Bacteriophage T4 ribonucleoside diphosphate reductase: on the defect causing decreased formation of the $\beta_{23}$ subunit encoded by the nrdB93 mutant gene

(dNTP synthesis; multienzyme complex; gene expression; transcription; translation; protein degradation)

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SUMMARY

Bacteriophage T4 ribonucleoside diphosphate reductase is composed of two proteins, $\alpha$ and $\beta$, encoded by the nrdA and nrdB genes, respectively. The expression of nrdB is the limiting factor for the assembly of the enzyme. A recently described mutation, nrdB93, may give new insight into the regulation of synthesis of the $\beta$ subunit encoded by nrdB. Infection by T4 nrdB93 produced only low concentrations of the $\beta_{23}$ protein. However, a site-specific mutation of phage T4 gene 39, encoding one of the subunits of T4 DNA topoisomerase, phenotypically suppressed the defect. The present work sought to characterize the nature of this defect. The mutation in nrdB93 was a single-base transition (G-A) resulting in a Gly$^{253}$→Asp change. In vivo and in vitro studies provided no evidence of degradation of the $\beta_{23}$ protein. Furthermore, the decrease in $\beta_{23}$ formation was not caused by a delayed onset of transcription, neither by a decreased rate of mRNA formation from the nrdB promoter, nor by a defective intron splicing of the nrdB gene or in the transcription of the terminal segments of the message. These findings are consistent with the concept that the nrdB93 lesion produces a defect at the level of translation.

INTRODUCTION

Bacteriophage T4-encoded ribonucleoside diphosphate reductase is required in the assembly of the dNTP synthetase complex (Chiu et al., 1982; Moen et al., 1988) and appears to be the last factor in place and the keystone in the assembly of the complex (Greenberg et al., 1994). The $\alpha$ and $\beta$ chains of this $\alpha_2\beta_2$ enzyme are encoded by the T4 nrdA gene and the intron-containing nrdB gene, respectively (Yeh and Tessman, 1969; Yeh et al., 1972; Tseng et al., 1988; 1990). The synthesis of the $\beta$ subunit is initiated after the synthesis of $\alpha_2$ protein begins (Tomich et al., 1974; Chiu et al., 1976; 1982; E.M. Kutter, personal communication to G.R. Greenberg).

Phage T4 nrdB93, a temperature-sensitive mutant (Cook et al., 1988; Wirak et al., 1988) provides a tool to study the regulation of $\beta_2$ synthesis. This mutant was isolated from tsG41, a T4 DNA delay mutant originally thought to contain a single mutation in gene 39 (Epstein et al., 1963), encoding the ATPase subunit of T4 DNA gene encoding one of the gyrase subunits; kb, kilobase(s) or 1000 bp; motA, T4 gene encoding protein modifier of transcription; NADH, reduced nicotinamide-adenine dinucleotide; nrdB, T4 gene encoding ribonucleoside diphosphate reductase $\beta$ subunit; nrdB93, T4 mutant gene encoding the ribonucleoside diphosphate reductase $\beta_{23}$ subunit; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; P$_{sec}$-promoter located immediately upstream from the nrdB gene; SDS, sodium dodecyl sulfate; wt, wild type.

Abbreviations: aa, amino acid(s); bp, base pair(s); Cm, chloramphenicol; denA, T4 gene encoding endonuclease A; dNTP, deoxyribonucleoside triphosphate; frd, T4 gene encoding dihydrofolate reductase; gyrB, host gene encoding one of the gyrase subunits; kb, kilobase(s) or 1000 bp; motA, T4 gene encoding protein modifier of transcription; NADH, reduced nicotinamide-adenine dinucleotide; nrdB, T4 gene encoding ribonucleoside diphosphate reductase $\beta$ subunit; nrdB93, T4 mutant gene encoding the ribonucleoside diphosphate reductase $\beta_{23}$ subunit; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; P$_{sec}$-promoter located immediately upstream from the nrdB gene; SDS, sodium dodecyl sulfate; wt, wild type.

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topoisomerase (Huang, 1986). Wirak and co-workers (1988) found that the tsG41 mutant actually harbored two mutations, nrdB93 in the nrdB gene and 39-01 in gene 39. Phage T4 39-01 shows a slight delay in DNA synthesis at either 30°C or 41°C. However, phage T4 nrdB93 grows very poorly at any temperature. The infection with the T4 double mutant is temperature-sensitive and results only in the typical delay in DNA replication.

The nrdB93 mutation produces two very different phenotypes. (i) $\beta_3$ activity is much more labile than $\beta_2$ activity, even at permissive temperatures. (ii) The level of $\beta_3$ chain synthesis varies from zero to about 10% of wt, even at 30°C, the permissive temperature employed. This defective synthesis is phenotypically suppressed by the second mutation, 39-01. In addition, the suppression requires the expression of the host gyrB gene, encoding the corresponding ATPase subunit of DNA gyrase (DNA topoisomerase II) (Wirak et al., 1988; for a review of the system, see Greenberg et al., 1994). To better understand the nature of these interactions, we sought to determine the location of the nrdB93 mutation and to characterize its physiological defect.

EXPERIMENTAL AND DISCUSSION

(a) Site of the nrdB93 mutation

Using the dideoxynucleotide chain-termination RNA sequencing technique (Tseng et al., 1990; Zimmern and Kaesberg, 1978), we examined the entire coding sequences, the exon-intron junctions, as well as the upstream and downstream regions of the mature nrdB and nrdB93 mRNAs isolated after infection by phage T4D and the double mutant, 39-01 nrdB93, respectively (Wirak et al., 1988; Cook et al., 1988). A restriction map of the nrdB gene and the sequencing strategy used to identify the nrdB93 mutation is presented in Fig. 1. nrdB93 was found to be a C$^{58}$T transition (G$\rightarrow$A in the mRNA) leading to a Gly$^{253}$$\rightarrow$Asp substitution (Fig. 2). The $\beta_3$ mutant subunit moves slightly more slowly than the wt $\beta$ chain in SDS-polyacrylamide gels (Cook et al., 1988; Tseng et al., 1992). No other mutations were found in this gene. In further support for the presence of only a single mutation, a nrdB93 clone constructed by site-directed mutagenesis of the cloned nrdB gene produced the $\beta_3$ protein exhibiting the same enzymatic and physical behavior as that generated after infection by phage T4 39-01 nrdB93 (Tseng et al., 1992).

When nrdB93 was discovered as a second mutation present in the gene 39 ts G41 mutant, the two functions were suspected of being associated (Wirak et al., 1988), though the original search employed a mutagen (Epstein et al., 1963). The direction of the transitional mutation in nrdB93 fits with the use of 5-bromodeoxyuridine mutagenesis (C$\rightarrow$T) in the isolation of the original ts G41 mutant. T4 nrdB93 grows very poorly whereas T4 39-01 shows wt growth, with only a slight delay in the initiation of T4 DNA replication. Therefore, we reasoned that nrdB93 was the initial lesion and that 39-01 had been selected as a suppressor of the nrdB93 defect.

(b) Comparison of transcriptional activities of nrdB and nrdB93

The low level of $\beta_3$ protein that accumulates during infection with T4 nrdB93 phage (Cook et al., 1988) might be explained by a decreased transcriptional activity. Recent work from this laboratory showed that transcription of the nrdB gene initiates from a motA-dependent
promoter, \( P_{\text{nrdB}} \), 2–2.5 min after infection at 30°C (Tseng et al., 1990). Primer extension analysis and RNA sequencing by a reverse transcriptase using primer 1 (Fig. 1) were employed to determine the kinetics of formation and the sequence of the 5' end of the mRNA encoding \( \text{nrdB} \). In Fig. 3, we demonstrate that the \( \text{nrdB93} \) mRNA initiated from \( P_{\text{nrdB}} \) was detectable at approx. 2 min after infection and quickly attained its highest level which was maintained throughout the time-course of the experiment. In addition, sequencing of the mRNA isolated at 8 min after infection demonstrates that the transcript is initiated at the same nt as the transcript synthesized during wt infection (Tseng et al., 1992).

During wt infection, transcripts originating from \( P_{\text{nrdB}} \) extend through the entire \( \text{nrdB} \) gene and continue on through the \( \text{denA} \) gene and gene 63 (Mileham et al., 1980; Sjöberg et al., 1986; Tseng et al., 1992). Since the lesion in the \( \text{nrdB93} \) gene might cause premature termination of transcription of the mutant gene, the relative levels of the mRNA upstream and downstream from the mutation site were measured by dot blot hybridization of the RNA prepared at 2, 5, 8, 11 and 14 min after infection by \( \text{nrdB} \) and \( \text{nrdB93} \) phage to radiolabeled DNA probes complementary to the two regions (Fig. 4). After infection with either \( \text{nrdB} \) or \( \text{nrdB93} \) phage, the mRNA segments corresponding to the two regions were formed with virtually identical kinetics, indicating no premature termination. Furthermore, the sequence of the intron splice junction, GCCGUGUAC, determined by RNA sequencing using primer 3, was the same in mRNA isolated after phage \( \text{nrdB93} \), 39-01 \( \text{nrdB93} \), and T4D infections (data not shown). Also, the sequence of the 5'-terminal segment of the intron, determined by RNA sequencing using primer 2 complementary to a sequence 75 nt inside the intron region (see Fig. 1), showed that the spliced 5' end of the intron coincided exactly with that of wt (data not shown). These results argue strongly that the transcription of the \( \text{nrdB93} \) gene and post-transcriptional processing of the \( \text{nrdB93} \) mRNA are not limiting the synthesis of \( \beta_3^3 \) protein chain.

(c) Stability of \( \beta_3^3 \) protein

One explanation for the low levels of the \( \beta_3^3 \) chain after infection by T4 \( \text{nrdB93} \) might be the rapid degradation of the \( \beta_3^3 \) protein. To examine its metabolic turnover, the proteins of T4 \( \text{nrdB93} \)-infected cells were labeled with \( [35S] \)Na\(_2\)SO\(_4\) (Cook and Seasholtz, 1982). To prevent
Fig. 4. Dot blot hybridization analysis of RNA prepared after infection with T4 nrdB93 and T4D, using exon I and exon II fragments as probes. Aliquots (5 μg) of total RNA prepared at various times after infection were subjected to dot blot hybridization using DNA probes derived from exon I or exon II fragments. The probe for exon I mRNA was the HindIII-BglII fragment and the probe for exon II mRNA was the KpnI-SnaBI fragment (see Fig. 1). Radioactivity in the hybridized fraction was determined with a Betagen Betascope 603 blot analyzer. An autoradiogram of the hybridization is shown in the upper left corner of each graph. The exposure time for the exon I autoradiogram was approx. 10 h and for the exon II autoradiogram about 36 h. Methods: RNA was prepared from cells infected at 30°C as previously described (Tseng et al., 1990). DNA probes were labeled by the random primer labeling technique according to the manufacturer’s specifications (Amersham, Arlington Heights, IL, USA). Hybridization was carried out with 5 x 10⁶ cpm of each DNA probe at a specific activity of 1 x 10⁸ cpm/μg of DNA using published procedures (Sambrook et al., 1989). The extent of hybridization was directly related to the amount of RNA used in the hybridization over a range from 1 to 10 μg of RNA (data not shown).

Further protein synthesis, Cm was added at 8.5 min after infection. Aliquots of infected cells were removed at various times afterward, and the proteins were subjected to two-dimensional gel electrophoretic analysis followed by autoradiographic exposure of the resulting gels for five or more days (Fig. 5). Because in the absence of the specific suppressor mutation of gene 39 the concentration of the β93 chain was so low, only minimal radioactivity signals corresponding to the β93 chain were detected at its position on the two-dimensional gel. However, direct measurement of radioactivity in the spots by scintillation spectrophotometry showed no decrease in concentration of the β93 chain after Cm addition. To determine whether the β93 protein was more prone than β2 to degradation in vitro, purified proteins were incubated for up to 1 h at 37°C with the 30 000 x g supernatants of extracts prepared from sonicates of T4-infected cells. No increased degradation as compared to the wt protein was observed (not shown). Crude membrane preparations, characterized by their NADH oxidase activity, an enzyme found only in the cytoplasmic membrane of E. coli, also caused no degradation (data not presented). Since after infection with 39-01 nrdB93 the β93 chain is found at wt levels, it could be argued that gene 39 protein is a protease. However, purified gene 39 protein had no effect on the integrity of β93 protein (data not shown). Taken together, these results rule out the possibility that the low level of β93 found upon nrdB93 infection is caused by an increased degradation of the mutationally altered subunit.

These studies demonstrate that the nrdB93 defect is not at the transcription nor the intron-splicing steps, nor is it a result of increased degradation of the mutant protein chain.

To better understand the nature of the nrdB93 defect, it is necessary to recount the factors involved in the expression of T4 nrdB93 during infection. Table I shows the relative levels of the β93 or β2 protein after infection of E. coli with the following phage; nrdB93, a gene 39 mutant, the double mutant, 39-01 nrdB93, or wt (Wirak et al., 1980; 1988; Wirak, 1981; Cook et al., 1988; G.R. Greenberg, personal communication). Infection with T4

<table>
<thead>
<tr>
<th>Phage</th>
<th>Temperature°C</th>
<th>β2 or β93 level</th>
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</thead>
<tbody>
<tr>
<td>39+ nrdB+ (wt)</td>
<td>30/41</td>
<td>++</td>
</tr>
<tr>
<td>39-01 nrdB+</td>
<td>30/41</td>
<td>++ +d</td>
</tr>
<tr>
<td>39+ nrdB93</td>
<td>30</td>
<td>+/−</td>
</tr>
<tr>
<td>39+ nrdB93</td>
<td>41</td>
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</tr>
<tr>
<td>39-01 nrdB93</td>
<td>30</td>
<td>+ + d</td>
</tr>
<tr>
<td>39-01 nrdB93</td>
<td>41</td>
<td>+ + d</td>
</tr>
<tr>
<td>39-01 nrdB93 (host gyrB)</td>
<td>30</td>
<td>+/−</td>
</tr>
</tbody>
</table>

* Phages are described elsewhere (Cook et al., 1988; Wirak et al., 1988).
+ Host gyrB mutant is hamB104 (Wirak et al., 1988).
+ 30/41 means at either temperature.
+ Relative levels of β2 or β93 protein formed after infection of E. coli by the T4 phages nrdB+ (wt), nrdB93, 39-01, and the double mutant 39-01 nrdB93, as measured by two-dimensional electrophoresis. +/− indicates slight synthesis. + + d indicates a delay of a few min in β2 and β93 protein formation upon infection by phage 39-01 as compared to β2 protein formation upon wt T4 phage infection (Wirak et al., 1980), based on the tritium release assay (Tomich et al., 1974).
Fig. 5. \( \beta^{\text{r}}_{93} \) stability in vivo; isotope turnover experiments. Two-dimensional electrophoretic gel patterns of \(^{35}\text{S}\)-labeled proteins synthesized after T4 \( \text{nrdB}^{93} \) infection at 30°C. \(^{35}\text{S}\)Na\(_2\)SO\(_4\) was added to T4 \( \text{nrdB}^{93} \)-infected cells 3.5 min after infection. Protein synthesis was stopped at 8.5 min by addition of Cm to a final concentration of 100 \( \mu \text{g/ml} \). 2-ml aliquots, equivalent to \( 1 \times 10^9 \) infected cells, were removed at (A) 8.5 min, (B) 12.5 min, (C) 16.5 min and (D) 25 min after infection. Samples equivalent to \( 2 \times 10^8 \) infected cells were analyzed on each two-dimensional gel. The arrows point to the indicated \( \alpha, \beta^\text{r} \) and gene 39 proteins. Methods: Infection of E. coli B with bacteriophage T4 was performed as described in earlier papers (Tomich et al., 1974; Tseng et al., 1988). Isotopic labeling of phage-encoded proteins with \(^{35}\text{S}\)SO\(_4\)\(^{2-}\) (New England Nuclear) essentially followed the method described previously (Cook et al., 1982, 1988). Two-dimensional, nonequilibrium, pH-gradient electrophoresis was carried out as described earlier (Cook et al., 1982), except that the infected cells were extracted directly with SDS to prevent possible degradation (Burre et al., 1983).

39\(^+\) \( \text{nrdB}^{93} \) at 30°C results in a very low level of \( \beta^{\text{r}}_{93} \) chain in the cell. By contrast, infection with the T4 39-01 \( \text{nrdB}^{93} \) double mutant results in a synthesis of its \( \beta^{\text{r}}_{93} \) protein equivalent to that of wt infection. However, upon infection by the double mutant at 41°C, the \( \beta^{\text{r}}_{93} \) is both inactivated and unable to bind to the \( \alpha_2 \) subunit to yield the active \( \alpha_3\beta_2 \) enzyme complex. Even though the \( \beta^{\text{r}}_{93} \) chain is formed at wt levels (Cook et al., 1983; 1988), a temperature-insensitive suppression of the synthetic defect of \( \text{nrdB}^{93} \) occurs by the site-specific mutation in gene 39 or by an amber mutation (amN116) in gene 39 (Wirak et al., 1988), but not by tsA41, a mutation at another site in the gene. This suppression is specific to the gene 39-encoded protein and does not involve T4 DNA topoisomerase activity (Cook et al., 1988).

These data suggest that translation of wt \( \text{nrdB} \) mRNA is regulated in a positive fashion by the \( \text{nrdB} \) gene product and in a negative manner by the gene 39 product. Thus, during \( \text{nrdB}^{93} \) infection, wt levels of the mutant protein can be formed only when gene 39 is either site-specifically mutated or absent. In addition, Table I shows that the host \( \text{gyrB} \) gene is an integral part of this system, since little \( \text{nrdB}^{93} \) protein synthesis is seen in the absence of an active host \( \text{gyrB} \) product. The concept of a translational mechanism controlling the \( \text{nrdB} \) expression is strengthened by recent findings that the proteins encoded by gene 39, \( \text{nrdB} \) (and \( \text{nrdB}^{93} \)) and the host \( \text{gyrB} \) gene have been shown to play roles in regulating the binding of the E. coli 30S ribosome/tRNAs\(^\text{Met} \) complex by the ribosome-binding region (see McCarthy and Gualerzi, 1990) of T4 \( \text{nrdB} \) mRNA through their specific interactions with the mRNA (P. He and G.R. Greenberg, data not shown).

(d) Conclusions

(1) The \( \text{nrdB}^{93} \) defect is a single nt change, from G\(^{258}\)\( \rightarrow \)A, leading to the Gly\(^{253}\)\( \rightarrow \)Asp change.

(2) The \( \text{nrdB}^{93} \) defect is not caused by a defect in transcription, post-transcriptional processing of \( \text{nrdB}^{93} \) mRNA, or an increased degradation of \( \beta^{\text{r}}_{93} \). The data presented in this report are consistent with the concept that the defect is at the level of translation.
(3) These results and previous findings (Cook et al., 1988; Wirak et al., 1988) provide strong argument that the products of the T4 \textit{nrdB} and 39 genes and the host \textit{gyrB} gene have roles in the control of the \textit{nrdB} translation.

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