB9 is vital for the expression of the *uvrB* gene on pNP5. Two arguments can be advanced to support this notion. First, cloning of fragment F on pBR322, in either orientation, does not result in UV resistance of transformed UvrB strains (Fig. 6). This observation is in accordance with the fact that the region between the Ap^r and Tc^r promotors of pBR322 does not contain a promotor at which the transcription of the cloned *uvrB* gene starts (29). Second, the comparison of the direction of transcription of the

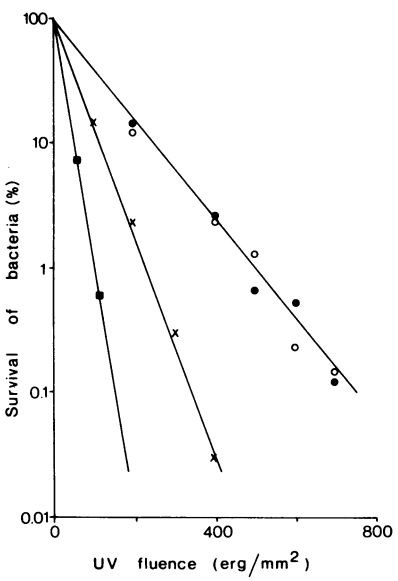

FIG. 6. *UV survival of UvrB strains transformed with either pNP5, pNP8, or pNP9 DNA. Strains HP3442 [=HP3435(pNP5)], HP3462 [=HP3435-(pNP8)], and HP3463 [=HP3435(pNP9)] were grown to the exponential phase, and the survival of the bacteria after irradiation with UV light was as outlined before (11). Symbols:* (○) *HP3442;* (●) *HP3462;* (×) *HP3463;* (■) *HP3435.*

uvrB gene on pNP7 (=pBH20 *uvrB*) with that on pNP5 indicated that *uvrB* mRNA synthesis proceeds counter-clockwise on the map of the latter plasmid (see Fig. 1). Hence, transcription should start between the *Eco*RI site (0%) and the Tc^r promotor (the Tc^r determinant is transcribed clockwise on this map) (29). To demonstrate that transcription of the *uvrB* gene on pNP5 is initiated on the 0.22-Mdal *Eco*RI-*Hin*dIII fragment of pMB9, we have cloned this fragment and fragment F on pBR322. Toward this objective, we digested both pNP5 and pBR322 DNA with *Eco*RI and *Hin*dIII, yielding fragments of 0.22, 3.3, 1.5, 2.8, and 0.02 Mdal. After ligation, UvrB cells were transformed and selection was for Ap^r Tc^r Uv^r clones. Selection of the Ap^r character guaranteed the presence of plasmid pBR322, and restoration of the Tc^r character meant that either the 0.22-Mdal or the 0.02-Mdal fragment had been inserted. Plasmid DNA was prepared from an Ap^r Tc^r Uv^r clone and analyzed with restriction enzymes (Fig. 7). This DNA, denoted pNP8, was cleaved with *Eco*RI and *Hin*dIII, and the restriction pattern was compared with those of pBR322 and pNP5 DNAs (Fig. 7, lanes 1, 2, and 3). It can be seen that pNP8 harbors the vector pBR322 (molecular weight 2.8 Mdal), fragment F (1.5 Mdal), and the small fragment of 0.22 Mdal, derived from pMB9. Inclusion of the 0.22-Mdal fragment is further illustrated after digestion of pNP5 and pNP8 with both *Bst*EII and *Hin*dIII (Fig. 7, lanes 8 and 9), resulting in the formation of a fragment with a molecular weight of 0.90 Mdal (=0.68 + 0.22 Mdal). Thus, fragment F is inserted in the same orientation on pNP8 as on pNP5. For a control experiment we also isolated plasmid DNA, denoted pNP9, from a clone which is phenotypically Ap^r Tc^r Uv^s. This plasmid lacks the 0.22-Mdal fragment (compare Fig. 7, lanes 5 and 6) and is composed of pBR322 DNA and fragment F. Apparently, the Tc^r locus of pNP9 was restored by insertion of the minute 0.02-Mdal *Eco*RI-*Hin*dIII fragment derived from pBR322.

The UV survival curves of strain HP3435 transformed with either pNP5 or pNP8 show that these strains are equally resistant to irradiation with UV light (Fig. 6). A transformant containing pNP9 DNA (lacking the 0.22-Mdal fragment) clearly is less UV resistant than pNP5- or pNP8-containing strains. Data shown in Fig. 6 and 7 demonstrate that the foreign promotor, which is required for expression of the *uvrB* gene on pNP5 DNA, is located on the 0.22-Mdal *Eco*RI-*Hin*dIII fragment of plasmid pMB9.

Direction of *uvrB* mRNA synthesis on the *E. coli* chromosome. We have discussed in a previous paper (23) that, due to the aberrant mode of excision of λ*b2int* prophages, transducing particles (λ*att*[2] phages) are generated which carry bacterial DNA from both sides of the attachment λ site. Actually, the segment to be packaged is located between the site X_r (to the right of *uvrB*) and the site X_l (within the gene

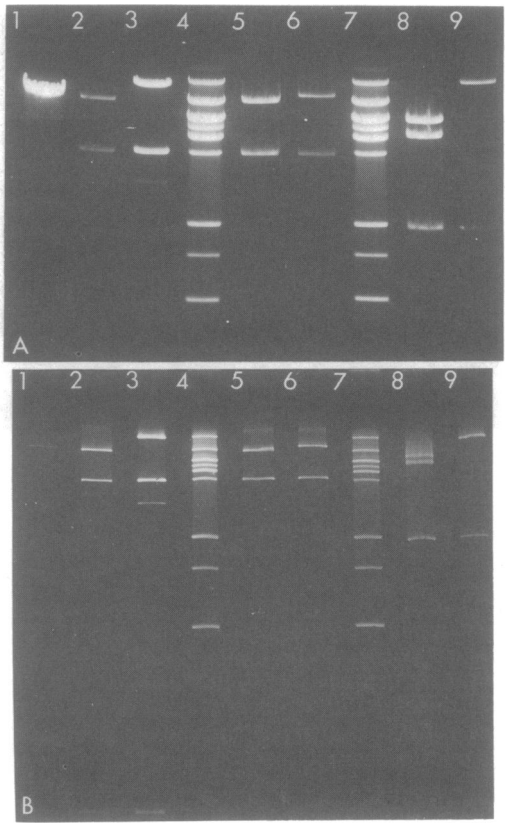

FIG. 7. *Restriction endonuclease analyses of pNP8 and pNP9 DNA. Molecular weight estimates were again based on the standards given in Fig. 2, being 3.6, 2.36, 2.15, 1.96, 1.5, 0.93, 0.73, and 0.52 Mdal. Lane 1, pBR322 digested with EcoRI and HindIII (2.85 Mdal, 0.02 Mdal [not visible]). Lane 2, pNP8 digested with EcoRI and HindIII (2.85, 1.5, 0.22 Mdal). Lane 3, pNP5 digested with EcoRI and HindIII (3.4, 1.5, 0.22 Mdal). Lane 4, marker DNA fragments. Lane 5, pNP9 digested with EcoRI (2.88, 1.5 Mdal). Lane 6, pNP8 digested with EcoRI (3.0, 1.5 Mdal). Lane 7, marker DNA fragments. Lane 8, pNP5 digested with BstEII and HindIII (2.26, 1.90, 0.90 Mdal). Lane 9, pNP8 digested with BstEII and HindIII (3.7, 0.90 Mdal). Electrophoresis was done both on a 0.8% agarose slab gel (A) to separate the larger fragments and on a 5% polyacrylamide slab gel (B) to visualize the 0.22-Mdal fragment.*

pgl). We have shown (23) that the sequence of loci on the physical map of λ*b2att*[2] is *-bio-uvrB-*X_r/X_l*-pgl-*. Restriction enzyme *Eco*RI cleaves between *bio* and *uvrB* and also within the *pgl* segment (*pgl*), yielding fragment F. The segments *Eco*RI-*uvrB*-X_r/X_l and X_r/X_l-*pgl-Eco*RI can be dissected by using *Bst*EII, which cleaves the *Eco*RI fragment F in two parts, i.e., 0.68 and 0.81 Mdal. The segment X_r/X_l-*pgl-Eco*RI is part of either the 0.68-Mdal or of the 0.81-Mdal fragment. Consequently, only that fragment which contains the segment X_r/X_l-*pgl* hybridizes with λp*gal*8 DNA, which contains bacterial DNA originating only from the *gal* side of the attachment λ site on the chromosome. For that purpose we performed DNA-DNA hybridization experiments with isolated ^{3}H-labeled 0.68- and 0.81-Mdal *Bst*EII-*Eco*RI restriction fragments and either λp*gal*8 DNA or λ*cb*2 DNA (Table 3). Our results show that only the 0.81-Mdal fragment hybridizes with λp*gal*8 DNA. Therefore, the 0.81-Mdal fragment harbors the segment X_r/X_l-*pgl-Eco*RI, derived from the *gal* side.

Based on these data we can deduce the direction of transcription of the *uvrB* gene on the *E. coli* chromosome. First, we have concluded in a previous paragraph that transcription of the *uvrB* gene on the pNP5 initiates on the 0.22-Mdal *Eco*RI-*Hin*dIII fragment of pMB9 and proceeds counter-clockwise on the map of this plasmid (see Fig. 1). Second, because the *uvrB* gene comprises most of fragment F, the 0.68-Mdal fragment is transcribed before the 0.81-Mdal fragment. The experiments reported with plasmid pNP7 (=pBH20 *uvrB*) support this conclusion. Third, an *Eco*RI site separates the *bio* genes from the *uvrB* gene; this site corresponds with the *Eco*RI site arbitrarily situated at 0% on the map of pNP5 (Fig. 1). From these arguments, we can conclude that the direction of transcription of the *uvrB* gene is from *bio* to X_r (or to *chlA*), i.e., clockwise on the chromosome.

TABLE 3. *Hybridization of ^{3}H-labeled fragments of pNP5 DNA, digested with BstEII and EcoRI, with λpgal8 DNA and λcb2 DNA*[a]

*Bst*EII-*Eco*RI fragment	Hybridization with:	
	λp*gal*8 DNA (cpm)	λ*cb*2 DNA (cpm)
0.81 Mdal	792	25
0.68 Mdal	50	54

[a] Both the protocols for the isolation of ^{3}H-labeled fragments from an agarose slab gel and for the DNA-DNA hybridization are given in detail in the text. The input of ^{3}H-labeled 0.81-Mdal *Bst*EII-*Eco*RI fragment was 4.7×10^3 cpm of 5% trichloroacetic acid-precipitable material, corresponding with 15.6 ng of denatured DNA. The input of the 0.68-Mdal fragment was 3.8×10^3 cpm (12.6 ng). The DNA segment from the *Eco*RI site at 70.6% of pNP5 DNA (see Fig. 1) to the cross-over site X_l/X_r (11) has a molecular weight of 0.30 Mdal. Consequently, only 37% of the 0.81-Mdal fragment is hybridizable. From these data we calculate that the hybridization efficiency of the DNA-DNA filter hybridization is approximately 46%. The values given are the average of duplicate or triplicate hybridizations.

DISCUSSION

Quantitative aspects of expression of cloned genes. Molecular cloning of *E. coli* genes without their promotor on ColE1-related plasmids allows expression of those genes regardless of their orientation of insertion. This is illustrated by our results and by those of Selker et al. (27), who have cloned the *Salmonella typhimurium trpA* gene, devoid of its promotor, on both ColE1 and pCR1. However, striking differences at the quantitative level of expression are observed when using opposite orientations of insertion or fusing the cloned gene to a different foreign promotor. The latter case is demonstrated by a comparison of the UV resistance of UvrB strains carrying either pNP5 (0.22-Mdal pMB9 promotor) or pNP7 (*lac* promotor) (see Fig. 3 and 4).

The difference in UV resistance could be due either to a more active transcription or to a more efficient translation. Based on the following arguments we favor the first possibility. Fusion of fragment F to the translational initiation elements of β-galactosidase, located on pBH20, probably leads to the synthesis of a hybrid protein composed of the *uvrB* gene product covalently coupled to a polypeptide chain encoded by the region between the ATG initiator codon of β-galactosidase and the *uvrB* gene. It is unlikely that such a hybrid protein is functional; enzymatic activity is only expected when the ATG initiator codon of the *uvrB* gene is utilized as a starting signal for translation of *uvrB* mRNA. A similar mode of expression has been proposed by Backman and Ptashne (1) for the λcI repressor synthesis when the *cI* gene was fused to the *lac* promotor. In the case of plasmid pNP5, the 0.22-Mdal *Eco*RI-*Hin*dIII region of pMB9 provides not only for a promotor, but also for translational initiation elements (J. Maat and H. Pannekoek, in preparation). Thus, in this system, a hybrid protein can be synthesized that probably is enzymatically inactive. According to this reasoning, a possible difference in efficiency of translational initiation on pBH20 and pMB9 cannot account for a difference in UV resistance displayed by pNP7 and pNP5. Only translation initiated at the initiation elements of the *uvrB*

gene can lead to a functional *uvrB* gene product. The difference in the levels of UV resistance evident in strains carrying pNP7 and pNP5 indicates that, under these circumstances, the 0.22-Mdal pMB9 promotor is more efficient for initiating *uvrB* mRNA synthesis than the *lac* promotor of pBH20. The reason for the relatively low efficiency of the *lac* promotor on the multicopy number plasmid pBH20 may be due to a deficiency of the required amount of catabolite gene activator protein factor (12).

When no apparent promotor is available to mediate the transcription of the *uvrB* gene, a low but significant expression of the *uvrB* gene is detected. Both pNP6 (pMB9 *uvrB*; inverted orientation) and pNP9 (pBR322 *uvrB*) render UvrB strains more UV resistant than the parental untransformed strains. This observation may be related to the properties of the enzyme, *E. coli* RNA polymerase. In vitro studies by Hinkle and Chamberlin (16) have shown that, although the association constants of RNA polymerase with promotor DNA and with nonpromotor DNA are quite different, due to the large excess of nonpromotor DNA over promotor sites, a substantial portion of the RNA polymerase molecules sticks to DNA nonspecifically. Assuming that the same observations hold in vivo, this could account for some leakage of RNA synthesis which is amplified for a particular gene when this gene is present in a multicopy number state. Our results show that a quantitative assay for gene expression is a prerequisite for the conclusion whether or not a cloned gene is expressed under control of its own promotor.

pMB9 promotor located on the 0.22-Mdal *Eco*RI-*Hin*dIII fragment. We have shown that the 0.22-Mdal *Eco*RI-*Hin*dIII fragment present on pMB9, but not on pBR322, is vital for expression of the cloned *uvrB* gene. Most of this fragment consists of an *Eco*RI* fragment, originally derived from plasmid pSC101 (molecular weight 5.8 Mdal; Tc^r) (11). Plasmid pMB9 (molecular weight 3.6 Mdal; Tc^r col^{imm}) was constructed by ligation of linear (*Eco*RI-digested) pMB8 (molecular weight 1.7 Mdal; col^{imm}) and *Eco*RI* fragments of pSC101 (24). The region surrounding the Tc^r locus of pSC101 is similar to that of pBR322, but entirely different from that of pMB9 (24). Clearly, an *Eco*RI* fragment of 0.22 Mdal has been inserted between the *Eco*RI and *Hin*dIII sites of pSC101, shifting the resulting *Eco*RI site to the left on the map of pMB9 (see Fig. 1). At present, experiments are in progress to locate the 0.22-Mdal fragment on plasmid pSC101 and to determine its original function.

The pMB9 promotor is an efficient promotor for cloned genes located on an *Eco*RI fragment whose promotor has been deleted. The transcription of genes located on an *Eco*RI fragment which do have their own promotors can probably be amplified by the addition of the pMB9 promotor. For these reasons we are interested in the characterization of this promotor. The DNA sequence of the *Eco*RI-*Hin*dIII fragment (approximately 350 base pairs), which we have determined, contains the structures that match the requirements for recognition and binding of RNA polymerase (14) (J. Maat and H. Pannekoek, in preparation). Preliminary experiments indicate that RNA polymerase specifically protects an *Alu*I restriction site within the promotor on the 0.22-Mdal fragment.

Genetic constitution of the cloned urvB locus. We assume that there are two possible modes of expression of the *uvrB* gene, coupled to a foreign promotor, like the *lac* promotor or the pMB9 promotor. First, translation starts at the ATG initiator codon of β-galactosidase or of pMB9 (18) and continues in the same reading frame to the terminus of the *uvrB* gene. This mode of expression requires that the hybrid protein synthesized be enzymatically active, which is not likely. Moreover, the chances that the reading frames of β-galactosidase and pMB9 fit into that of the *uvrB* gene are rather low (in both cases one out of three). We have argued in the preceding paper (22) that, at least in the case of pNP5, translation initiated on pMB9 is "out of phase" with that of the *uvrB* gene.

The second possible mode of expression of the cloned *uvrB* gene is mediated by its own ATG initiator codon and other start sequences (28). Based on the arguments presented above we feel that this second possibility for the expression of the *uvrB* gene is more likely to occur.

Provided our interpretation for the mode of *uvrB* expression is correct, speculation on the genetic content of the *Eco*RI fragment F suggests two possibilities: (i) *Eco*RI cleaves between the ATG initiator codon and the genuine *uvrB* promotor or (ii) *Eco*RI cuts within an unknown gene preceding the *uvrB* gene, located between the *uvrB* gene and its promotor. In general, the distance between a promotor, defined as the region recognized and subsequently bound by RNA polymerase, and its ATG initiator codon is fairly short and spans approximately 50 to 60 base pairs (14). An exception to this rule are the regulatory regions of some operons involved in the biosynthesis of amino acids (2, 3, 13, 21, 32). In those cases a "leader sequence" of about 150 base pairs separates the promotor from the ATG initiator codon. To decide between these two possibilities we are currently attempting to insert a transposon into the region between the ATG initiator codon of the *uvrB* gene and the *Eco*RI site at 0/100% (Fig. 1). Furthermore, we

are trying to clone the region located between the *bio* operon and the *uvrB* gene, to elucidate the function of this region in relation to the *uvrB* gene.

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