Overproduction of the B1 Subunit of Ribonucleotide Reductase with Gene Amplification *

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Ribonucleotide reductase catalyses the reduction of ribonucleotides to their corresponding deoxyribonucleotides. The reaction is essential to all living cells and it is the first unique step in the biosynthesis of DNA. The *Escherichia coli* enzyme contains two nonidentical subunits, B1 and B2, which are weakly associated to an active 1:1 complex.[1] The B1 subunit contains two 87 kd polypeptide chains, $\alpha\alpha'$, which differ at the N-termini. B1 has four allosteric sites, two regulating the total activity and two the substrate specificity. At the active site B1 contributes with oxidation-reduction active dithiols. The B2 subunit is also a dimer consisting of two identical 43 kd polypeptide chains, β_2. B2 contains two Fe^{3+} ions and a tyrosine radical, the radical is needed for enzyme activity and is stabilized by the iron center. The B1 and B2 subunits are coded for by the *nrdA* and *nrdB* genes, respectively, located at 48 minutes on the *E. coli* chromosome.[2] The genes are expressed as a single polycistronic transcript with the *nrdA* gene at the 5′ end and the *nrdB* gene at the 3′ end.[3] Regulation occurs at the transcriptional level and shows variation within the cell cycle.[1,4] The intriguing active site and allosteric control of ribonucleotide reductase have led to advanced structural and mechanistic studies. In order to obtain large amounts of protein each *nrd* gene was cloned separately. The genes were first transferred to a pBR322 plasmid via a λ transducing bacteriophage,[5,14] and the *nrdB* gene was further subcloned in a "run-away" vector.[6] This paper describes the cloning of the *nrdA* gene in a "run-away" vector and a preliminary characterization of the gene product.

Experimental procedure. The strain used is *E. coli* C600 (*F⁻ hsr$_K$⁺ hsm$_K$⁺ gal thr leu thi pro lac str^r*).[5] The 15-kb pPS2 plasmid harbouring the complete *nrd*-operon was constructed by Platz *et al.*[5] and is a *tet*⁺ recombinant pBR322 derivative. The 13.5-kb "run-away" vector pBEU17 is a *bla⁻ cop^{ts}* derivative of R1.[7]

E. coli C600, was grown in Luria broth[8] with glucose, 1g/l, in aerated cultures at 37 °C. Bacteria harbouring *cop^{ts}* plasmids were grown at 30 °C. When included 10 μg/ml of tetracycline or 40 μg/ml of ampicillin was used.

Plasmid DNA was prepared from 50-ml cultures by alkaline extraction,[9] followed by treatment with RNase and proteinase K. DNA samples were analyzed by submarine mini agarose gel electrophoresis.[10] Digestion, ligation and transformation were performed according to Maniatis *et al.*.[10]

Immunological screening was performed on Ap^r colonies.[11] The colonies were first treated with polyclonal rabbit anti-B1 antibodies and then with [^{125}I]-protein A.

Two types of bacterial extracts were made by using ultrasonic treatment or using lysozyme.[12] Extracts were analyzed qualitatively by sodium dodecyl sulfate gel electrophoresis.[13] Rocket immunoelectrophoresis was used for quantitation of B1 protein.[14] Protein determinations were made according to Lowry *et al.* [15] or Bradford *et al.*.[16] Specific activity is expressed as units per mg of immunologically determined protein B1.[14]

Purification of protein B1 on dATP-Sepharose has been described by Eriksson *et al.*.[14]

Results and discussion. Digestion of pPS2 DNA (15-kb) with restriction endonuclease *KpnI* resulted in a 4.7-kb fragment containing the *nrdA* gene. Digestion of pBEU17 (13.5-kb) with *KpnI* preserves its ampicillin resistance. The ligation mixture contained 1.5 μg of pPS2/*KpnI* and 0.8 μg of pBEU17/*KpnI*. The transformation mixture (0.5 ml) contained 2.8×10^8 viable cells and 0.5 μg of ligated DNA. The transformation frequency was 1.5×10^3 transformants/μg of DNA. 300 transformants were chosen for immunological screening and 15 were clearly positive with B1 antiserum. Plasmid DNA was isolated from two of these positive clones, pLSH1 and pLSH3. After *KpnI* digestion, both DNA preparations were found to contain a 4.7-kb and a 13.5-kb fragment. The shorter fragment comigrated with the pPS2 fragment and the longer fragment with the linearized vector. pLSH1 was chosen for further studies.

To determine the amount of protein B1 produced in cells containing pLSH1, a culture was shifted from 30 to 43 °C and cells were withdrawn for analysis at different times after heat induction. Upon heat induction the doubling time initially decreased from 43 to 27 min but after approximately four doublings the growth levelled off (Fig. 1). Extracts from uninduced culture (0 h

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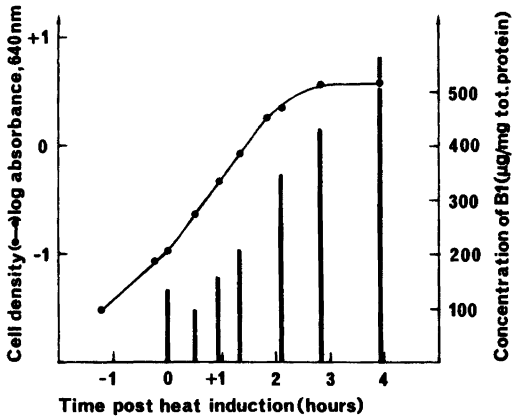

Fig. 1. Heat induction of clone C600/pLSH1. The bars indicate the concentration of protein B1 in cell extracts. At time zero the culture was shifted from 30 to 43 °C.

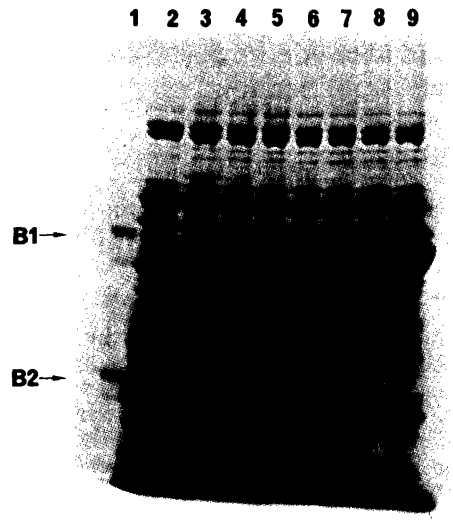

Fig. 2. SDS gel electrophoresis on extracts from the heat induction experiment. In each slot 64 µg of protein was loaded. Lane 1, homogeneous proteins B1 and B2; lane 2, C600; lane 3, C600/pLSH1 0 hours post heat induction (h); lane 4, 0.50 h; lane 5, 0.92 h; lane 6, 1.33 h; lane 7, 2.10 h; lane 8, 2.83 h; lane 9, 3.92 h.

after heat induction) contained 0.13 mg of protein B1 per mg of total protein. Shortly after heat induction (0.5 h) the concentration of B1 dropped transiently in the extract to 0.10 mg/mg of total protein. After this initial decrease the B1 concentration gradually increased until a level of 0.5 mg protein B1/mg of total protein was reached in the stationary phase (3.9 h after heat induction), *i.e.*, protein B1 constituted 50 % of total protein in the extract. Each extract was also compared to the others by sodium dodecyl sulfate gel electrophoresis (Fig. 2) which qualitatively shows the same result as Fig. 1. For comparison, an extract from the recipient strain C600 was included (lane 2, Fig. 2). This extract had a protein B1 content of 0.0024 mg/mg of total protein. Thus, heat induction of pLSH1 cells resulted in a 200-fold overproduction of protein B1 as compared to wild type cells.

Protein B1 from heat induced cells was purified on dATP-Sepharose. Sodium dodecyl sulfate gel electrophoresis showed that the eluate consisted of apparently homogeneous B1 protein and that it comigrated with B1 standard. The specific activity of protein B1 before and after heat induction is 800–1000 U/mg Bl, which is equivalent to good earlier preparations.[14]

Even before heat induction cells harbouring pLSH1 have a 60-fold increase in B1 content relative to cells without the plasmid. The copy number of pBEU17 derivatives is usually less than 10 before heat induction.[7] From the recently deduced nucleotide sequence covering the *nrd*-operon, it is obvious that the 4.7-kb fragment cloned in pLSH1 includes 1.7-kb upstream of *nrdA*; the *nrd*-promotor is within this 1.7-kb.[17] The high uninduced level of B1 suggests that the *nrd*-promotor is very efficiently transcribed in the pLSH1 recombinant.

In summary, gene amplification with the "runaway" derivative pBEU17 has proven excellent for overproduction of both subunits of *E. coli* ribonucleotide reductase (Ref. 6 and this report) to yield simplified purification procedures and sufficient amounts of protein for X-ray crystallographic analyses.[18]

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