JOURNAL OF BACTERIOLOGY, Nov. 1990, p. 6323–6332
0021-9193/90/116323-10$02.00/0

Vol. 172, No. 11

Bacteriophage T4 *nrdA* and *nrdB* Genes, Encoding Ribonucleotide Reductase, Are Expressed Both Separately and Coordinately: Characterization of the *nrdB* Promoter

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Received 30 April 1990/Accepted 18 August 1990

We examined the expression of the bacteriophage T4 *nrdA* and *nrdB* genes, which encode the α_2 and β_2 subunits, respectively, of ribonucleoside diphosphate reductase, the first committed enzyme in the pathway of synthesis of the deoxyribonucleoside triphosphates. T4 *nrdA*, located 700 bp upstream from *nrdB*, has been shown previously to be transcribed by two major transcripts: a prereplicative, polycistronic message, T_U, originating at an immediate-early promoter, P_E, that is 3.5 kb upstream from *nrdA*, and a postreplicative message commencing from a late promoter in its 5′ flank. We have found a third promoter initiating a transcript at 159 nucleotides upstream from the reading frame of *nrdB*. P_{nrdB} functions only in the presence of the T4 *motA* gene product, which is required for middle (time) promoters, and therefore the onset of *nrdB* transcription is delayed more than 2 min after infection. Because of the distance of *nrdA* from P_E, the inception of *nrdA* transcription (delayed early) coincides closely with that of *nrdB*. An apparent termination site, t_A, occurs about 80 bp downstream from *nrdA*. Some of the polycistronic mRNA reading through the site after 5 min contributes to *nrdB* transcription. *nrdA* and *nrdB* genes in an uninfected host have been reported to be transcribed only coordinately. In contrast, T4 *nrdA* and *nrdB* are initially transcribed separately onto the P_E and P_{nrdB} transcripts, respectively, but at about 5 min after infection are transcribed both coordinately and on separate transcripts. Evidence is presented that T_U coordinately transcribes a deoxyribonucleotide operon in the order: *frd*, *td*, gene 'Y,' *nrdA*, *nrdB*. Since the β_2 subunit is known to be formed after the α_2 subunit, the expression of the *nrdB* gene determines the onset of deoxyribonucleoside triphosphate synthesis and thus of T4 DNA replication.

Bacteriophage T4 ribonucleoside diphosphate reductase appears to be the limiting factor in the initiation and rate of deoxyribonucleotide synthesis in infected cells (11, 12, 15). In turn, the onset of phage DNA replication, which occurs at about 5 min after infection, is dependent on the turning on of deoxyribonucleotide synthesis (13, 33, 45, 51). Accordingly, workers in our laboratory are investigating the control of expression of the T4 *nrdA* and *nrdB* genes, which encode the α_2 and β_2 subunits, respectively, of ribonucleotide reductase (4, 5, 16, 36, 39, 43, 46). *nrdC*, which encodes phage thioredoxin, is unlikely to be limiting in the initiation of ribonucleotide reductase synthesis (7, 46). It may be mentioned that after infection by wild-type phage T4 the ribonucleotide reductase of the host ceases to function (6). However, after infection with *nrdA* or *nrdB* mutants of phage T4, the host enzyme continues to function and provides about 25% of the enzyme activity found after wild-type phage infection (12).

Recently, we reported the sequence of T4 *nrdA* and its flanking regions (46). Figure 1 shows a map of this region of the phage genome. *nrdA*, lying between two intron-containing genes, *td* (thymidylate synthase) and *nrdB* (14, 43), is transcribed onto two major mRNAs (46). One, T_U, is a prereplicative, polycistronic message that is considered to initiate at an immediate-early promoter, P_E, upstream from the *frd* gene (32, 42), and the other transcript, T_L, originating from a late promoter, P_L, in the 5′ flank of *nrdA*.

In this paper we describe a third promoter, P_{nrdB}, which is located upstream from *nrdB* and is dependent on the product of the T4 *motA* gene. An apparent mRNA termination site following *nrdA* is also reported. While *nrdA* and *nrdB* are coordinately expressed in *Escherichia coli* (21, 22, 47, 48), the corresponding phage T4-encoded genes are expressed initially from separate messages, as in eucaryotic systems (8, 19). We demonstrate that some readthrough of the RNA termination site occurs, thus giving rise later not only to simultaneous separate expression of the two genes but, to a lesser degree, to coordinate expression. Finally, the kinetic aspects of the onsets of expression of *frd*, *nrdA*, and *nrdB* are compared.

(Some of the results are taken from a Ph.D. thesis submitted by M.-J.T. to the University of Michigan in 1989.)

MATERIALS AND METHODS

Biological components. The bacterial and phage strains and plasmids employed have been described previously (46). Phage T4 *amG1* (*motA* [35]), its suppressor strain, *E. coli* B^E NapIV *supD*, and *E. coli* B^E NapIV (38) were kindly provided by David Parma, University of Colorado, Boulder.

Enzymes and chemicals. Restriction enzymes were purchased from New England BioLabs, Bethesda Research Laboratories, and Boehringer Mannheim. Vaccinia guanylyltransferase and T4 polynucleotide kinase were obtained from Bethesda Research Laboratories. The Klenow fragment of DNA polymerase I, DNase I, dideoxyribonucleoside triphosphates, deoxyribonucleoside triphosphates (dNTPs), avian reverse transcriptase, and calf intestinal phosphatase were from Boehringer Mannheim. RNAsin was from Promega Corp. Radioactive nucleotides and GeneScreen Plus membranes were from Du Pont-New England Nuclear. The 17-mer oligodeoxyribonucleotide primer, GGG

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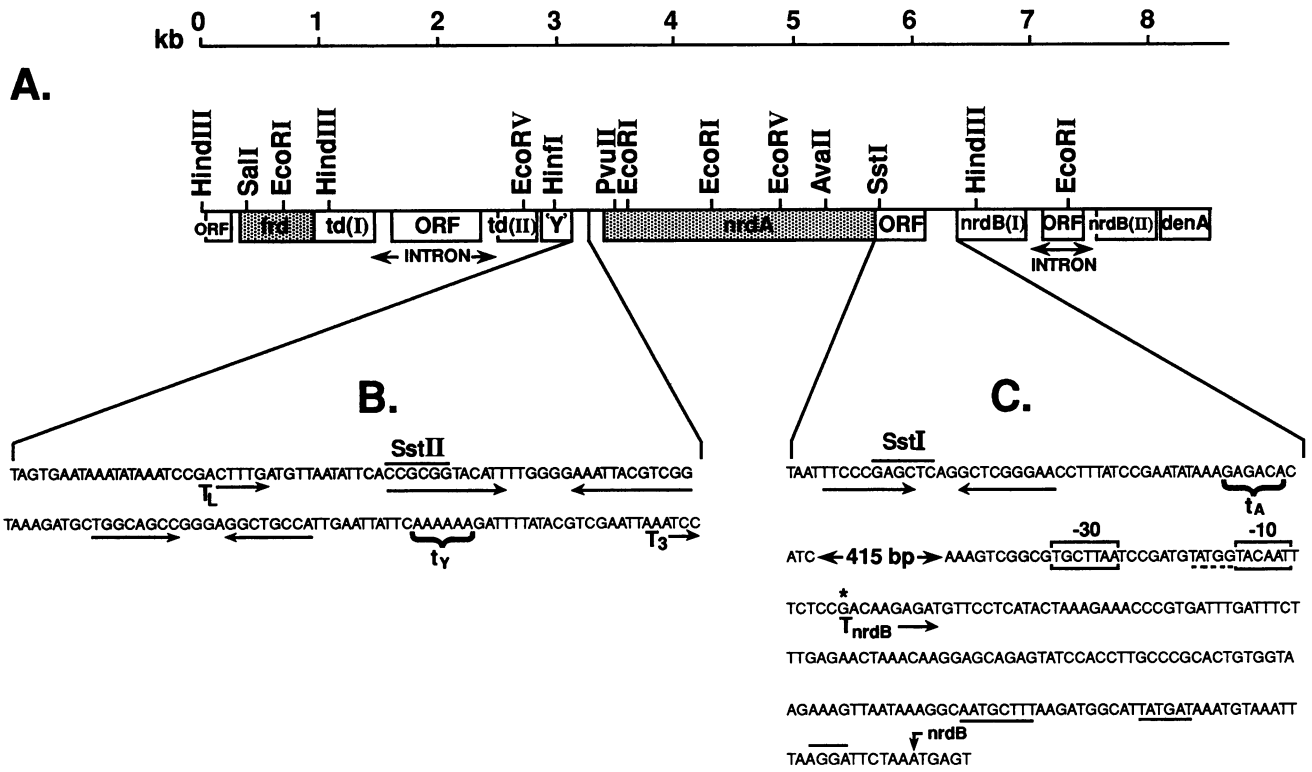

FIG. 1. *frd-td-'Y'-nrdA-nrdB-denA* region of the phage T4 genome. (A) As determined by sequence analysis, the distance between *Hin*dIII at 0 kb and *Hin*dIII in exon I of *nrdB* is 6.499 kb. At the present time the *Hin*dIII site at 0 kb is taken to be at 144.154 kb relative to zero (assigned to a point between the rIIA and rIIB genes) and progresses clockwise on the 166-kb genetically circular genome; this value varies as other sequences in the genome are determined (31; E. Kutter, B. Guttman, G. Mosig, and W. Rüger, unpublished data; E. Kutter, personal communication). Transcription is from left to right (counterclockwise). Only restriction sites mentioned in the text are shown. The 'Y' gene (not to be confused with the *uvsY* gene) and its protein product are described elsewhere. (46; Hilfinger, Tseng, and Greenberg, unpublished data). The ORF at 0 kb is *frdorf1* (42). (B) Sequence of a segment of the region between the *td* and *nrdA* genes. The site of a possible processing site, t_Y, is indicated. Potential hairpin structures are indicated by convergent arrows. The sites of initiation of the late transcript, T_L, and the minor, transient transcript, T_3, are indicated (46). (C) Site of initiation of the *nrdB* transcript. The asterisk at the G at −159 bp indicates the initiation site of T_{nrdB}. The *motA* −10 and −30 promoter sequences proposed in this paper for P_{nrdB} are indicated by both solid underlines and overlines, while the extended −10 site of Liebig and Rüger (32) is indicated by dashed underlines. The *nrdB* ribosome-binding site is indicated by overlines, and solid underlines indicate the −30 and −10 sites of an apparent *motA* promoter sequence observed previously (43). The limits of the 141-residue open reading frame following the *nrdA* gene are indicated (see Discussion). t_A is a termination site of *nrdA* transcripts (Fig. 7). A potential hairpin structure is indicated by convergent arrows. ORF, Open reading frame.

CAAGGTGGATACTC, was synthesized by Charles Mountjoy, DNA Synthesis Facility, University of Michigan.

Infection by bacteriophage T4. *E. coli* B was grown at 37°C to a concentration of 3×10^8 to 4×10^8 cells per ml in Vogel-Bonner medium (49) containing 0.2% glucose and a 0.1% enzymatic hydrolysate of casein, and the cells were infected at 30°C by phage T4D at a multiplicity of 8 (46). After rapid cooling in ice or pouring over crushed, frozen medium, infected cells were harvested at the times indicated in the legends to the figures.

Isolation and purification of nucleic acids. Plasmid DNA, M13 DNA, and RNA formed after T4 infection were prepared as described previously (46). In the primer extension analysis an extraction with phenol at 68°C preceded the treatments with phenol and chloroform at room temperature.

Labeling of 5′ and 3′ termini of DNA fragments. DNA fragments generated by restriction enzymes were labeled at their 3′ ends by incubation in 25 μl of 10 mM Tris-chloride (pH 8.0)–5 mM $MgCl_2$ with 50 μCi of an [α-^{32}P]dNTP complementary to the first base in the 5′ extension of the termini, using 2 U of the Klenow fragment of DNA polymerase I at 37°C for 30 min. Labeled fragments were separated from the labeling nucleotides by using Elutip columns (Schleicher & Schuell), as described by Davis et al. (17).

The 5′ ends of dephosphorylated DNA fragments were labeled with [γ-^{32}P]ATP (3,000 Ci/mmol) and isolated as described previously (46).

Uniform labeling of DNA probes. For labeling of DNA probes we used a random primer labeling kit from Boehringer Mannheim (20).

Hybridization and S1 nuclease mapping. Hybridization and S1 mapping were carried out essentially as described previously (26). For experiments with RNA labeled at the 5′ terminus, 15 μg of capped T4 RNA was incubated with 10 μg of a single-stranded M13mp19 DNA containing a segment from the *Sst*I site just downstream from the *nrdA* gene to the *Hin*dIII site in the 5′-terminal region of the *nrdB* gene. The reaction mixtures contained 140 U of S1 nuclease for end-labeled DNA and 3,150 U for radioactively capped RNA.

Primer extension analysis of mRNA initiation site. The procedure used for primer extension analysis of the mRNA initiation site was that of Guild et al. (26), with minor modification.

Capping of initiation termini of RNA from T4-infected cells.

To determine whether 5′ termini initiated with di- or triphosphate groups, RNA capping studies were carried out by using 40 μg of RNA from T4-infected cells as described previously (26). The resulting reaction mixture was subjected to hybridization with specific T4 DNA fragments and to S1 nuclease mapping as described above and in Results.

Kinetic analysis of appearance of mRNAs by hybridization. For the dot blot hybridization technique of Kafatos et al. (30), we used GeneScreen Plus membranes and direct measurement of the radioactivity of the bound probe. Details are given in the legend to Fig. 6.

Computer searches. DNA sequences were searched for secondary structure, specific sequences, and −10 and −35 promoter sites as reported previously (46).

RESULTS

Initiation site of phage T4 *nrdB* mRNA: S1 nuclease analysis. The 5′-end-labeled DNA probe used to study the transcript for the *nrdB* gene was an 853-bp restriction fragment (Fig. 2). Figure 2 shows a protected fragment of about 356 bp detected at 5, 8, and 15 min after infection. The size of the fragment corresponded to an initiation site at about 162 bp from the *nrdB* reading frame. A *motA* promoter sequence pointed out by Sjöberg et al. (43), which has its −30 site centered about −44 bp from the *nrdB* translatable frame, appears to be nonfunctional.

In the same gel a fragment equal in size to the original probe was found at 8 and 15 min, but not at zero time and only in trace amounts at 5 min. Accordingly, this band was not derived by self-hybridization of the probe, but represented RNA transcribed upstream from the *nrdB* promoter site (i.e., polycistronic mRNA reading through the t_A termination site) (see below). At 8 and 15 min the quantities of this fragment were estimated by using density scanning to be about 30 and 40%, respectively, of the quantities of the transcript from the *nrdB* promoter. The detection of the large fragment at about 5 min after infection was in agreement with the time required for the polycistronic mRNA, T_U, to reach the t_A site. Determination of the initiation site of the *nrdB* transcript by primer extension analysis confirmed the readthrough of the polycistronic message (see below).

Primer extension analysis of the *nrdB* mRNA initiation site. A 17-mer oligodeoxyribonucleotide complementary to the mRNA between 76 and 92 nucleotides upstream from the *nrdB* translatable frame, was used as the primer. Figure 3 shows the results at 3.5 and 10 min after infection. At both times the mRNA was found to initiate with a guanylate residue 159 nucleotides before the *nrdB* translatable frame. Figure 3 also shows the sequence in the region of the initiation site. Uninfected controls showed no initiation sites (data not presented).

Notice that at 3.5 min after infection no evidence of RNA was observed upstream from the *nrdB* initiation site. By 10 min the reverse transcriptase was able to continue beyond the initiation site and revealed the sequence of an RNA transcribing immediately upstream (Fig. 3, lower-case letters). Thus, mRNA originating upstream is entering into the *nrdB* region and most certainly represents polycistronic messenger that has failed to terminate at the terminator, t_A (see below). These experiments do not suggest that T_U competes with initiation at the *nrdB* promoter, P_{nrdB}.

Capping of *nrdB* transcript. To determine whether T_{nrdB} was a primary transcript possessing a 5′ triphosphate terminus or was a product of processing, the T4 mRNA was treated with the vaccinia capping system, guanylyltransferase, and [α-^{32}P]GTP and then subjected to S1 nuclease analysis (37). The capped T4 RNA was hybridized with M13mp19 DNA containing the same restriction fragment employed as the probe in the analysis of the 5′ terminus of the *nrdB* gene (Fig. 2), and the product was subjected to S1 nuclease treatment. Protected bands with the same sizes as those shown in Fig. 2 were found at 5, 8, and 15 min after infection (Fig. 4), establishing that T_{nrdB} is a primary transcript. From these studies we judge that significant portions of the S1 nuclease-protected *nrdB* transcripts possess 5′ triphosphate initiation structures at 5, 8, and 15 min. Figure 1 summarizes the findings shown in Fig. 2 through 4.

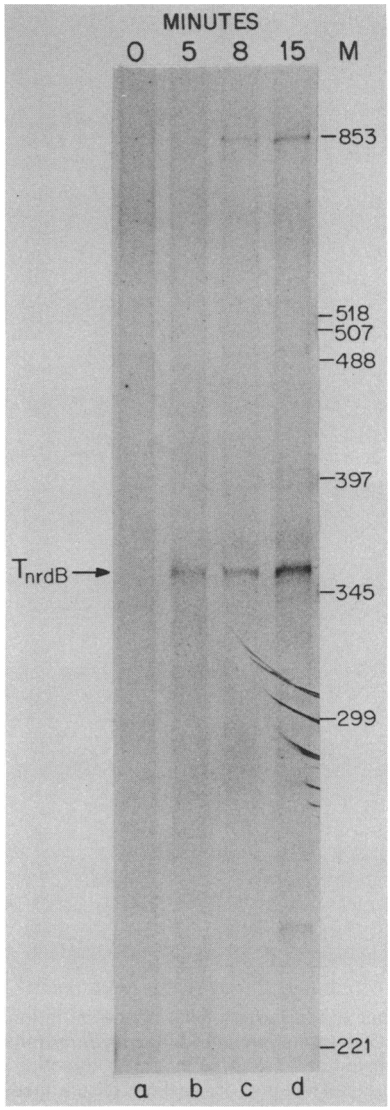

FIG. 2. Mapping of wild-type T4 mRNA by the S1 nuclease protection method, showing a 5′ end upstream from the *nrdB* gene. *E. coli* B cells were collected (following rapid cooling of the cultures in ice) before infection and at 5, 8, and 15 min after infection. Each of the RNA preparations from these cells was hybridized with the 853-bp restriction fragment extending from the *Sst*I site just downstream from the *nrdA* gene to the 5′-terminal region of the *nrdB* gene and labeled with $^{32}P_i$ at the *Hin*dIII 5′ terminus. One protected fragment of about 356 nucleotides, (T_{nrdB}) was present after S1 digestion of the 5-, 8-, and 15-min mRNA-DNA hybrids (lanes b, c, and d, respectively).

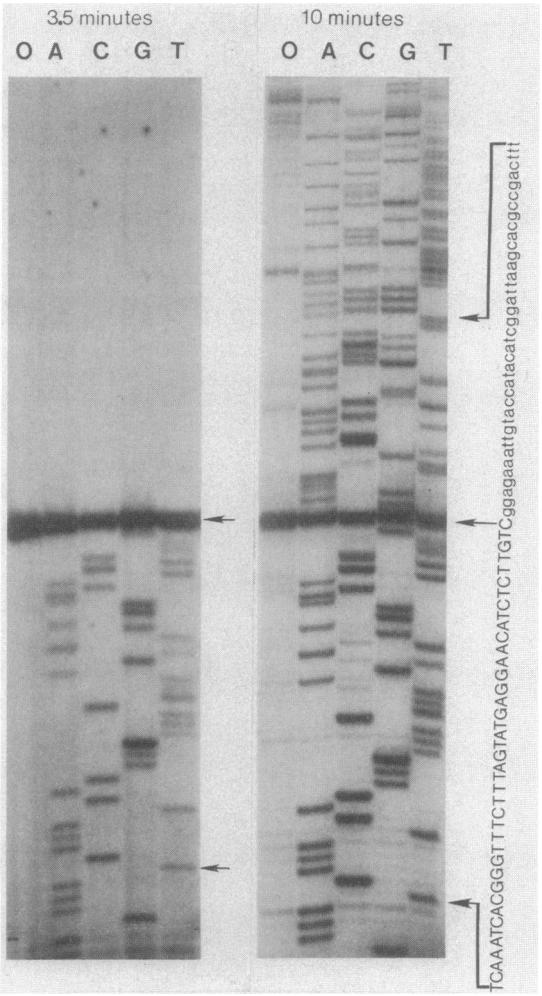

FIG. 3. Primer extension analysis of the initiation site and sequencing of the 5'-terminal segment of the *nrdB* promoter transcript. Cells were collected at 3.5 and 10 min after infection by pouring a culture over an equal volume of crushed, frozen medium (−20°C). At each time point, 80 μg of isolated RNA (about 5×10^9 cells) was hybridized with 1 pmol of primer. The reaction was carried out with reverse transcriptase in the presence dNTPs and dideoxyribonucleoside triphosphates. The very dark bands at the center represent the 5' initiation site. The lowest arrows indicate the positions of the same thymidylate residues in the 3.5- and 10-min assays. In lanes O the runoff in the absence of dideoxyribonucleotides is shown. More extensive exposure of films to these gels showed no evidence of bands beyond the initiation at the 3.5-min site (data not shown; see text).

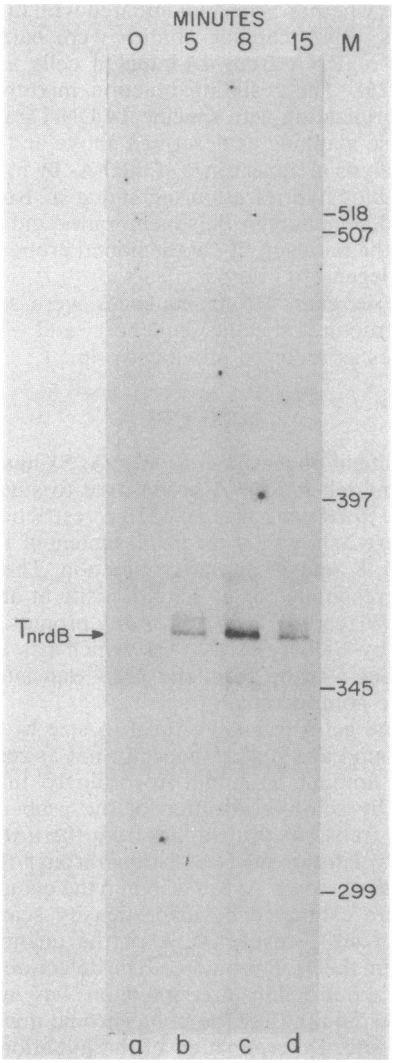

FIG. 4. T_{nrdB} is a primary transcript based on S1 nuclease mapping of guanylyl-capped wild-type T4 mRNA. RNA prepared from an *E. coli* B culture before infection and at 5, 8, and 15 min after infection was labeled at the 5' triphosphate initiating end by using [α-^{32}P]GTP and the vaccinia capping enzyme, guanylyltransferase. Each capped RNA preparation was hybridized with phage M13mp19 single-stranded DNA containing the *Sst*I-*Hin*dIII fragment extending from the *Sst*I site just downstream from the *nrdA* gene to the 5'-terminal portion of the *nrdB* gene, and the product was digested with S1 nuclease. One protected fragment of about 356 nucleotides, T_{nrdB}, was found at 5, 8, and 15 min after infection (lanes b, c, and d, respectively).

P_{nrdB} is a *motA*-dependent promoter. Our finding that P_{nrdB} activity is dependent on the product of the *motA* gene was determined as follows. When chloramphenicol, which blocks protein synthesis and gives rise to shorter transcripts (9), was added at the time of infection, the initiation of *nrdB* transcription was greatly decreased. The *frd* transcript was essentially unaffected. The *frd* reading frame is about 0.42 kb from its promoter. On the other hand, the transcript probed by a fragment in the 5' segment of *nrdB* was absent after chloramphenicol treatment. Therefore, it was clear that a protein factor was required for transcription of the *nrdB* gene from P_{nrdB} (He, data not shown). Analysis of the P_{nrdB} region revealed a plausible *motA*-dependent promoter sequence (Fig. 1C) (see Discussion). Since P_{nrdB} does not initiate its transcripts until after 2 min of infection and since the *motA* product has been shown to be required for T4 middle-promoter function (9, 26, 35), the appearance of the P_{nrdB} transcripts was examined after infection by a T4 phage carrying an amber mutation of the *motA* gene. Figure 5 shows the results of an experiment comparing the P_{nrdB} messages by the primer extension method after wild-type T4 infection and after infection by the *amG1 motA* mutant. The *motA* mutant formed little or no P_{nrdB} message (Fig. 5A). In contrast, the wild-type phage formed the message, which again originated at −159 bp from the *nrdB* frame. As a control, we determined the effect of *motA* infection on the expression of *frd*, which is known to be dependent on an

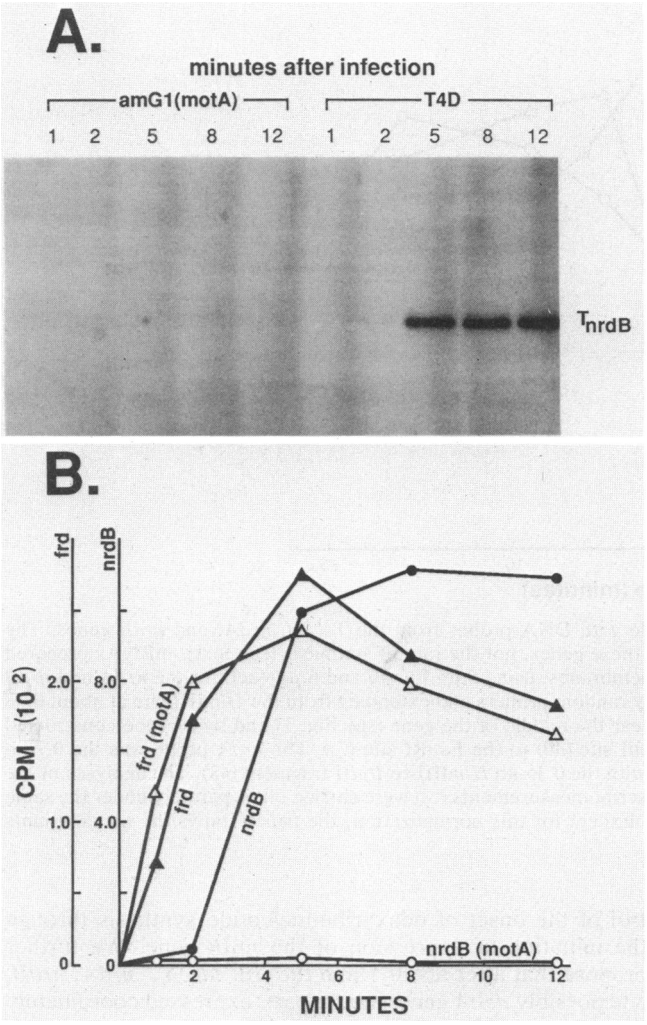

FIG. 5. P_{nrdB} is a *motA*-dependent promoter. (A) Comparison of the formation of P_{nrdB} transcripts after infection by T4D and by *amG1*, a *motA* mutant. The isolated mRNAs were analyzed by primer extension analysis, as described for lane O in the legend to Fig. 3. The bands found in the electrophoretic analysis of the mRNA from T4D infection are at the site of initiation of the P_{nrdB} transcript (see Fig. 3), but these bands were not observed after infection by the *motA* mutant. (B) Comparison of the effects of infection by a *motA* mutant on the transcription of the *frd* and *nrdB* genes (*frd* transcription was not affected by *motA* infection). The *frd* values were obtained by hybridization through dot blotting. The radioactive hybrid spots, located on the membrane by exposure to X-ray film, as well as the bands from panel A, were cut out and counted in a liquid scintillation spectrophotometer. Essentially the same results were obtained after infection of *E. coli* B or *E. coli* B^E NapIV (see reference 26), the parent of the amber suppressor strain (38).

immediate-early promoter. Indeed, as measured by dot blot hybridization, infection by the *motA* mutant appeared to have no effect on *frd* expression or the degradation of the transcript (Fig. 5B). We conclude that P_{nrdB} is a *motA*-dependent promoter.

Kinetic analysis of the onset of transcription of the *frd*, *nrdA*, and *nrdB* genes after phage infection. RNA was analyzed at various times after infection by dot blot hybridization with separate labeled DNA probes covering the 5′ segments of *frd*, *nrdA*, and *nrdB* (Fig. 6). Because of the normally rapid rate of degradation of procaryotic mRNAs, the data in Fig. 6 are estimates of the timing of events, primarily initiation, and are not meant to be used to compare the concentrations of the RNAs. The *frd*-hybridizable transcript was clearly present by 1 min, as expected from the distance of this gene from its early promoter (see Discussion), and reached an apparent maximum level at about 5 or 6 min. In the experiment shown in Fig. 6 the *nrdB* message, assayed by using a probe within exon I of the gene, was not apparent until after 2 min and did not reach its maximum net rate of increase until about 4 min. The *nrdA* message also did not appear to be significantly elevated above the counts in the uninfected culture until after 2 min of infection and reached its maximum net rate of increase by about 3 min. A second run gave essentially the same results as those shown in Fig. 6.

The *nrdA* probe began at about 3.6 kb downstream from the beginning of the *frd* probe. The apparent maximum rates of increase for the *frd* and *nrdA* transcripts were separated by 2.1 min at 30°C, which corresponded to a transcription rate of about 29 nucleotides per s.

Finally, this experiment functionally verifies the conclusion that the polycistronic mRNA, T_U, cannot be involved in the initial phases of the transcription of *nrdB*. That is, both *nrdA* transcription and *nrdB* transcription initiated at essentially the same time, although the two transcripts were detected by using probes which had their 3′ ends at genomic positions separated by about 3.4 kb or some 2 min of transcription.

Evidence for an mRNA termination site, t_A, following the T4 *nrdA* gene. The mRNAs transcribing *nrdA* were analyzed by the S1 nuclease protection method. The DNA probe, labeled at its 3′ end, was a fragment extending from the 3′-terminal region of the *nrdA* gene into the 5′-terminal segment of the *nrdB* gene. On examination of the digested hybridization mixtures by gel electrophoresis and autoradiography, a protected fragment, referred to as t_A, was found (Fig. 7) at about 80 nucleotides downstream from the translatable frame of the *nrdA* gene, directly after a potential G·C-rich loop (Fig. 1C). Since no mRNA 5′ terminus resulting from a processing cleavage was found (Fig. 2), we tentatively consider t_A to be a termination site. Termination was found at 8 and 15 min after infection, but was just detectable at 5 min. Thus, the polycistronic transcript, T_U (see introduction and Discussion), approaches the termination site at about 5 min.

Possible mRNA processing site between the *td* and *nrdA* genes. A DNA probe revealed a 3′ terminus between the *td* and *nrdA* genes. This probe was a *Hin*fI-to-*Pvu*II restriction fragment, extending from the center of the 'Y' gene to a site within the 5′-terminal region of the *nrdA* gene (Fig. 1A). A protected fragment of about 236 nucleotides was found at relatively constant net levels at 5, 8, and 15 min after infection (Fig. 8) and corresponded to a site designated t_Y. Its 3′ end was estimated to fall within a run of five adenine residues centered 106 nucleotides downstream from the reading frame of the 'Y' gene, just beyond two potential hairpin loop structures (Fig. 1B). The discrete bands of smaller sizes are most probably products of 3′ RNA exonuclease activity (3). Note that the intensity of the bands with lengths of about 222 nucleotides increased with time. The sizes of these bands suggest that the exonuclease activity ceased at the 3′ terminus of the potential loop centered at about 192 nucleotides upstream from the *nrdA* frame. Taken together, the 236- and 222-nucleotide fragment fractions represent a significant level of 3′ ends. Our previous work

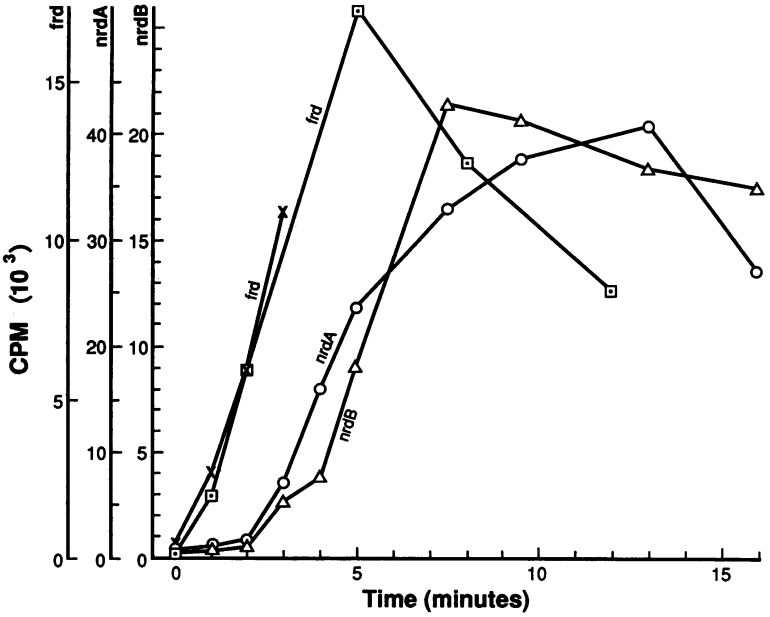

FIG. 6. Kinetics of appearance of the mRNA transcripts hybridizable with DNA probes from the T4 *frd*, *nrdA*, and *nrdB* genes. The purpose of this experiment was to compare the onset of transcription in these genes, not the rate of synthesis (see text). mRNAs prepared at the indicated times after infection were applied to GeneScreen Plus membranes, using 5 μg for *frd* and 6 μg each for the *nrdA* and *nrdB* probes. *frd* messenger was probed with a 0.7-kb DNA fragment labeled by random primers and extending from the *Hin*dIII site at about 0.38 kb upstream from the translatable element of *frd* to the *Eco*RI site (×) near the middle of the gene (see Fig. 1) and by a probe constructed from a 0.32-kb fragment falling entirely within the *frd* frame from the *Sal*I site (40) to the *Eco*RI site (□). The *nrdA* probe was the 0.7-kb segment that ran between the two *Eco*RI sites. *nrdB* mRNA was probed with the 0.35-kb *Hin*dIII-to-*Bgl*II fragment (43). The analyses of the dot blots were conducted as described in the legend to Fig. 5. The *frd* transcript measurements (□) were carried out separately under the same conditions, and the values were normalized (×) to the results at 2 min. Except for this normalization, the figure shows the actual counts obtained with each probe, uncorrected for size and specific activity.

(46) showed an uncappable, minor, transient transcript, T_3, with its 5′ end just downstream from t_Y. The significance of T_3 remains unknown, but it is possible that t_Y is a processing site and that the resulting transcript, T_3, after further processing, is degraded. t_A, located upstream from *nrdB*, and possibly t_Y, located upstream from *nrdA*, may regulate the levels of T_U and T_L that reach the downstream genes (Fig. 1).

DISCUSSION

Bacteriophage T4 *nrdB* is initially transcribed only by its own promoter, P_{nrdB}. The transcript initiates with a capable guanylate residue 159 nucleotides upstream from the translatable element, and either it is quite stable or its synthesis is extended over a period of at least 13 min (Fig. 2 through 6). Our evidence supports the concept that *nrdA* is initially transcribed by a polycistronic mRNA, T_U, which appears to derive primarily from a strong early promoter (32, 42) located upstream from *frd* (Fig. 1A). In addition, *nrdA* is transcribed from a late promoter in its 5′ flanking region. Perhaps one-third of the T_U transcripts read through the terminator, t_A, and after about 5 min of infection reach and transcribe *nrdB* (Fig. 9). Thus, *nrdA* and *nrdB* are expressed onto separate transcripts through at least 15 min, but after about 5 min the two genes also undergo coordinate transcription, although to a lesser degree (Fig. 2 and 3). With time, T_L may be expected to contribute a larger proportion of the transcripts of both *nrdA* and *nrdB*. Similar complex, overlapping transcription patterns have been described elsewhere in the T4 genome (2, 9, 10, 27, 28). This study relates such overlapping transcripts to development of precise control of the onset of deoxyribonucleotide synthesis through the initiation of expression of the *nrdB* gene. We further propose that after about 5 min the *frd*, *td*, 'Y,' *nrdA*, *nrdB*, and possibly *denA* genes are, in part, expressed coordinately (i.e., they represent an operon).

P_{nrdB} is a *motA*-dependent promoter (Fig. 5). A recent perceptive compilation of 14 *motA*-dependent promoters by Guild et al. (26) produced the consensus (a/t)(a/t)TGCTT(t/c)A—11 to 13 bp—TAnnnT, with n being an unspecified nucleotide. These promoters are distinguished by "−30" rather than "−35" sites and therefore by less space between the two segments of the promoter. The spacing between the −10 sites and the initial RNA nucleotide is mostly 5 to 7 bp, but some spaces vary between 3 and 12 bp. P_{nrdB} has the following comparable (underlined) structure (Fig. 1C): CGT GCTTAA—12 bp—TACAAT—6 bp—G.

The T4 *motA* gene gives rise to a protein that is required for the activity of T4 phage middle promoters (9). Middle promoters appear to be devices to regulate the timing of expression of genes in a given system and to be part of the overall clock in T4 infection (9, 26), in this instance as an integral part of the timing of *nrdB* expression.

Our findings are consistent with the idea that the transcript, T_U, initiates at a strong immediate-early promoter, P_E, which is about 0.42 kb upstream from *frd*, as described by Liebig and Rüger (32) and by Ruckman et al (42). Liebig and Rüger described a set of 29 early T4 promoters that are considerably more active than the *lacZ* promoter. Initially at least, such promoters have to function with unmodified *E. coli* RNA polymerase holoenzyme (23), although their −10 and −35 sites differ widely in sequence and in length from

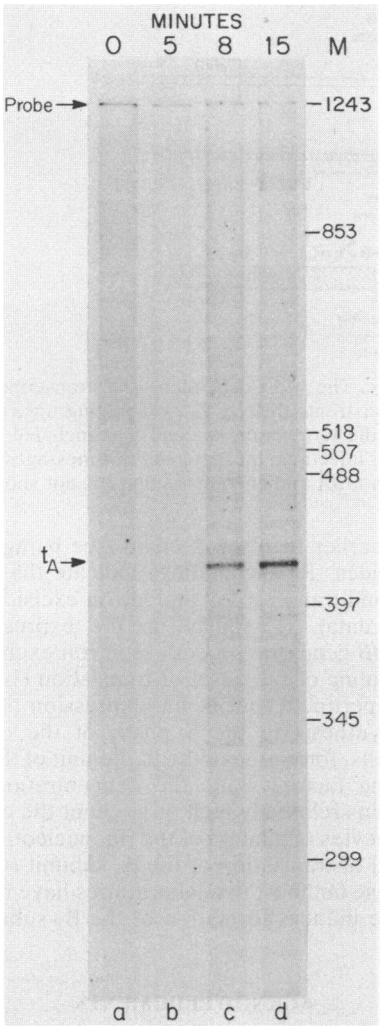

FIG. 7. S1 nuclease protection mapping of wild-type phage T4 mRNA reveals a termination site after *nrdA*. RNAs prepared from an *E. coli* B culture before infection and at 5, 8, and 15 min after infection with T4D were hybridized with the 1,243-bp *Ava*II-*Hin*dIII restriction fragment extending from the 3′-terminal region of the *nrdA* gene to the 5′-terminal portion of the *nrdB* gene. The fragment was labeled with [α-^{32}P]dGTP at the *Ava*II 3′ end. One protected fragment of about 428 nucleotides, t_A, was found after S1 digestion of the 8- and 15-min mRNA-DNA hybrids (lanes c and d, respectively). At 5 min a band is just perceptible (lane b). No fragments were protected by RNA isolated from the culture before infection (lane a).

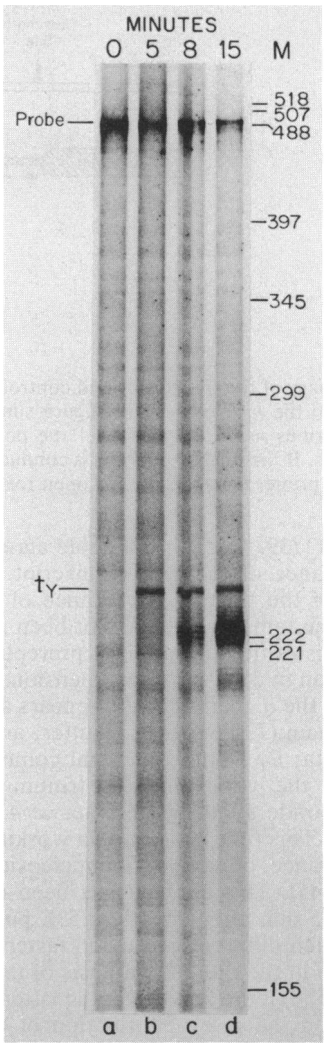

FIG. 8. S1 nuclease mapping of wild-type T4 mRNA shows a possible processing site between the *td* and *nrdA* genes. RNAs prepared from an *E. coli* B culture before infection and at 5, 8, and 15 min after infection with T4D were subjected to hybridization with the 488-bp *Hin*fI-*Pvu*II restriction fragment extending from the center of the 'Y' gene to the 5′-terminal region of *nrdA*. The analysis was performed as described in the legend to Fig. 7. The fragment was labeled with [α-^{32}P]dATP at its *Hin*fI 3′ end. One protected fragment of about 236 nucleotides, t_Y, was found after S1 digestion of the 5-, 8-, and 15-min mRNA-DNA hybrids (lanes b, c, and d, respectively). The discrete, smaller bands may be digestion products.

those of *E. coli* in 42 of the 43 such promoters identified in phages T4 and T6. The proposed promoter for T_U has the −35 sequence AAAACGGTTTAC and the −10 sequence TATGGTACTATA (the underlining indicates correspondence with the consensus sequence) (32). Parenthetically, we note that P_{nrdB} also includes a large part of the −10 early promoter consensus sequence (Fig. 1C, dashed underlined extension of the −10 box).

The proposal that a polycistronic message transcribes the *frd*, *td*, '*Y*,' *nrdA*, *nrdB*, and possibly *denA* (43) genes as an operon is supported by the presence of an early promoter with the appropriate strength and location (see above), which is consistent with the kinetics of appearance of the transcripts shown in Fig. 2, 3, 5, and 6 and with the previous evidence that *nrdA* transcription is dependent on an early promoter (39, 46). Hilfinger, using Northern blot analysis with probes from segments of either *nrdA* or *nrdB*, has found that mRNA from phage-infected cultures reached sizes of about 6 kb (unpublished data), supporting our previous findings (46), those of Gram et al. (25), and those of Ruckman and co-workers (42). That the *frd* and *td* genes overlap slightly (14, 40) and that the translational frames of *nrdB* and *denA* are separated by only 30 bp (43) argue against promoters in these interfaces. Furthermore, no obvious additional promoters were uncovered by a computer-based analysis of non-intron DNA from 0 kb in Fig. 1A to within *denA*. However, late and *motA*-dependent promoters have been described within the *td* and *nrdB* introns (24).

The α chain has been found between 3 and 4 min after

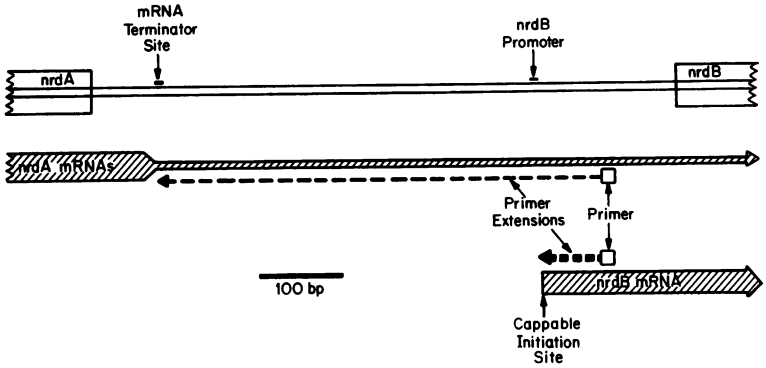

FIG. 9. Summary of the transcripts and controls operating in the *nrdA-nrdB* interface. The *motA*-dependent *nrdB* transcript begins at 159 bp upstream from the *nrdB* gene. This figure summarizes the evidence that the polycistronic mRNA T_U, originating upstream from *frd*, ultimately transcribes *nrdB*. A portion of the polycistronic *nrdA* transcript escapes the terminator, t_A, and was included in the primer extension analysis. If the mRNA analysis is conducted prior to approximately 5 min after infection , the residual *nrdA* message does not reach the region of the primer (see Fig. 3). An open reading frame directly downstream from *nrdA* and encompassing t_A is not shown.

infection at 30°C (39, 46), in reasonable agreement with the time of appearance of the *nrdA* transcript. A comparable measurement of the time of appearance of the β chain of *nrdB* at the same temperature has not been reported. However, an analysis of the formation of prereplicative proteins after T4 infection at 37°C by two-dimensional electrophoresis showed that the α chain of *nrdA* appears about 1 to 2 min before the β chain (J. Cowan, E. Kutter, and B. Guttman, unpublished data; E. Kutter, personal communication). As determined by the very sensitive tritium release assay, deoxyribonucleotide synthesis initiates at 4.8 min after infection at 30°C. This time is taken as a working value for the in vivo appearance of active ribonucleoside diphosphate reductase (13, 45). This enzyme has been detected in extracts at about 5 min after infection (53), but its kinetics of formation is much different and much faster than the initial exponential rise in the rate of synthesis of the deoxyribonucleotides (13, 45, 51); the kinetics of the latter is presumed to be dependent on the rate of formation of the dNTP synthetase complex (11, 34, 41, 52), which contains ribonucleotide reductase as a central component (the term dNTP synthetase complex is an amalgamation of earlier designations used in our publications and in those of C. K. Mathews and co-workers [1, 11, 34, 45, 51]).

What is the significance of an initial separation of the transcription of the T4 *nrdA* and *nrdB* genes? First, a separate promoter may be necessary for the regulation of *nrdB* expression or for a second function of the *nrdB* protein. Second, if *nrdA* and *nrdB* were exclusively transcribed by the same polycistronic messenger, the distance between *nrdA* and the 3′ end of *nrdB* and the time for intron excision (24) are such that the β chain would not be formed until at least 8 min after infection (i.e., after deoxyribonucleotide synthesis is known to initiate). Finally, the α_2 subunit is known to be degraded in the absence of the β_2 subunit (15).

We were originally led to examine the expression of T4 *nrdB* by findings obtained with *nrdB93*, a temperature-sensitive mutant defective in the formation of the *nrdB93* protein. gp39, a subunit of T4 DNA topoisomerase, is involved in the expression of the *nrdB93* gene. Since the two other genes encoding T4 DNA topoisomerase are not involved, the effect is not mediated by the enzyme (16, 50). Because of this intriguing relationship, we reasoned that in wild-type infection the synthesis of the β chain is the limiting factor in the initiation of synthesis of ribonucleotide reductase activity. The finding of Cowan et al. that the α chain is synthesized earlier than the β chain (see paragraph above) verified this idea. Recent findings indicate that the *nrdB93* block is beyond transcription and intron excision (Hilfinger, unpublished data). Obviously, in the expression of the wild-type *nrdB* gene, the process of intron excision contributes to the timing of the onset of translation (14, 24).

It may be pertinent that in the expression of deoxyribonucleotide synthesis in the S phase of the cell cycle in eucaryotic cells, formation of the β_2 subunit of the *nrdB* gene is the limiting factor, while the concentration of the α_2 subunit remains relatively high throughout the cycle (19). In sea urchin oocytes regulation of the ribonucleotide reductase is at the level of translation of the β_2 subunit (44). Possibly related to these findings, two laboratories have reported that DNA damage induces formation of the β_2 subunit in yeast cells (18, 29).

ACKNOWLEDGMENTS

These studies were supported in part by Public Health Service grant GM29025 from the National Institutes of Health.

We are grateful to Betty Kutter, The Evergreen State College, Olympia, Wash., for allowing us to quote the results from her laboratory before publication, to David Parma, University of Colorado, Boulder, for the *amG1* mutant and its suppressor strain, and to Ruby A. Hogue and Patricia M. Wilson for help in preparing the manuscript.

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