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Construction and Characterization of Hybrid Plasmids Containing the *Escherichia coli nrd* Region

ANTON PLATZ AND BRITT-MARIE SJÖBERG*

Medical Nobel Institute, Department of Biochemistry, Karolinska Institute, S-104 01 Stockholm, Sweden

Recombinant plasmids containing all or part of the genetic region of *Escherichia coli* coding for the two subunits of ribonucleoside diphosphate reductase (proteins B1 and B2) were constructed with the aid of the multicopy plasmid pBR322. Two of these plasmids (pPS1 and pPS2) appeared to carry both a regulator and the complete structural information for the enzyme and, after transformation of *E. coli*, directed a 10- to 20-fold overproduction of both proteins B1 and B2. The other plasmids (pPS101 and pPS201) carried structural information for only protein B2. Cells carrying pPS1 and pPS2 showed a 5- to 500-fold increased resistance against the drug hydroxyurea. This establishes that in *E. coli* the inhibition of deoxyribonucleic acid synthesis by hydroxyurea is fully explained by its action on ribonucleotide reductase.

Ribonucleotide reductase catalyzes the formation of deoxyribonucleotides from their corresponding ribonucleotides (31). The enzyme is an essential constituent of all living cells and plays an important role in the control of DNA synthesis.

In *Escherichia coli*, two adjacent genes, *nrdA* and *nrdB*, code for the two nonidentical subunits of ribonucleoside diphosphate reductase, proteins B1 and B2 (14–16). The *nrd* genes are located at 48 min on the map of the *E. coli* chromosome between the *ubiG* gene, which is involved in the metabolism of ubiquinone (17, 28), and the *glpT* gene, which codes for the transport protein of α-glycerophosphate (9).

In earlier work by Karlström, a segment of *E. coli* DNA containing the *nrd* genes was integrated in a defective λ bacteriophage (unpublished results reviewed in reference 13). This recombinant phage was used by Collins et al. (8) to construct ColE1 plasmid derivatives containing the *nrd* genes. These hybrid plasmids were partially mapped by cleavage with *Eco*RI and *Sma*I restriction enzymes (8).

We are studying the organization and regulation of ribonucleotide reductase of *E. coli* and for this purpose wish to obtain mutants in the *nrd* region of the DNA. In this connection, hybrid *nrd* plasmids with the following properties would be of value: (i) the vector should have an easily scorable phenotype and should be of minimal size; (ii) the segment of inserted *E. coli* DNA should preferably code only for the *nrd* genes; and (iii) the products of the *nrd* genes should be produced in large quantities in bacteria transformed with the hybrid plasmids.

In this communication, we describe the construction of such plasmids by the transfer of a *Pst*I fragment containing the *nrd* region from the ColE1 recombinant into the multicopy plasmid vector pBR322 (4). Recombinant plasmids with the *nrd* insertion in both possible orientations were isolated and after transformation of *E. coli* gave rise to a 10- to 20-fold overproduction of both subunits of ribonucleotide reductase. We also constructed smaller plasmids which carry only a functional *nrdB* gene. Transformation of *E. coli* with the two larger plasmids conferred to the cells a 5- to 500-fold increased resistance against hydroxyurea. This finding definitely establishes that the inhibition of *E. coli* by this drug is caused by its effect on ribonucleotide reductase.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* strains C600 (*hsdR hsdM*$^+$, obtained from G. Magnusson of this department) and KK535 (*thr leu thi deo tonA lacY supE44 recA nalA nrdA nrdB*, obtained from O. Karlström, Department of Microbiology, University of Copenhagen) were used for propagation and maintenance of plasmids. Strain KK535 carries conditional lethal mutations in both structural genes of ribonucleotide reductase (*nrdA,B*) resulting in a temperature sensitivity phenotype. Strain CSR603 (*recA uvrA6 phr-1*; 26) was used to study in vivo transcription and translation of genes carried on plasmids.

Recombinant *nrd* ColE1 plasmids were constructed by Collins et al. (8). Plasmids designated pJC808 (ColE1 *nrdA*$^+$ *nrdB*$^+$ *nalA*$^+$) and pJC812 (ColE1 *nrdA*$^+$ *nrdB*$^+$), maintained in KK535, were used in this study. Plasmid pBR322 (4) was propagated in C600.

Media. Minimal medium (10) was used for KK535 strains and was supplemented with 0.4% glucose, 0.1% Casamino Acids, and leucine (80 μg/ml), threonine (50

μg/ml), thymine (20 μg/ml), and thiamine (1 μg/ml). L broth (22) was used for C600 strains. In appropriate cases, tetracycline (10 μg/ml) and ampicillin (40 μg/ml) were added. Hydroxyurea was obtained from E. R. Squibb and Sons, Ltd., Twickenham, England, and was dissolved in water and sterilized by filtration immediately before use.

Labeling medium for CSR603 strains was composed of 2 volumes of modified Hershey medium (35) supplemented with glucose (0.4%) and thiamine (1 μg/ml) and 1 volume of methionine assay medium (Difco Laboratories, Detroit, Mich.).

Purification of plasmid DNA. Plasmid DNA was isolated after amplification in the presence of chloramphenicol (170 μg/ml) as described by Clewell (6). A cleared lysate was prepared according to Guerry et al. (18), and the DNA was purified by two consecutive centrifugations in CsCl-ethidium bromide gradients. Ethidium bromide was removed by passing the DNA through a Dowex (AG50 W-X4, Bio-Rad Laboratories, Richmond, Calif.) column or by extraction with isopropyl alcohol. The DNA was then precipitated with ethanol and finally dissolved in 50 mM Tris-hydrochloride, 10 mM NaCl, and 1 mM EDTA, pH 7.4. DNA concentrations were determined by measuring the absorbance at 260 nm (1.0 absorbance unit corresponds to a DNA concentration of 50 μg/ml; 3).

Preparations of plasmid DNA for rapid analyses of restriction endonuclease patterns were performed according to Bernard and Helinski (2).

Enzymes. Restriction endonucleases *Pst*I, *Eco*RI, *Bgl*I, and *Alu*I were from BioLabs, Beverly, Mass. *Bam*HI endonuclease was a gift of G. Winberg, Department of Virology, Karolinska Institute, Stockholm, Sweden. Alkaline phosphatase was obtained from Worthington Biochemicals Corp., Freehold, N.J. T4 ligase was a kind gift of G. Magnusson of this department.

Electrophoretic analyses. Electrophoresis was performed in Tris-EDTA-borate buffer (3). The size of DNA fragments was determined from plots of molecular weight versus relative mobility (32). The following markers were used for agarose gel electrophoresis: the six *Eco*RI-generated fragments of the bacteriophage λ genome (13.7, 4.68, 3.71, 3.56, 3.03, and 2.01 megadaltons [Md]; 32), two of the three *Bgl*I fragments of the plasmid pBR322 (1.58 and 1.19 Md; 29, 34), and two of the *Alu*I fragments of pBR322 (0.60 and 0.44 Md; 4).

Preparation and ligation of DNA fragments. To prepare pPS1 and pPS2 plasmids, 15 μg of pBR322 and 10 μg of pJC808 DNA, respectively, were digested with *Pst*I endonuclease. Restricted pBR322 DNA was then treated with alkaline phosphatase as described by Ullrich et al. (33). Donor and vector fragments were then extracted with phenol, precipitated with ethanol, mixed, and ligated at 15°C overnight. For isolation of pPS101 and pPS201 plasmids, 10 μg each of *Bam*HI-restricted pPS1 and pPS2 DNA, respectively, were directly incubated with T4 ligase. Total DNA concentrations ranged from 5 to 125 μg/ml, and incubation mixtures contained 50 mM Tris (pH 7.6), and 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 to 1.0 mM ATP, and excess of T4 polynucleotide ligase.

Transformation procedure. For transformation, competent cells were prepared with the $CaCl_2$ method according to Cohen et al. (7). Plasmid DNA was added to the competent cells (1 to 2 μg per 2×10^9 to 6×10^9 cells), and the mixture was incubated for 15 min at 0°C, 2 min at 42°C, and 30 min at 0°C. Portions (0.1 ml) of the suspension were directly plated in 3 ml of soft agar on plates containing tetracycline (10 μg/ml).

Phenotypic characterization of constructed plasmids. Hydroxyurea resistance was determined by plating equal portions of appropriately diluted exponential-phase cultures on L agar (C600 strains) or minimal agar plates (KK535 strains) containing increasing amounts of hydroxyurea. Temperature sensitivity of KK535 [*nrd*(Ts)] strains was determined on minimal agar plates.

Analyses of ribonucleotide reductase subunits in crude extracts. Cultures of C600 cells carrying different plasmids were grown in 1 liter of L broth, harvested by centrifugation during exponential growth, and extracted by the alumina method as described by Eriksson et al. (13). The crude extracts used in this study were obtained after precipitation with streptomycin sulfate and ammonium sulfate (13) and were analyzed for protein B1 and protein B2 content by rocket immunoelectrophoresis (13) and for enzyme activity (5, 13). One unit is defined as the amount of enzyme which catalyzes the formation of 1 nmol of dCDP per min at 25°C. Specific activity is expressed as units per milligram of protein. Total protein content was determined by the method of Lowry et al. (21).

Protein labeling in maxicells. The maxicell system of Sancar et al. (26) was used for specific labeling of plasmid-coded proteins with little background synthesis of total cellular proteins. The total UV irradiation dose used was approximately 40 J/m^2. [^{35}S]methionine was added to a final concentration of 50 μCi/ml.

Containment. All experiments were performed with PI containment.

RESULTS

Mapping of pJC808 and pJC812. As starting point for our experiments, we chose the recombinant *nrd* ColE1 plasmids pJC808 and pJC812 (Fig. 1), which had been obtained by transfer of *Eco*RI fragments from a λ d*nrd* phage (8, 13). These two plasmids had earlier been mapped partially, and we therefore first determined their cleavage patterns with *Bam*HI and *Pst*I. Earlier work had already established the recognition sites for *Eco*RI and *Sma*I in the recombinants (8). In addition, the recognition sites for *Pst*I within the *Col*E1 genome (1) and a *Bam*HI site of the λ *att* position (19, 24) were known.

The results of single digestions of pJC808 or pJC812 with *Bam*HI or *Pst*I are shown in Table 1, and fragments common to both plasmids are indicated in italics. These data were used to construct the map for pJC812 (Fig. 1). The results of double digestions of pJC808 or pJC812 with combinations of *Bam*HI, *Pst*I, and *Eco*RI

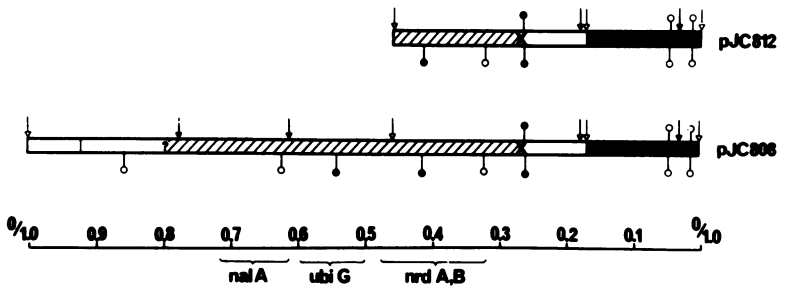

FIG. 1. *Cleavage maps of hybrid nrd ColE1 plasmids. The different origins of the DNA are indicated as follows: E. coli, ▨; phage λ, □; plasmid ColE1, ■. Cleavage sites indicated above the bars are known from earlier work (1, 8, 19, 24). EcoRI, ↓ ; SmaI, ↓ ; PstI, ⚲ ; BamHI, ⚲ . Below the bars are shown PstI and BamHI specific cuts described in this work. For pJC808, the resulting fragments are expressed as fractions of total molecular weight (approximately 25 × 10⁶). For simplicity, the fragments obtained for pJC812 are also expressed as fractions of 25 × 10⁶. (The molecular weight of pJC812, 11.4 × 10⁶, is 0.45 fractional length of pJC808.)*

are also shown in Table 1. As expected, when *Eco*RI digestion was used, all fragments obtained from pJC812 were also present in the digest of pJC808. The double digestions were used to construct the map for pJC808 (Fig. 1).

Construction of plasmids pPS1 and pPS2. The purpose of our mapping experiments was to find a suitable fragment of pJC808 for transfer of the entire *nrd* region to a small multicopy plasmid. Earlier data had demonstrated that the *nrd* region is close to the *Eco*RI site of pJC808 at 0.45 map units (8). In fact, it seems likely that this *Eco*RI site separates the *nrd*-coding region from a regulatory sequence (8). The amount of DNA required to code for the amino acid sequence of ribonucleotide reductase cannot exceed 4 × 10⁶ daltons, which positions the other end of the *nrd* structural genes not further than 0.16 map units to the right of the *Eco*RI site. Thus, the *Pst*I fragment of pJC808 which is located between 0.32 and 0.64 map units (Fig. 1) is expected to contain the complete *nrd* region. This fragment has a molecular weight of approximately 7.8 × 10⁶ and was chosen for the transfer of the *nrd* region into the plasmid pBR322.

For this purpose, pBR322 and pJC808 were first digested separately with *Pst*I. Linearized pBR322 was then treated with alkaline phosphatase to prevent religation of vector DNA and was then mixed and ligated with the pJC808 fragments. The ligated mixture was introduced into competent C600 cells, and 79 Tc^r Ap^s colonies were obtained after phenotypic selection. Plasmid DNA from these clones was further analyzed after *Pst*I digestion by the rapid extraction procedure of Bernard and Helinski (2). Two plasmids, designated pPS1 and pPS2, contained a 7.8 × 10⁶-dalton fragment from pJC808 DNA. Plasmid pPS1, in addition, contained a second 0.8 × 10⁶-dalton *Pst*I fragment from pJC808. Further restriction enzyme analyses with endonucleases *Eco*RI and *Bam*HI showed that both plasmids contained the identical *nrd*-containing *Pst*I fragment of pJC808, the difference between the two being the orientation of the *nrd* fragment with respect to pBR322. The restriction maps for pPS1 and pPS2 are given in Fig. 2.

TABLE 1. *Fragments obtained after single or double restriction endonuclease digestion of pJC808 and pJC812*

Plasmid	*Bam*HI	*Pst*I	*Bam*HI/ *Eco*RI	*Pst*I/ *Eco*RI	*Bam*HI/ *Pst*I
pJC808[a]	0.70	0.32	0.46	0.24	0.23
	0.15	*0.28*[b]	*0.18*	0.17	*0.23*
	0.15	0.21	*0.14*	*0.15*	0.16
		0.16	*0.09*	*0.14*	0.13
		0.03	0.08	*0.13*	*0.08*
			0.05	0.12	0.07
				0.03	*0.06*
					0.03
pJC812[c]	0.30	*0.28*	*0.18*	*0.15*	*0.23*
	0.15	0.14	*0.14*	*0.14*	*0.08*
		0.03	*0.09*	*0.13*	*0.06*
			0.05	*0.03*	0.05
					0.03

[a] Total length of pJC808 (25 × 10⁶ daltons) is set at 1.0.
[b] Fragments which are common to both plasmids are italicized.
[c] Total length of pJC812 (11.4 × 10⁶ daltons) is set at 0.45 for ease of comparison between pJC808 and pJC812.

Construction of plasmids pPS101 and pPS201. The *Bam*H site at position 0.41 is close to the start of the *nrd* region. Cleavage at this site might therefore involve the beginning of the coding part of the region and occur within the *nrdA* gene. It should therefore be possible to use this site for the construction of smaller plasmids containing the *nrdB* region but not the complete *nrdA* region. Digestion of pPS1 or pPS2 with

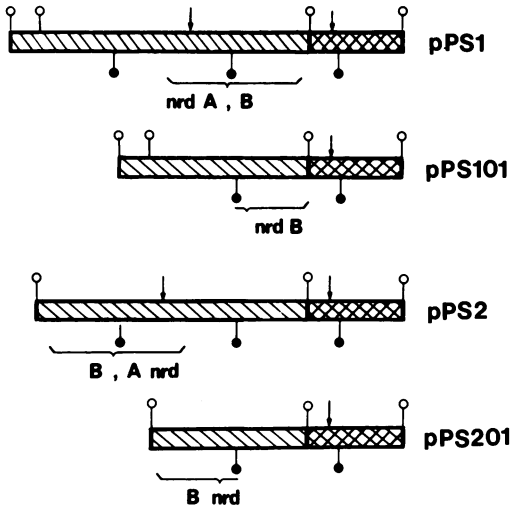

FIG. 2. *Restriction endonuclease cleavage map of hybrid nrd pBR322 plasmids pPS1, pPS101, pPS2, and pPS201. Different origins of the DNA are indicated as follows: plasmid pJC808, ▨; plasmid pBR322, ▩. The symbols are explained in Fig. 1.*

*Bam*HI followed by religation should theoretically give three different products of the Tc^r Ap^s phenotype: (i) the reconstituted parental plasmid, pPS1 or pPS2; (ii) plasmids of parental size but with the 3.5-Md *Bam*HI fragment common to both pPS1 and pPS2 inverted; and (iii) smaller plasmids lacking the 3.5-Md *Bam*HI fragment.

Phenotypic selection of *Bam*HI digested and religated pPS1 DNA resulted in 22 Tc^r Ap^s colonies. After rapid screening (2) with *Bam*HI restriction, one of the small plasmids (= pPS101) containing only two *Bam*HI fragments (corresponding to the last alternative mentioned above) was chosen for further analyses. An analogous plasmid (= pPS201) was isolated after *Bam*HI treatment of pPS2. Restriction maps of pPS101 and pPS201 with endonucleases *Bam*HI, *Pst*I, and *Eco*RI are included in Fig. 2. Each small plasmid (pPS101 and pPS201) lacked the 3.5×10^6-dalton *Bam*HI fragment of its parent (pPS1 or pPS2) (Fig. 3).

Phenotypic characterization of plasmids pPS1, pPS2, pPS101, and pPS201. (i) Hydroxyurea resistance. The plating efficiency of different *nrd* plasmids as a function of hydroxyurea concentration was studied in two types of cells: those that carry temperature-sensitive mutations in the *nrd* genes (KK535), and those that can be considered wild type in this context (C600). The *nrd* mutations in KK535 give the cells a hydroxyurea-sensitive phenotype.

With KK535, plasmid-containing cells were plated on minimal medium at 30°C at hydroxyurea concentrations between 10 and 6,000 μg/ml. The presence of either pPS1 or pPS2 increased the resistance of KK535 to hydroxyurea about 500-fold (Fig. 4B). Whereas the controls (KK535 with or without pBR322) were inhibited at concentrations of 10 to 20 μg/ml, pPS1- and pPS2-containing cells showed no decreased plating efficiency at 2 mg/ml. The presence of the smaller plasmids, pPS101 and pPS201, gave only a very slight increase in the resistance of the cells.

Plasmid containing C600 cells were plated on rich medium at 37°C at hydroxyurea concentrations between 2 and 20 mg/ml. The presence of pPS1 or pPS2 conferred a four- to fivefold increased hydroxyurea resistance to the cells (Fig. 4A). The smaller plasmids (pPS101 or pPS201) did not give an increased resistance.

(ii) Temperature resistance. Table 2 shows the effects of the different *nrd* plasmids on the temperature-sensitive phenotype of KK535, which is caused by its *nrd* lesions. Cells were plated on minimal medium at 25 to 45°C. Cells containing pPS1 and pPS2 still grew at 40°C, whereas control cells and cells carrying pPS101 and pPS201 gave no colonies above 30°C (Table 2). Thus, the presence of pPS1 or pPS2, but not pPS101 or pPS201, conferred heat resistance to KK535 cells. Apart from the heat sensitivity,

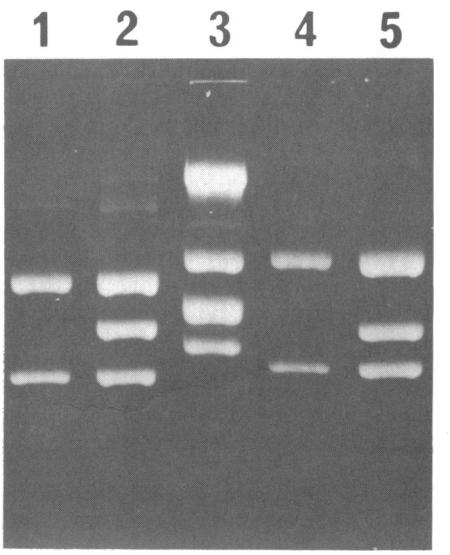

FIG. 3. *Gel electrophoresis of fragments obtained by BamHI cleavage of hybrid nrd pBR322 plasmids. (1) pPS201; (2) pPS2; (4) pPS101; (5) pPS1 DNA. (3) λDNA digested with EcoRI, which was used as marker DNA.*

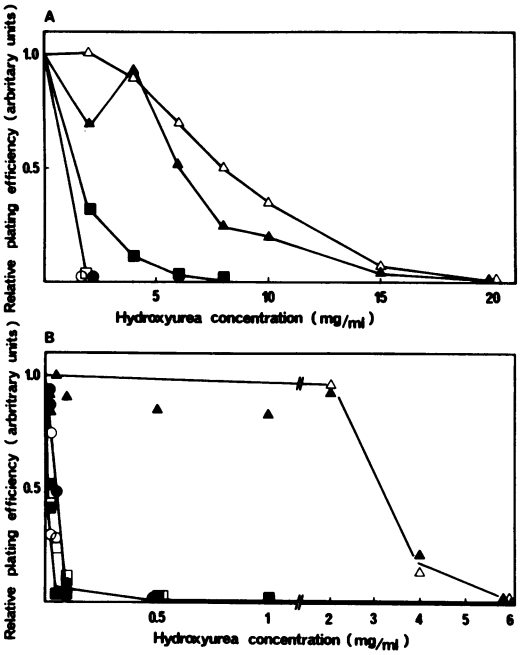

FIG. 4. *Effect of hydroxyurea on the plating efficiency of strains containing different nrd pBR322 plasmids. The recipient strains are (A) C600 (nrd⁺) and (B) KK535 (nrd). Symbols: no plasmid, □; pBR322, ■; pPS1, △; pPS2, ▲; pPS101, ○; pPS201, ●.*

TABLE 2. *Effect of temperature on the relative plating efficiency[a] of KK535 (nrd) cells containing different nrd pBR322 plasmids*

Temp (°C)	Plating efficiency					
	No plasmid	pBR322	pPS1	pPS2	pPS101	pPS201
25	0.3	0.9	1.0	0.7	1.0	0.9
30	1.0	1.0	1.0	1.0	1.0	1.0
37	0	0	1.0	0.6	0	0
40	0	0	1.0	0.3	0	0
45	0	0	0.4	0	0	0

[a] Plating efficiency at 30°C is set at 1.0 arbitrary unit.

KK535 also showed a cold-sensitive phenotype that was not characterized further. One possible explanation would be a disturbed complex formation of the defective *nrd* subunits, which is suppressed upon transformation with the *nrd* plasmids (Table 2).

Induction of ribonucleotide reductase subunits by *nrd* plasmids. Synthesis of plasmid-encoded *nrd* products was studied by three different methods: rocket immunoelectrophoresis (13), enzymatic assay (5, 13) and sodium dodecyl sulfate-gel electrophoresis of labeled products in the maxicell system (26).

With rocket immunoelectrophoresis, the levels of the two subunits of ribonucleotide reductase (proteins B1 and B2) were measured in crude extracts prepared from C600 cells carrying the appropriate plasmid. Extracts from cells containing plasmids pPS1 and pPS2 contained a 10-fold excess of each subunit. In cells carrying pPS101 and pPS201, only the level of protein B2 was increased, and this increase was marginal.

Both proteins B1 and B2 were also assayed by their enzymatic activity in crude extracts (Table 3). Cells carrying plasmid pPS1 or pPS2 showed levels of B1 and B2 which were approximately 20 times higher than those of control cells. Plasmid pPS101 or pPS201 again gave a slight increase in the level of B2, whereas the activity of B1 was actually lower than in the control.

So far our data for plasmids pPS1 and pPS2 clearly show that each plasmid contained and expressed both *nrd* genes. For plasmids pPS101 and pPS201, the results suggest the presence of an intact *nrdB* gene, but in this case the evidence is weaker. We therefore further investigated this point with the maxicell system of Sancar et al. (26). In this case, the expression of host genes is suppressed by UV irradiation resulting in a preferential labeling of plasmid-coded proteins. After gel electrophoresis, autoradiography of the gels, as expected, showed that cells carrying plasmid pPS1 or pPS2 synthesized two labeled polypeptides with mobilities corresponding to those of protein B1 or B2 (Fig. 5). Extracts from control cells carrying pBR322 did not show any labeled bands in this part of the gel. Cells carrying plasmid pPS101 or pPS201 gave a labeled band corresponding to protein B2, but not to protein B1 (Fig. 5). It is thus clear that plasmids pPS101 and pPS201 direct only the synthesis of the *nrdB* product of the reductase, though at much lower levels than plasmids pPS1 and pPS2, which contain the complete *nrd* region and direct the synthesis of both subunits.

TABLE 3. *Levels of proteins B1 and B2 in crude extracts of C600 cells containing different nrd pBR322 plasmids*

Plasmid	Protein content			
	μg/mg of protein		U/mg of protein[a]	
	B1	B2	B1	B2
pPS1	31	13	10	23
pPS2	32	14	17	39
pPS101	5.3	2.4	<0.5	4.7
pPS201	2.1	4.9	<0.5	3.3
pBR322	4.2	0.8	0.5	1.6

[a] Unit = nanomoles of dCDP formed per minute at 25°C.

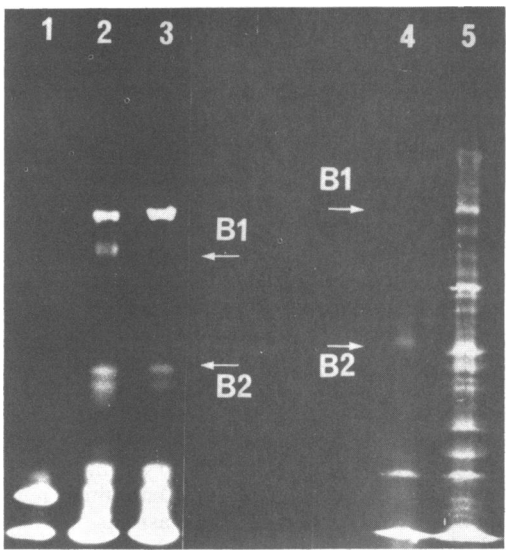

FIG. 5. *Gel electrophoresis of proteins formed in maxicells containing different nrd pBR322 plasmids. (1) pBR322; (2) pPS1; (3) pPS1; (4) pPS201; (5) pPS2. The mobilities of purified proteins B1 and B2 are also shown.*

DISCUSSION

The *nrdA* and *nrdB* genes of *E. coli*, which code for the two subunits of ribonucleotide reductase (proteins B1 and B2; 14–16), were earlier integrated into a lysogenic λ phage and subsequently recombined with the DNA of ColE1 (8, 13). Such recombinant plasmids were of two size classes, 25×10^6 daltons (represented in Fig. 1 by pJC808) and 11.4×10^6 daltons (represented by pJC812). The two classes had the following properties: (i) both plasmids contained a 7.2×10^6-dalton *Eco*RI fragment from the hybrid phage which carried the *nrdA* and *nrdB* genes; (ii) the large plasmids (e.g., pJC808) also contained a second *Eco*RI fragment, of 13.6×10^6 daltons, adjacent to the 7.2×10^6-dalton fragment, as a result of incomplete digestion of the hybrid phage; (iii) the large plasmids were of two kinds, differing only in the orientation of the inserted DNA fragment with respect to the ColE1 genome, whereas the small plasmids (e.g., pJC812) all contained their inserted fragment in the same orientation; (iv) all plasmid-carrying strains had higher levels of ribonucleotide reductase than did cells without plasmids, but this level was four to six times higher for the large plasmids. All these results were explained by the hypothesis that the 7.2×10^6-dalton fragment carrying the structural *nrdA* and *nrdB* genes lacked a regulatory sequence which was present on the 13.6×10^6-dalton fragment (8).

We have now constructed several new recombinant plasmids containing all or part of the *nrd* region combined with the multicopy plasmid, pBR322. For this purpose, we first introduced a 7.8×10^6-dalton *Pst*I fragment of pJC808, expected to contain the complete *nrd* region, into the penicillinase gene of pBR322 (4). Two classes of plasmids, corresponding to the two possible insertion directions, were isolated. Representatives for both classes (pPS1 and pPS2) directed an equally efficient synthesis of both subunits of the reductase, suggesting that the integrated *Pst*I fragment carries both structural and regulatory information. A less likely explanation would be that the *nrd* genes are transcribed in pPS1 from a promoter localized on the 0.8-Md *Pst*I fragment and in pPS2 from the β-lactamase promoter.

We then deleted a 3.5×10^6-dalton large *Bam*HI fragment from both pPS1 and pPS2 and obtained the two smaller plasmids, pPS101 and pPS201. Extracts from cells carrying either of these small plasmids showed a small increased synthesis of protein B2 but not of protein B1. With the maxicell system (26), which specifically measures plasmid-directed protein synthesis, we could demonstrate that the smaller plasmids contain an intact *nrdB* gene but lack a functioning *nrdA* gene.

These data suggest that the *Bam*HI fragment which was removed from pPS1 and pPS2 during the construction of pPS101 and pPS201 contains not only part of the *nrdA* gene but also a regulatory function, in its simplest case a *nrd* promoter. In cells transformed with pPS1 or pPS2, the *nrd* region then would utilize its own promoter, making the level of enzyme dependent only on the plasmid copy number and independent of the direction of insertion. With pPS101 and pPS201, the transcription of the *nrdB* gene then must start from a vector promoter.

The molecular weights of proteins B1 and B2 are 160,000 and 78,000, respectively (30, 31). Both proteins are dimers; protein B1 is of the general structure $\alpha\alpha'$, whereas protein B2, containing two apparently identical polypeptides, is of a β_2 structure (30). The two polypeptide chains of protein B1 are of similar size with identical COOH termini, but differ at their NH_2 termini (30). It is not clear whether the difference between α and α′ is caused by preparation artifacts or by post-translational modification, or whether α and α′ are products of separate structural genes. At least three possible genotypic constellations of the *nrd* region on the *E. coli* genome can thus be considered: (i) *nrdAA′ nrdBB*, (ii) *nrdAA′ nrdB*, or (iii) *nrdA nrdB*, requiring 4×10^6, 3.3×10^6, or 2×10^6, respec-

tively, of double-stranded DNA. We can now ask the question of whether our knowledge of the organization of the *nrd* region on the different plasmids gives preference to any of the above constellations. It is obvious that the *nrdA* gene is located to the right of the *Eco*RI site at 0.45 units in Fig. 1 (8). Furthermore, the *nrd* region cannot extend further than to 0.32 on the map since this point marks the end of the *Pst*I fragment incorporated into pPS1 and pPS2 and both plasmids overproduce proteins B1 and B2 to the same extent. It follows that the DNA piece which codes for the ribonucleotide reductase of pPS1 and pPS2 has a maximal molecular weight of 3.5×10^6, a fact which excludes the first possibility above but does not distinguish between (ii) and (iii). Future work involving mutagenesis of the integrated *nrd* genes should resolve this point.

Finally, our results concerning the effect of *nrd* plasmids on the sensitivity of *E. coli* toward inhibition by hydroxyurea is important for understanding the mechanism of action of this drug. Work from this laboratory has earlier established that hydroxyurea inhibits purified ribonucleotide reductases from mammalian and bacterial sources (12, 20). With the *E. coli* enzyme, it was shown that the inhibition specifically involves the destruction of the tyrosine radical of the B2 subunit required for enzyme activity (11, 27). Together with results demonstrating an immediate decrease of deoxyribonucleotide pools on addition of hydroxyurea to cells (23), these results strongly suggest that the in vivo-observed inhibition of DNA synthesis (25) is a secondary effect caused by the lack of supply of deoxyribonucleotides. The present experiments show that the introduction of functional *nrd* genes by pPS1 and pPS2 gives a dramatic increase in the resistance of *E. coli* to hydroxyurea. Since essentially no other genetic information was introduced by these two plasmids, our data represent convincing evidence that the hydroxyurea inhibition of DNA synthesis, at least in *E. coli*, is fully explained by the action of the drug on ribonucleotide reductase.

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ADDENDUM

Plasmids pPS101 and pPS201 have been transformed into a *nrdA*$^+$ *nrdB*(Ts) *recA* strain. Such transformants have a temperature-resistant phenotype and an increased resistance against hydroxyurea, which confirms the presence of an intact *nrdB* gene in plasmids pPS101 and pPS201.

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