

Regulation of the Elongation-Termination Decision at Intrinsic Terminators by Antitermination Protein N of Phage λ

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The mechanisms that control N-protein-dependent antitermination in the phage λ life cycle have counterparts in the regulatory systems of other organisms. Here we examine N-dependent antitermination at the intrinsic *tR'* terminator of λ to elucidate the regulatory principles involved. The *tR'* terminator consists of a sequence of six base-pairs along the template at which the transcription complex is sufficiently destabilized to make RNA release possible. Within this "zone of opportunity" for termination the termination efficiency (*TE*) at each template position is determined by a kinetic competition between alternative reaction pathways that lead either to elongation or to termination. *TE* values at each position within *tR'* have been mapped as a function of NTP concentration, and it is shown that N protein (in the presence of NusA and a *nut* site; the minimal system for N-dependent antitermination) can offset increases in *TE* that are induced by limiting the concentrations of each of the next required NTPs. By limiting NTP concentrations or working at low temperature we show that a significant effect of N within the minimal system is to increase the rate of transcript elongation three- to fivefold at most positions along the template. Assuming that a comparable increase in elongation rate applies at template positions within the terminator, we show that an increase of this magnitude is not sufficient to account for the antitermination efficiency observed and that an ~ 100 -fold stabilization of the transcription complex at intrinsic termination sites as a consequence of binding the N-containing antitermination sub-assembly must be invoked as well. A general method for partitioning *TE* effects in antitermination between changes in elongation rate and termination complex stability is demonstrated, based on competing free energy of activation barriers for the elongation and termination reactions. The analysis and utility of such mixed modes of transcriptional regulation are considered in general terms.

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Keywords: transcription; transcript elongation; transcript termination; RNA polymerase; transcription factors

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Introduction

Bacteriophage λ -coded antitermination protein N activates gene expression downstream of the early *pL* and *pR* phage promoters by inhibiting transcrip-

tion termination (for reviews see Friedman, 1988; Das, 1992, 1993; Greenblatt *et al.*, 1993; Friedman & Court, 1995). In cooperation with a *cis*-acting sequence element called *nut* and several host cell proteins, the N protein of phage λ renders transcription complexes resistant to both intrinsic and rho-dependent terminators (Gottesman *et al.*, 1980; Das & Wolska, 1984; Mason *et al.*, 1992). A transcription unit must carry a *nut* sequence within the transcribed region upstream of the terminator to permit N to work as an antiterminator *in vivo* under normal conditions (de Crombrugge *et al.*,

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Abbreviations used: *TE*, termination efficiency; *RE*, read-through efficiency.

1979; Lazinski *et al.*, 1989; Franklin & Doelling, 1989). The *nut* sequence is expressed as RNA and functions within the nascent transcript as a specific high affinity stem-loop binding site (called boxB) for N (Whalen & Das, 1990; Nodwell & Greenblatt, 1991; Chattopadhyay *et al.*, 1995). Highly efficient, processive N-mediated antitermination also requires *Escherichia coli* transcription elongation factors NusA, NusB, NusG, and S10, as well as another RNA component of the *nut* sequence called boxA (Friedman *et al.*, 1990; Mason *et al.*, 1992; DeVito & Das, 1994). These factors allow N to be tightly bound to the elongating transcription complex and lead to efficient *in vitro* and *in vivo* suppression of terminators located thousands of base-pairs downstream of the *nut* site (Barik *et al.*, 1987; Horowitz *et al.*, 1987; Mason & Greenblatt, 1991; Mason *et al.*, 1992; DeVito & Das, 1994).

The N-dependent antitermination system can be simplified. N and NusA, together with the *nut* sequence, are sufficient to induce effective antitermination *in vitro* at many terminators located within a few hundred base-pairs downstream of a *nut* site (Whalen *et al.*, 1988; Whalen & Das, 1990; Mason *et al.*, 1992; DeVito & Das, 1994). Finally, at high N concentrations and somewhat lower salt concentrations, even the requirements for NusA and the *nut* sequence are obviated and N alone can work with Δ *nut* transcripts to greatly reduce the efficiency of termination at intrinsic terminators (Rees *et al.*, 1996). This shows that a direct interaction between N and the transcription complex, supported by non-specific binding to the nascent transcript (Rees *et al.*, 1996; Van Gilst *et al.*, 1997), is ultimately sufficient to mask termination signals.

A working model for N-dependent antitermination suggests that N, NusA, and *nut* RNA form a metastable complex with an elongating RNA polymerase (Friedman, 1988; Das, 1992, 1993; Roberts, 1993; Greenblatt *et al.*, 1993). N is thought to modulate targeted transcription complexes by binding to the *nut* site within the nascent transcript, and then to the transcription complex by *cis* RNA looping. NusA stabilizes this association. In support of this model, direct pairwise interactions have been demonstrated *in vitro* between N and NusA (Greenblatt & Li, 1981a), between NusA and RNA polymerase (Greenblatt & Li, 1981b; Gill *et al.*, 1991a,b), between N and *nut* RNA (Chattopadhyay *et al.*, 1995), and between the N-boxB complex and NusA (Van Gilst & von Hippel, 1997). Multiple interactions between *nut* site sequences on the nascent RNA and the various proteins of the N-antitermination complex have also been demonstrated (Mogridge *et al.*, 1995; Chattopadhyay *et al.*, 1995).

Little is known about how the binding of N alters the transcription complex to mask termination signals. This study is concerned with the mechanism of this termination suppression. A simple kinetic model for the regulation of termination suggests that the efficiency of termination (and antitermination) at terminator sites is the result of a competition between the rate of tran-

script elongation to template positions beyond the terminator and the rate of transcript release at template positions within the terminator (von Hippel & Yager, 1991, 1992; Jin *et al.*, 1992; Wilson & von Hippel, 1994; McDowell *et al.*, 1995). One prediction of this model is that the efficiency of termination at any given template position should increase as the concentration of the next nucleotide triphosphate required for elongation is decreased below saturating levels, but that this change in efficiency should be relatively insensitive to changes in the concentrations of the other NTPs. It is known that the dwell-time of the transcription complex at a pause site generally increases as the concentration of the next nucleotide to be added to the transcript is decreased below saturation levels (Landick & Yanofsky, 1987; Levin & Chamberlin, 1987; Wilson & von Hippel, 1994). Termination efficiency (*TE*) at intrinsic terminators *in vitro* also generally increases as the total NTP concentration is decreased (Reynolds *et al.*, 1992; Wilson & von Hippel, 1994). *In vitro* studies on the determinants of termination efficiency at rho-dependent and intrinsic terminators are consistent with the notion that this parameter is governed by a competition between the rate of transcription elongation and the rate of RNA release (Jin *et al.*, 1992; Wilson & von Hippel, 1994; McDowell *et al.*, 1995).

A further prediction of the kinetic competition model is that an antiterminator that masks the ability of RNA polymerase to respond to termination signals must bias the relative rates to favor elongation at the expense of termination by either increasing the rate of elongation, decreasing the rate of termination, or a combination of both. The N protein of phage λ shares with the λ Q antitermination protein the ability to increase the rate at which the transcription complex passes through biologically relevant pause sites (Yang & Roberts, 1989; Das, 1992; Mason *et al.*, 1992). It has been speculated that this pause suppression activity may represent the fundamental modification of transcription complexes that is responsible for antitermination (von Hippel & Yager, 1991, 1992; Jin *et al.*, 1992; Das, 1992, 1993; Roberts, 1993; Greenblatt *et al.*, 1993).

We have studied, and describe here, the dependence of *TE* on NTP concentrations at various positions within the intrinsic terminator *tR'* in the presence and absence of N. In keeping with the predictions of the kinetic competition model (von Hippel & Yager, 1991) we show that *TE* in the presence and absence of N under conditions of limiting NTP concentration is inversely related to the concentration of the next NTP required to elongate the transcript at each template position within the *tR'* terminator, while *TE* is relatively independent of the concentration of the other three NTPs. We then show that N operates as a general enhancer of the rate of transcript elongation at virtually all template positions, regardless of whether the rate is limited by biologically relevant pause signals, by artificially low NTP concentrations, or by low tem-

perature. However, we also conclude that this measured general elongation rate enhancement by N is not sufficient to explain the magnitude of the N-dependent antitermination effect at the terminators that we have investigated, and that a major stabilization of the transcription complex to decrease the rate of the competing RNA release process is also required. The implications of this type of biological regulation are discussed. Elsewhere (Van Gilst *et al.*, 1997) some of the molecular mechanisms and interactions that actually underlie N-dependent antitermination are described and evaluated.

Results

The $\lambda tR'$ terminator contains multiple RNA release sites

The effect of kinetic competition between nucleotide addition and RNA release on termination efficiency was investigated at the intrinsic terminator tR' . This terminator is highly efficient *in vitro*, becomes still more efficient in the presence of NusA, and can be effectively suppressed either by a minimal antitermination system containing N, NusA, and operating on a DNA template carrying a *nut* site located a few hundred base-pairs upstream of tR' (Whalen *et al.*, 1988), or by high concentrations of N alone, with or without the *nut* site, at somewhat reduced salt concentrations (DeVito & Das, 1994; Rees *et al.*, 1996). Transcription on each of the templates used in this study (see Figure 1 and Experimental Procedures for a description of these templates) was initiated at the λpL promoter and terminated either at tR' or at the end of the template to form a run-off transcript.

The vast majority of intrinsic terminator sites found in the genomes of *E. coli* and its phages consist of an interrupted G·C-rich palindromic sequence followed on the template strand by a run of dA residues (Yager & von Hippel, 1987; Carafa *et al.*, 1990). These features result in the formation of a stable stem-loop hairpin in the transcript that is followed by a run of rU residues (Figure 2A). The positions within intrinsic terminators at which transcript release occurs tend to be clustered at a few adjacent positions in the rU-rich region (Yager & von Hippel, 1991; Wilson & von Hippel, 1994, 1995; McDowell *et al.*, 1995). To see if this is true of tR' , we have placed this terminator within less than 100 bp of the promoter and have determined the 3' ends of the terminated transcripts by direct RNA sequencing (Figure 2B). These terminated transcripts can be easily resolved by denaturing gel electrophoresis. We define template positions within the transcript with a one-letter code for the RNA residue, followed by a position index number. For example, the first nucleotide of the run of Us that follow the CGGGA sequence at the end of the stem is designated as position U1 (Figure 2A).

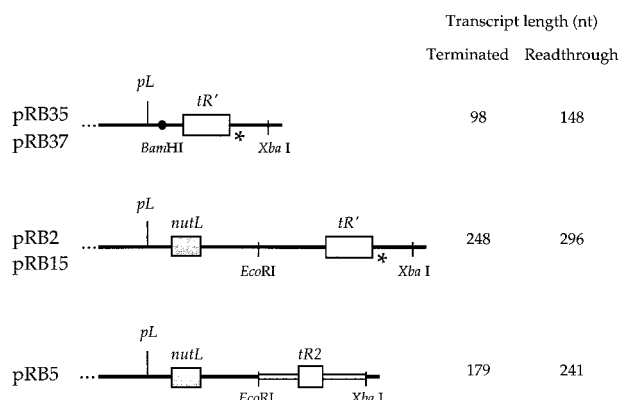


Figure 1. Template constructs used in this study. Plasmid construction is described in Experimental Procedures. The five templates shown are identical upstream of the transcription start site. The thick open boxes represent sequences of hyphenated dyad symmetry in the tR' and $tR2$ terminators; the stippled boxes represent the *nutL* sequence; the thin open bars represent the sequences surrounding the $tR2$ terminator in pRB5 that replaced the *EcoRI*- tR' -*XbaI* fragment from pRB2. An asterisk (*) represents the approximate location of the sites of termination in tR' and the location of the single base mutation that distinguishes pRB2 from pRB15, and pRB35 from pRB37. The lengths of the terminated and readthrough transcripts are shown.

In reactions that contain 0.5 or 1.0 mM concentrations of each of the four NTPs, transcription terminates primarily at positions A7 and U8 within tR' , with termination at U8 predominating (Figure 2B). An earlier RNA "fingerprinting" study of the 3' ends of tR' -terminated transcripts generated on an intact λ DNA template with purified RNA polymerase *in vitro* (Rosenberg *et al.*, 1975) showed that most of the transcripts terminated at position A7, and the rest at U8. We assume that this difference reflects somewhat different transcription conditions, perhaps including differences in the NTP concentrations used (see below).

The pattern of 3' ends obtained is not an invariant property of the terminator. Rather this pattern changes in response to changes in the availability of the substrate NTPs (Figure 2C). At approximately physiological (1 mM) concentrations of all four NTPs, transcription terminates at the A7 and U8 template positions as described above (Figure 2C, lane 1). However, significant levels of termination are realized at the previously silent U6 position when the ATP concentration is reduced 100-fold (lane 2). Transcription is terminated almost exclusively at A7 in the presence of 10 μ M UTP and 1 mM ATP, CTP and GTP (lane 3). In contrast, the pattern of 3' ends obtained is perturbed very little by drastic changes in the concentrations of CTP or GTP (lanes 4 and 5).

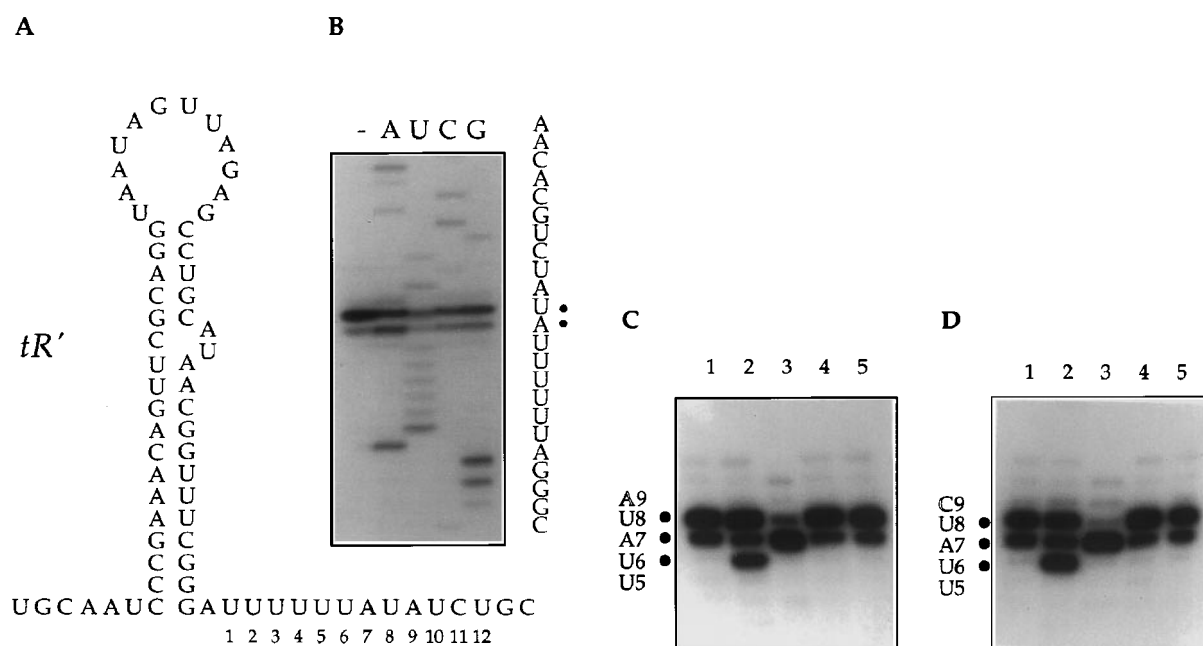


Figure 2. Termination efficiencies vary with NTP concentration. A, RNA sequence and idealized stem-loop structure of *tR'*. RNA sequencing was performed using a method analogous to the dideoxy-sequencing procedure of Sanger *et al.* (1977) for DNA. The index numbers for each base in the region of termination are shown. The U1 position corresponds to base 44774 in the DNA sequence of phage λ (Daniels *et al.*, 1983). The positions of the "zone of opportunity" for termination (see the text) are underlined at the bottom. B, Determination of the 3' ends of transcripts terminated at *tR'*. A18 complexes on the pRB35 DNA template were allowed to continue chain elongation for three minutes at 37°C in transcription buffer containing 300 mM potassium acetate, as described in Experimental Procedures. In the first lane we show A18 complexes that were elongated in the presence of 0.5 mM of each of the four substrate NTPs. The reactions in the lanes marked A, U, C, and G all contained 0.25 mM concentrations of ATP, UTP, CTP and GTP, 40 mg/ml of an antisense DNA oligomer (5'-CCTGCGAACTGTTTCGGAT) that is complementary to the 5' arm of the *tR'* stem-loop sequence and inhibits termination (data not shown), and 0.125 mM of the 3'-dNTP indicated above each lane in the Figure. The 3' ends of the *tR'*-terminated transcripts that were determined experimentally are indicated with dots. C and D, Analysis of the *tR'*-terminated transcripts generated in the presence of limiting substrate NTP concentrations: C shows terminated transcripts on the pRB35 (wild-type *tR'*) template; D shows terminated transcripts on the pRB37 (C9 mutant *tR'*) template. A18 complexes formed on the pRB35 and pRB37 DNA templates were permitted to resume chain elongation in the presence of the following (final) concentrations of substrate NTPs: lanes 1, 1 mM ATP, UTP, CTP and GTP; lanes 2, 10 μ M ATP, 1 mM UTP, CTP and GTP; lanes 3, 10 μ M UTP, 1 mM ATP, CTP and GTP; lanes 4, 10 μ M CTP, 1 mM ATP, UTP and GTP; lanes 5, 10 μ M GTP, 1 mM ATP, UTP and CTP. Reactions were incubated in transcription buffer containing 50 mM potassium acetate for two minutes at 37°C before quenching and processing as described in Experimental Procedures.

The concentration of the next required NTP is a critical determinant of termination efficiency

Termination is not an all-or-none process and the definition of a termination position under a given set of conditions is ultimately a quantitative issue. In Table 1 we summarize our measurements of the overall termination efficiency (*TE*) for the entire *tR'* terminator and the *TE* at each position within the terminator in reactions containing three NTPs at 1 mM concentrations and the fourth at either 1 mM or 10 μ M, using the Δ *nut* (pRB35) and *nut*⁺ (pRB37) templates (see Figure 1).

In accord with previous results of others (Wilson & von Hippel, 1994; McDowell *et al.*, 1995), we find that *TE* at various positions within *tR'* increases as the concentration of the next NTP

required to elongate the transcript is decreased. Thus, as seen in Figure 2A, reduction of the ATP concentration from 1 mM to 10 μ M is accompanied by an increase in *TE* from 0.04 to 0.24 at template position U6, and an increase in *TE* from 0.85 to 0.90 at U8 (chain elongation at each of these positions requires the addition of an rA residue to the transcript; see Figure 2A). Similarly a 100-fold reduction in UTP concentration resulted in an increase in *TE* from 0.34 to 0.70 at A7, where elongation requires the addition of an rU residue. In contrast, the value of *TE* at most positions was relatively insensitive to changes in the concentrations of NTPs not required to elongate the transcript to the next template position. Thus, *TE* at U6 increases at low ATP concentrations, but is rela-

Table 1. Termination efficiencies at individual template positions and overall at wild-type (A9) and mutant (C9) *tR'* terminators

Substrate concentrations during chain elongation	Positions of termination				
	U6	A7	U8	A9/C9	Total
1.0 mM NTPs	0.04 (0.05) ^a	0.03 (0.30)	0.85 (0.63)	— (0.20)	0.95 (0.80)
10 μ M ATP, 1.0 mM UTP, CTP and GTP	0.24 (0.30)	0.27 (0.28)	0.90 (0.55)	— (0.15)	0.99 (0.83)
10 μ M UTP, 1.0 mM ATP, CTP and GTP	0.06 (0.05)	0.07 (0.70)	0.58 (0.40)	— (0.26)	0.91 (0.88)
10 μ M CTP, 1.0 mM ATP, UTP and GTP	0.03 (0.02)	0.27 (0.27)	0.84 (0.88)	— (0.20)	0.93 (0.95)
10 μ M GTP, 1.0 mM UTP, CTP and ATP	0.02 (0.02)	0.24 (0.07)	0.81 (0.56)	— (0.11)	0.92 (0.72)

Transcription reactions were performed and analyzed as described in Experimental Procedures. The average error in determining *TE* was ± 0.02 . (—) means large fitting errors prevented accurate determination of *TE* at this template position.

^a *TE* at each position in the mutant (C9) terminator is indicated in parentheses below the corresponding values for the wild-type (A9) terminator.

tively independent of the concentrations of UTP, CTP, or GTP.[†]

This dependence of *TE* on the concentration of the next required nucleotide holds only over a narrow cluster of template positions within the rU-rich region of the *tR'* terminator. As shown by Wilson & von Hippel (1994) for the *tR2* terminator of phage λ , this cluster of "next-nucleotide-concentration-sensitive" positions falls within a zone of opportunity for termination along the template that represents the portion of the terminator sequence within which the transcription complex is sufficiently destabilized to permit transcript release during the time the transcription complex remains at this template position. Defined operationally as the template positions at which *TE* can be made to exceed 0.05 by manipulation of NTP concentration, we have shown that the upstream border of this zone for the *tR'* falls at position U6, and the downstream border at position C11 (see Figure 2A). Under all conditions tested *TE* was greatest at position U8, and most of the observed release occurred at positions U6, A7, and U8, with only trace amounts of RNA being released at positions A9, U10, and C11. Low UTP concentrations yielded no detectable termination at either positions U5 or U12 under these experimental conditions.

To demonstrate that this inverse relationship between nucleotide concentration and *TE* at a given position holds true in general, we constructed a mutant template (pRB37) in which residue A9 of *tR'* was replaced by an rC residue. If the next-required-nucleotide rule is general, then this replacement should alter the NTP concentration dependence of *TE* observed at U8 from ATP to CTP. As seen in the autoradiograms shown in

Figure 2C and D, the overall patterns of release for the two terminators are strikingly similar, although the mutant (C9) terminator is somewhat weaker than the wild-type *tR'* terminator (due primarily to a reduced *TE* at U8; see Table 1). The C9 mutation has little quantitative effect on termination efficiency at U6 or A7. In contrast, and as expected, the C9 mutation makes termination at U8 inversely dependent on CTP concentration and essentially independent of ATP concentration.

Changes in the concentrations of the next required nucleotide alter the efficiency of N-mediated antitermination at *tR'*

We next asked whether and how the concentrations of NTPs influence termination in the presence of N. For this purpose a pair of templates containing a *nutL* site inserted between *pL* and *tR'* were constructed from templates pRB35 and pRB37 (templates pRB2 and pRB15 shown in Figure 1). The pRB2 and pRB15 templates are identical except for the C9 in the run of U residues within the *tR'* terminator of pRB15. *TE* values for these two *nut*⁺ templates were measured first in the absence of N and NusA, to allow a comparison of the results with those of Table 1. The results

Table 2. Net termination efficiency at wild-type (A9) and mutant (C9) *tR'* terminators on *nut*⁺ DNA templates pRB2 and pRB15

DNA template	1 mM NTPs	Low [ATP] ^a	Low [UTP]	Low [CTP]	Low [GTP]
pRB2 (wild-type <i>tR'</i>)	0.69	0.91	0.74	0.71	0.67
pRB15 (C9 mutant <i>tR'</i>)	0.62	0.71	0.69	0.82	0.57

Transcription reactions were performed as described in Experimental Procedures (and Table 1), except the reactions were run in transcription buffer containing 300 mM potassium acetate. The average error in determining *TE* was ± 0.02 .

^a Low [NTP] denotes transcription reactions performed in the presence of 10 μ M of the indicated NTP and 1.0 mM of the other NTPs.

[†] An apparent exception to this "next-nucleotide-rule" is seen with UTP (Table 1), in that lowering the concentration of UTP from 1 mM to 10 μ M reduces the observed *TE* at U8 from 0.85 to 0.58 (ATP is the next required nucleotide at U8).

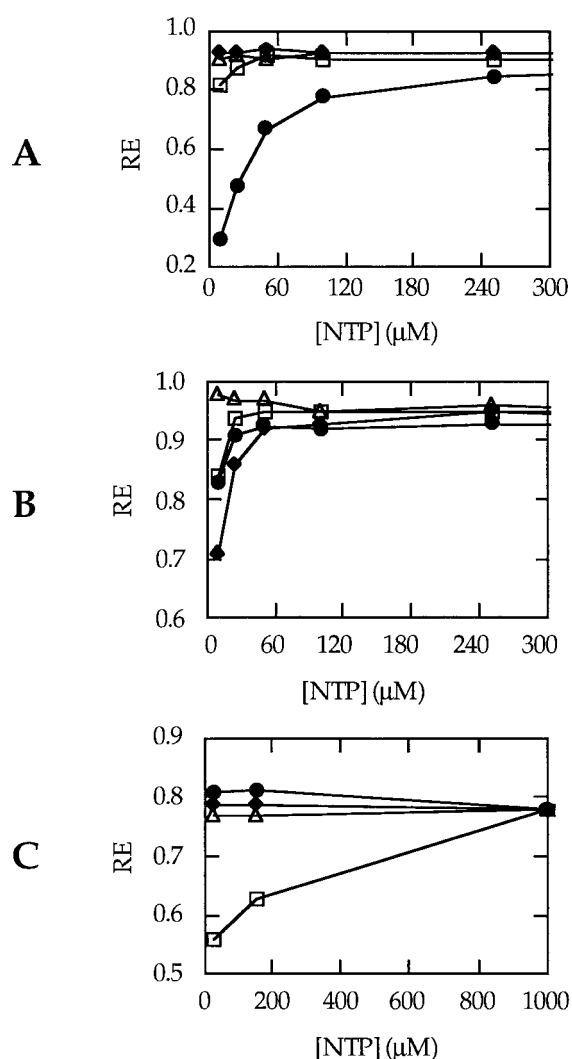


Figure 3. Nucleotide concentration dependence of N-mediated antitermination at intrinsic terminator tR' , the C9 mutant of terminator tR' , and intrinsic terminator $tR2$. C15 complexes formed on DNA templates: (A) pRB2; (B) pRB15; and (C) pRB5 were permitted to continue chain elongation for two minutes at 37°C in transcription buffer containing 100 mM potassium acetate, 120 nM NusA, 100 nM N, 1.0 mM of three NTPs and a variable concentration of the fourth NTP. Filled circles, [ATP] varied; open squares, [UTP] varied; filled diamonds, [CTP] varied; open triangles, [GTP] varied. Reactions in C were performed in buffer containing glutamate rather than acetate salts.

summarized in Table 2 show that the overall TE of the wild-type tR' terminator is strongly (inversely) dependent on ATP concentration, slightly increased at low UTP concentrations, and relatively insensitive to changes in the concentration of CTP or GTP. (The lower values of TE shown for tR' in the pRB2 construct (Table 2), relative to the TE values at the same terminator in the pRB35 construct (Table 1 and Figure 2), reflect largely the higher concentrations of salt (potassium acetate) used in the former reactions.) TE with the C9

mutant nut^+ template is most strongly dependent on CTP concentration, relatively insensitive to GTP concentration, and intermediate in its dependence on ATP and UTP concentrations. Thus, we conclude that the insertion of the nut site into these templates has little effect on the NTP concentration dependencies of the tR' terminator.

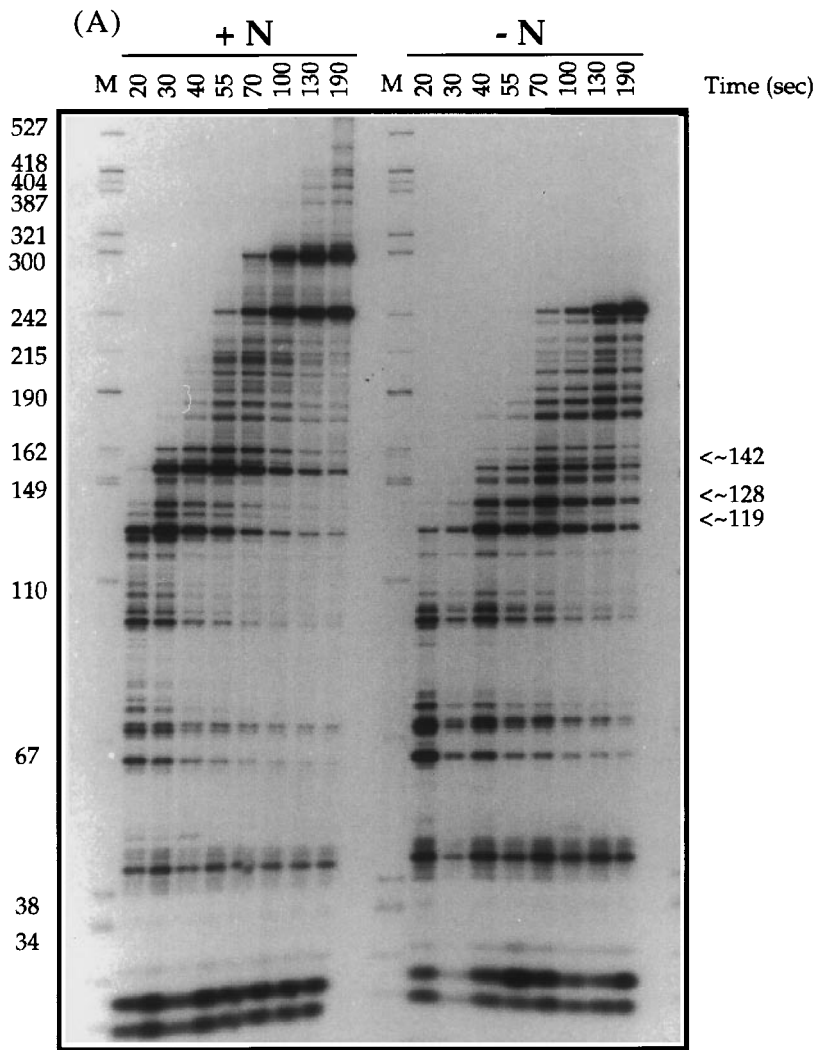
In the presence of saturating concentrations of N, NusA, and NTPs, the read-through efficiency (RE ; equals $(1-TE)$) was increased to greater than 0.9 for both the pRB2 and pRB15 templates (Figure 3A and B). As these Figures show, the efficient antitermination at the wild-type terminator that is induced by N protein was not changed when the concentration of GTP or CTP was lowered to 10 μ M. However, N function at the wild-type terminator was severely compromised at low ATP concentrations. Half-maximal antitermination was realized with ~ 60 μ M ATP. Low UTP concentrations only slightly reduced the efficiency of antitermination.

If this extreme ATP concentration dependence of N function is a manifestation of the intrinsic ATP concentration dependence of termination efficiency at tR' , antitermination at the C9 mutant of tR' should be primarily CTP concentration-dependent and only marginally ATP concentration-dependent (reflecting the low ATP concentration-dependence of termination at the U6 position). As expected, antitermination at both terminators shows a similar dependence on the UTP concentration, but suppression of the C9 mutant terminator is strongly CTP-dependent and only moderately ATP-dependent.

To test the generality of this observation, we investigated the nucleotide concentration dependence of N-mediated antitermination at another intrinsic terminator ($tR2$ of phage λ ; for a detailed characterization of this terminator see Wilson & von Hippel, 1994) on template pRB5, which contains the $tR2$ terminator preceded by a nut site (Figure 1). Previous work had shown that termination at $tR2$ occurs primarily at two adjacent template positions that require UTP for further elongation, and the efficiency of termination at these sites is inversely dependent on UTP concentration (Wilson & von Hippel, 1994). As shown in Figure 3C, RE for the $tR2$ terminator is 0.85 in the presence of saturating N, NusA, and NTPs, and 0.10 under the same conditions in the absence of N. Consistent with the results obtained with tR' , N function at the $tR2$ terminator is strongly UTP concentration-dependent and is insensitive to changes in the concentration of the other three NTPs.

The rate of transcript elongation as a function of N and NusA

One prediction of the kinetic competition model is that a decrease in TE (i.e. antitermination) can be induced by an increase in the rate of elongation (induced by an increase in the limiting concentration of the next required NTP), a decrease in the



B

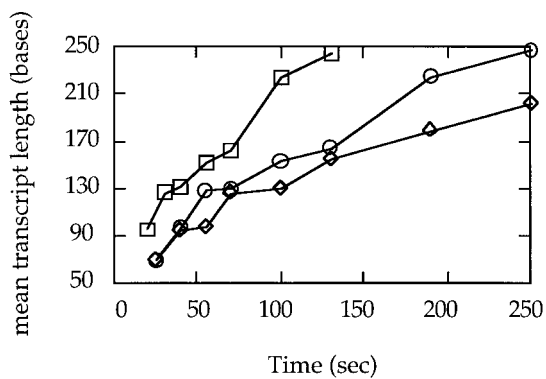
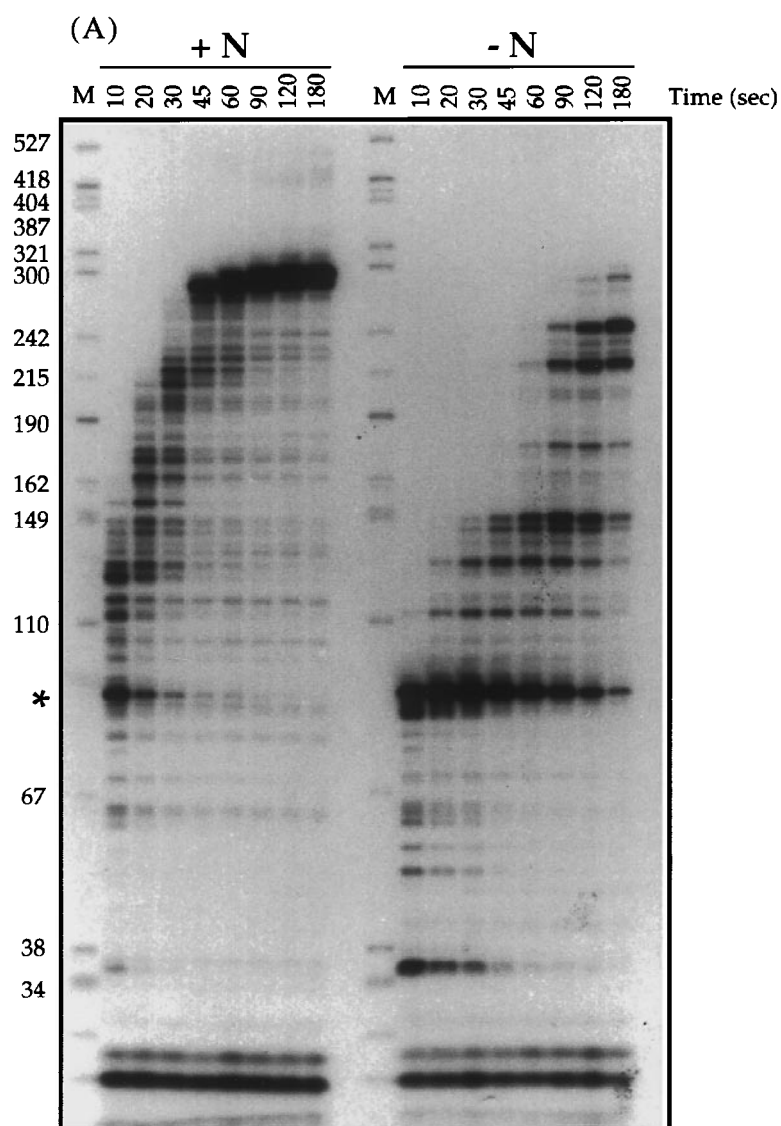


Figure 4. Transcription elongation time courses at low ATP concentrations. (A) Autoradiogram of a transcription elongation time course at low [ATP]. Transcription elongation reactions were performed as described in Experimental Procedures in the presence of 10 μ M ATP, 1.0 mM UTP, CTP, and GTP, and contained 90 nM N, 120 nM NusA (+N panel) or 120 nM NusA only (-N panel). Elongation reaction times are indicated above each lane. The lengths of the DNA size markers (lane M) are indicated to the left of the autoradiogram. B, Plots of the mean positions of transcription elongation complexes versus time in the presence or absence of N protein and/or NusA. Reactions were performed as described and illustrated above and contained: RNA polymerase alone (circles) ; RNA polymerase plus NusA (diamonds); or RNA polymerase plus N and NusA (squares).

rate of termination (increased stabilization of the transcription complex to slow the rate of RNA release), or a combination of both (see Wilson & von Hippel, 1995). As a direct test of this prediction for N-dependent antitermination, we have examined the effects of N on the rate of transcript

elongation catalyzed by RNA polymerase on the pRB2 template (which contains both wild-type *nut* and wild-type *tR'* sites; see Figure 1). Because the elongation rate was monitored by manual sampling of the reaction at short intervals, it was necessary to reduce the average rate of elongation,



B

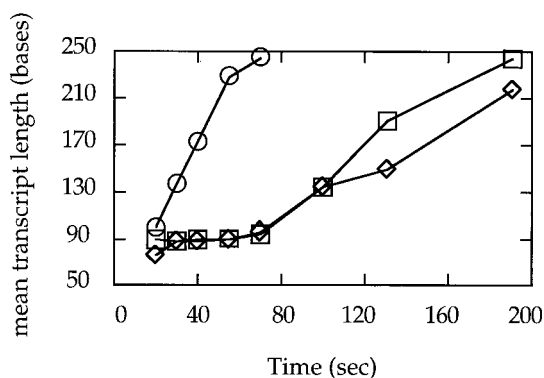


Figure 5. Transcription elongation time course at low GTP concentrations. (A) Autoradiogram of a transcription elongation time course at low [GTP]. Transcription elongation reactions were performed as described in the legend to Figure 4, in the presence of 10 μ M GTP, 1.0 mM ATP, UTP, and CTP. Elongation reaction times are indicated above each lane. The lengths of the DNA size markers (lane M) are indicated to the left of the autoradiogram. An asterisk (*) marks the location of the strong GTP concentration-dependent transcriptional pause site (see the text). B, Plot of the mean position of the transcription elongation complexes *versus* time in the presence or absence of N protein and/or NusA. Reactions were performed as described and illustrated above and contained: RNA polymerase alone (squares); RNA polymerase plus NusA (diamonds); or RNA polymerase plus N and NusA (circles).

which is typically ~ 30 nt/s at 37°C in the presence of saturating NTP concentrations (Levin & Chamberlin, 1987). In the experiments described in the following sections, the transcription rate in the

absence of N and NusA was limited to an average of less than 5 nt/s, either by carrying out the reactions at low temperature or by using low concentrations of one of the four NTPs.

The distribution of elongating polymerases on the template was monitored by resolving RNAs on a sequencing gel. After 30 seconds of chain elongation in the presence of physiological concentrations of substrate (1.0 mM of each NTP), virtually all the transcription complexes had reached or transcribed beyond the *tR'* terminator (data not shown). When the concentration of any one of the four NTPs was reduced to 10 μ M while holding the concentration of the other NTPs at 1 mM and running the reaction at 22°C, quenching after 30 seconds of transcription resulted in a distribution of complexes located well short of the *tR'* and run-off template sites (near positions 245 and 310, respectively; see Figures 4(A) and 5(A)).

Thus, although these experiments were started with a homogeneous population of stalled complexes, transcription elongation becomes highly asynchronous in less than 30 seconds, resulting in the generation of a heterogeneous population of complexes. Within these populations complexes pause at some positions significantly longer than at others, generating pausing patterns that are typical of the template sequence and the limiting NTP concentrations used (for example, see Kassavetis & Chamberlin, 1981; Reisbig & Hearst, 1981; Turnbough *et al.*, 1983; Fisher *et al.*, 1985; Barik *et al.*, 1987; Levin & Chamberlin, 1987). Pausing of complexes at some template positions occurs even at saturating concentrations of all the NTPs, while the lengths of other pauses are artificially increased at low NTP concentrations. Other pause sites appear at low NTP concentrations at template positions at which no pausing was observed with saturating NTP concentrations. In general, decreasing the concentration of a particular NTP enhanced pausing at positions for which that NTP is the next required nucleotide (Levin & Chamberlin, 1987; Landick & Yanofsky, 1987; Wilson & von Hippel, 1994), but the duration of the observed pause depended also on the apparent Michaelis constant (K_M) for the addition of the next required NTP, which may vary significantly at different positions on a natural DNA template (Kingston & Chamberlin, 1981; Levin & Chamberlin, 1987).

Figure 4(A) shows a time course of elongation on the *nut*⁺ pRB2 template at low ATP concentration in the presence and absence of saturating N protein. Both sets of reactions contained saturating levels of NusA. The group of gel bands located at template positions 39 to 44 were pronounced only at low ATP concentration, and correspond to a stretch of five dT residues on the template. Figure 5(A) shows a comparable experiment carried out at low GTP concentrations (the symbol * located near template position 90 in Figure 5(A) marks a collection of pauses that were strongly enhanced at low GTP concentrations). Qualitative examination of Figures 4(A) and 5(A) shows that the "waves of pausing" move through the template more quickly in the presence of N than in its absence; thus, the addition of N appears to increase the net rate of transcription at virtually all template

Table 3. Modulation of average transcription elongation rates (in nucleotide residues/s) by saturating concentrations of NusA and N

N	NusA	Low [ATP]	Low [UTP]	Low [CTP]	Low [GTP]	2°C 1 mM NTPs
-	-	0.8	0.8	2.4	1.0	0.6
-	+	0.5	0.6	1.5	0.8	0.6
+	+	1.9	2.0	3.5	3.7	1.1

Reaction conditions are defined in Experimental Procedures. Low [NTP] denotes experiments performed in the presence of variable concentrations of the indicated NTP and 1.0 mM concentrations of the other NTPs. Low [ATP]: 7.5 μ M; low [UTP]: 4.5 μ M; low [CTP]: 3.0 μ M; low [GTP]: 7.5 μ M. Rates were measured over a >100 bp segment of the pBr2 template located between 50 and 250 bp downstream of the transcription start site.

positions. Plots of the mean position (the position on the gel at which half the radioactivity is in larger and half in smaller bands) of the elongation complexes as a function of time for these experiments at low ATP and low GTP concentrations are shown in Figures 4B and 5B, respectively. The strong deviations from linearity in many of the plots represent the positions of strong pause sites induced by low concentrations of a particular NTP; thus, the strong GTP-dependent pause close to template position 90 (*) in Figure 5(A) corresponds to the flat section in the plot of mean band position at this template region in Figure 5B.

Mean transcription elongation rates over defined regions of the DNA template have been calculated from the data of Figures 4B and 5B, as well as from similar plots of the kinetics of RNA chain elongation at low UTP and low CTP concentrations (data not shown), and are summarized in Table 3. As can be seen in Figures 4B and 5B, the addition of NusA alone decreases the net rate of elongation under all conditions tested at 22°C. This is consistent with previous findings that NusA increases pausing and decreases the rate of transcription elongation (Schmidt & Chamberlin, 1984; Kassavetis & Chamberlin, 1981; Arndt & Chamberlin, 1990; Levin & Chamberlin, 1987; Gill *et al.*, 1990; Wilson & von Hippel, 1995). In contrast, elongation on a *nut*⁺ template in the presence of saturating N and NusA (NusA is here required as part of the minimal N-dependent antitermination complex) proceeds at a higher rate under all conditions tested. For example, compare the times at which the mean elongation complex reaches template position 130 in the presence (~35 seconds) and in the absence (~100 seconds) of N and NusA for the low ATP reactions (Figure 4(A) and B). In general, the addition of saturating N (with saturating NusA) increases the average elongation rate of the transcripts on the *nut*⁺ template from two- to fourfold beyond the average measured with polymerase alone, and from three- to fivefold above the average measured with polymerase and saturating NusA (Table 3).

We note that the N-mediated increase in the overall (average) rate of elongation is not the result of an equal effect at every template position, since Figures 4 and 5 show that N has a greater effect on the rate of elongation at some positions than at others. Thus the addition of N alters somewhat the overall shapes of the pausing patterns, as well as moving these patterns more rapidly along the template. For example, while the transcription complex is seen to hesitate markedly at positions +119, +128, and +142 after 100 seconds of transcription in the absence of N, pausing at position +128 is nearly abolished in the presence of N. A quantitative assessment (by the method of Theissen *et al.*, 1990) of transcriptional pausing at the collection of bands (marked by *) in Figure 5(A) indicates that the addition of N protein reduces the strength of this pause about tenfold relative to that measured in the presence of NusA. We conclude that the average (two- to fivefold) increase of the rate of elongation mediated by N (plus saturating NusA) above the rates seen with polymerase alone (and with polymerase plus saturating NusA) must reflect the sum of larger and smaller effects at different template positions and, of course, could also result in either larger or smaller effects at specific positions within terminator sites.

To investigate the extent to which the rate-enhancing effects of N might be limited to those positions at which NTP concentrations are sub-saturating, transcription reactions were performed in the presence of saturating (1 mM) concentrations of each NTP substrate at a reaction temperature of 2°C, since working at this low temperature also allows the elongation time course to be monitored by manual methods. Under these conditions saturating N protein (with a *nut*⁺ transcript and saturating NusA) increases the overall elongation rate ~1.8-fold (Table 3), showing that the N-dependent increase in the rate of transcript elongation observed above is not simply due to the presence of sub-saturating concentrations of one of the NTPs.†

† We note, in contrast to our observations at 22°C with sub-saturating concentrations of individual NTPs, that NusA alone shows no effect on the apparent rate of elongation at 2°C with saturating NTP concentrations. This apparent loss of the slowing effect of NusA on elongation is not due to a failure of NusA to bind elongation complexes under these conditions, since NusA does increase the efficiency of termination under these conditions ($TE = 0.51$ in the absence of NusA, and 0.73 in the presence of NusA at 2°C). Perhaps NusA also increases the rate of dissociation of the transcription complex under these low temperature conditions? This observation at 2°C also differs from an earlier observation at 37°C, where NusA was shown to decrease the elongation rate on a T7 Δ D111 DNA template at saturating NTP concentrations (Kassavetis & Chamberlin, 1981). Finally, NusA (in the absence of N protein) has been shown to increase TE at a variety of *tR2* terminator variants (Wilson & von Hippel, 1995).

Discussion

Previously we have shown that high concentrations of N protein alone can cause *E. coli* transcription complexes to read through intrinsic termination signals *in vitro* (Rees *et al.*, 1996). This effect does not require that the terminators be preceded by a *nut* sequence, even though the presence of this sequence is generally essential for the manifestation of N-dependent antitermination *in vivo*. Thus, the basic antitermination phenotype can be attributed to a direct interaction between N and the core elongation complex, which means that the other components of the full N-dependent antitermination complex (the *boxA* and *boxB* sequences of the nascent RNA, and protein factors NusA, NusB, NusG, and S10) must function primarily to modulate the interaction of N with the elongation complex in order to make antitermination processive and operon (and lambdaoid phage)-specific (Rees *et al.*, 1996; Van Gilst *et al.*, 1997).

N protein increases the overall rate of transcript elongation

Here we have shown that a major effect of N protein on the *E. coli* transcription complex (in the presence of a *nut* site and a saturating concentration of NusA) is to increase the average rate of transcript elongation three- to fivefold. By slowing transcript elongation (either by lowering the concentration of individual NTPs or by carrying out elongation at physiological NTP concentrations at very low temperature) we have shown that this effect occurs at all template positions at which pausing bands can be seen, rather than reflecting only the relief of specific (and perhaps biologically relevant) pauses along the template. On the other hand the data of Figures 4 and 5 show that the effects of N on elongation rates are not entirely uniform; clearly some sequence specificity of the rate increase is manifested along the template and the measured rate increase at individual template positions can range from two- to tenfold or more. Furthermore, it is important to point out that these experiments do not measure directly the putative rate increase within the *tR'* terminator itself, though the fact that Figures 4(A) and 5(A) (and their low CTP and UTP counterparts, data not shown) show neither measurable pauses nor rate enhancements at *tR'* for the transcription complexes that read through suggests that the putative N-dependent rate enhancement within the terminators is likely to be comparable to that seen at other positions.

The extent of N-dependent antitermination depends on the relative rates of elongation and termination

We ask now whether such N-induced variations in the elongation rate of the transcription complex can provide a quantitative explanation of the anti-

termination effects that we have measured. Elsewhere (von Hippel & Yager, 1991, 1992) a theoretical formulation of the efficiency of termination at any template position has been presented in terms of a rate competition between alternative

elongation and termination reaction pathways. This formulation shows, at template positions in the vicinity of intrinsic terminators where the transcription complex is sufficiently destabilized to make the rates of elongation and termination com-

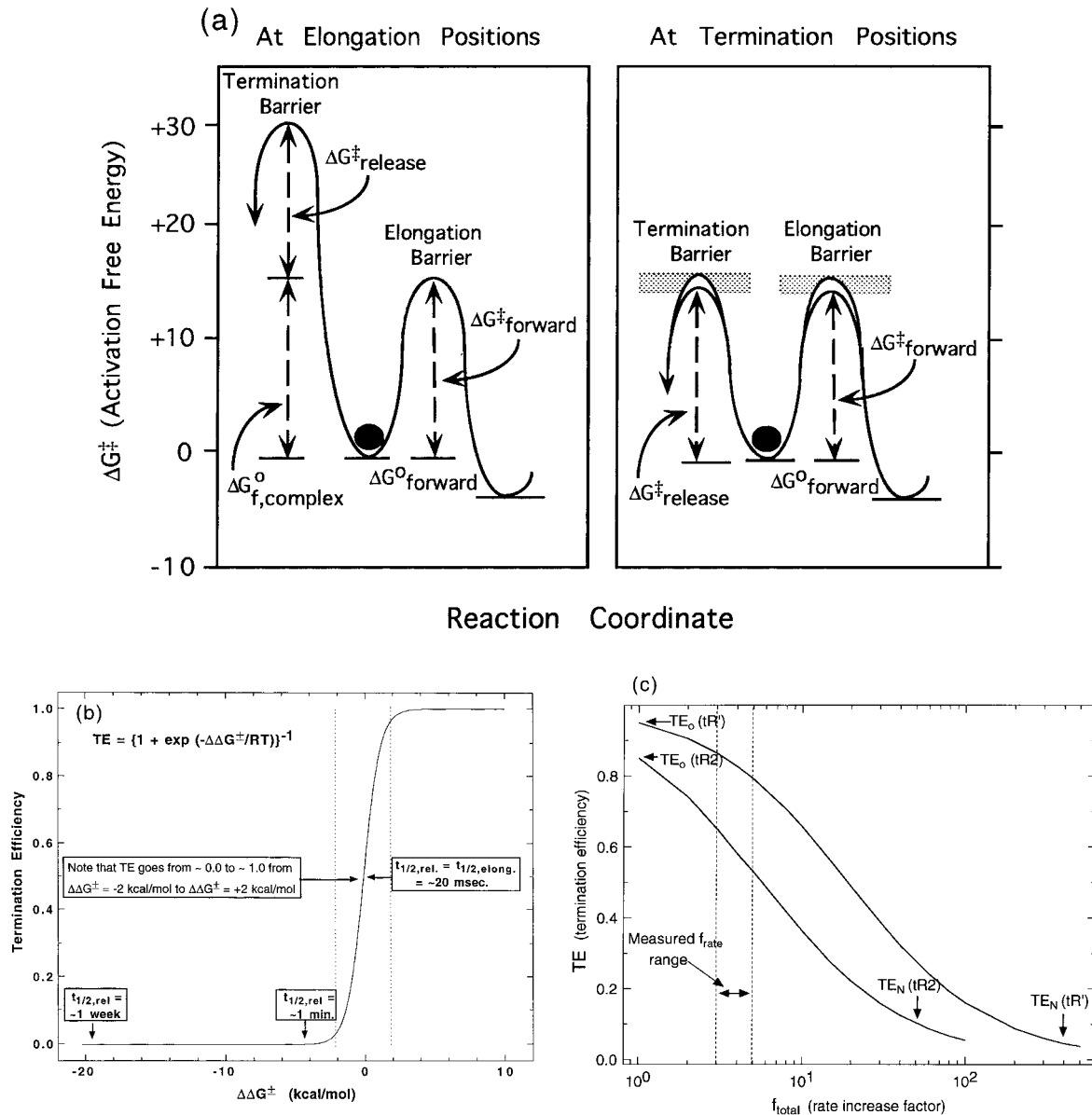


Figure 6. The elongation-termination decision as a competitive kinetic process. (a) Schematic of the relative heights of the free energy of activation barriers to elongation and to termination at a typical elongation position (left) and at a typical terminator position (right). The zero-point on the y-axis is set equal to the free energy of the reactant state of the transcription complex at template position I. The total height of each barrier is the sum of a thermodynamic (stability) component ($\Delta G_{f,complex}^{\circ}$) and a kinetic component ($\Delta G_{forward}^{\ddagger}$ or $\Delta G_{release}^{\ddagger}$). Barrier heights corresponding to tenfold changes in rate are shown as alternative heavy lines at the tops of the peaks in the right-hand panel; the stippled areas corresponding to peak height variations of ± 1.4 kcal/mol over which range the termination efficiency (TE) goes from ~ 0.01 to ~ 0.99 (see (b)). (b) Termination efficiency (TE) as a function of the difference in the heights of the free energy of activation barriers to termination and to elongation at template position I ($\Delta\Delta G^{\ddagger}$). Some important features of this relationship are indicated by numerical examples (see the text and annotations on the graph). (c) Theoretical plots of the overall termination efficiency (TE) at intrinsic terminators tR' and $tR2$ as a function of the calculated increase in net elongation rate (f_{total}), assuming an invariant rate of termination (RNA release). The plots are normalized to the experimentally measured TE values for these terminators in the absence of N (TE_0 values indicated). Observed TE values for each terminator in the presence of saturating N and NusA (and on nut^+ templates) are also shown, and the corresponding values of f_{total} are indicated (see the text). (a) and (b) have been modified, with permission, from von Hippel & Yager (1991).

parable (Wilson & von Hippel, 1994), that the expected termination efficiency should depend on the difference in heights of the free energy of activation barriers to elongation and termination ($\Delta\Delta G^\ddagger$).

The left-hand panel of Figure 6(a) represents the competitive situation at non-terminator positions along the template.† Here the height of the free energy of activation barrier to elongation is approximately +16 kcal/mol, which corresponds (via the Eyring relation, see von Hippel & Yager, 1991) to a forward rate constant of about 30 to 50 s⁻¹ (i.e. to an average “dwell-time” of 20 to 40 ms at each template position). In contrast, the height of the free energy of activation barrier to termination is typically greater than +30 kcal/mol under these conditions, corresponding to a characteristic dissociation time of the complex (and thus an RNA release rate) that is measured in hours or days. Thus, this representation simply shows, in activation barrier terms, that termination is not a possibility at most template positions and that significant changes in the rate of elongation or in the stability of the complex as a consequence of sequence or factor-dependent effects can be used to control elongation at these positions without risk of premature RNA release.

In contrast, the competitive situation shown in the right-hand panel of Figure 6(a) represents events at terminators where the free energy of activation barrier to RNA release has been significantly lowered, either in a sequence-dependent fashion at intrinsic terminators (von Hippel & Yager, 1991; Wilson & von Hippel, 1994, 1995) or by the action of transcription termination protein rho (A.Q. Zhu & P.H. von Hippel, unpublished results). In this situation the rates of elongation and termination are comparable, as shown by the fact that the free energy of activation barriers are of comparable height, and that small changes in the relative heights of the barriers (within the stippled zones shown) can tip the regulatory balance almost entirely toward either elongation or termination. In Figure 6(b) these effects are illustrated quantitatively by plotting termination efficiency (TE) as a function of the difference in height of the competing free energy of activation barriers ($\Delta\Delta G^\ddagger$). We note that the entire range of TE is covered by a change of $\Delta\Delta G^\ddagger$ from -2 to +2 kcal/mol, meaning that at termination sites a very small change in either the rate of elongation of the transcript or the stability (with respect to dissociation) of the complex, or a combination of both, can effec-

tively move the termination efficiency across the entire regulatory range ($TE \approx 0.01$ to 0.99).

The properties of this relationship are further illustrated by annotations on Figure 6(b) that emphasize that if (e.g.) the average dwell-time of the elongation complex at each template position is ~ 20 ms, then the termination efficiency for the complex will be effectively zero, whether the stability of the elongation complex (the $t_{1/2}$ for RNA release) is one week or one minute. On the other hand, we also note that within the zone of $\Delta\Delta G^\ddagger$ over which TE is sensitive to differences in the relative barrier height, the experimental change in TE observed at a given terminator as a consequence of (e.g.) the addition of N protein can be used with Figure 6(b) to calculate the value of $\Delta\Delta G^\ddagger$ to which this TE change corresponds. As a consequence this tells us the total change in rate or complex stability (or a combination of both) that is required to bring about the change in TE observed experimentally, and thus tells us the magnitude of the effect that we should be looking for. (This general point is developed further below.)

Thus, if we assume that the binding of N to the transcription complex alters only the elongation rate constant of the transcription complex (k_{elong}), where k_{elong} is related to the height of the elongation barrier to elongation by the Eyring equation (von Hippel & Yager, 1991), we can write:

$$(k_{\text{elong,N}}/k_{\text{elong,0}}) = \exp(\Delta\Delta G_{\text{N-0}}^\ddagger/RT) \quad (1)$$

where $(k_{\text{elong,N}}/k_{\text{elong,0}})$ represents the ratio of the elongation rates in the presence and absence of N, respectively, and $\Delta\Delta G_{\text{N-0}}^\ddagger$ represents the decrease in the height of the free energy of activation barrier to elongation to which this rate change corresponds. If we further assume that the N-dependent enhancement in elongation rate of the transcription complex within terminator sequences is comparable with the average rate enhancement over the entire template (this assumption is reasonable (see above) but not easy to test directly because of the instability of the transcription complex while traversing terminator sites), we can calculate that the three- to fivefold increase in average elongation rate in the presence of saturating N (and NusA) concentrations that we have measured experimentally corresponds to a change in the height of the activation barrier to elongation of 1.0 to 1.6 kcal/mol. These values, in turn, can be related to the corresponding change in termination efficiency (ΔTE) by:

$$\Delta TE = (1 + \exp\{-\Delta\Delta G_{\text{N-0}}^\ddagger/RT\})^{-1} \quad (2)$$

Using these equations and assuming that the rate of dissociation of the complex does not change, we can generate plots of the apparent overall TE as a function of the increase in elongation rate induced by the addition of saturating levels of N and NusA. Such plots, for the tR' and $tR2$ terminators, are shown in Figure 6(c). The experimental values of the overall termination efficiencies obtained for each terminator in the absence of N (but in the pre-

† These “one-hump” activation barriers should not be taken to represent the actual reaction pathways to either elongation or termination, since these reactions must consist of multiple steps. Rather these barriers as presented simply serve to show the relative magnitudes of the free energies of activation needed to reach the transition state for the rate-limiting step in each of the competing processes.

sence of saturating NusA) are plotted on the theoretical lines (0.95 for tR' and 0.85 on $tR2$) on the y -axis, and the values obtained in the presence of saturating N and NusA (and a functional *nut* site) are plotted on the theoretical curves at the TE values observed experimentally (i.e. 0.05 for tR' and 0.10 for $tR2$).

The range of positions along the theoretical curves at which these experimental points would be expected to fall if the entire N-dependent anti-termination effect were due to the observed three- to fivefold increase in average elongation rate are indicated by vertical lines in Figure 6(c). Clearly this rate-enhancement effect is not sufficient to account for the observed changes in TE if the rate acceleration of the elongation reaction at the terminator positions is comparable to that seen at other template positions. An additional ~ 100 -fold decrease in the termination rate, presumably reflecting an ~ 100 -fold stabilization of the transcription complex brought about by the binding of N and the boxB hairpin of the transcript to the complex with NusA, is also required to account for the degree of antitermination observed. We note that such an ~ 100 -fold stabilization of the transcription complex by binding the minimal antitermination subassembly (N, NusA, and boxB RNA, delivered to the transcription complex by looping of the nascent transcript chain with 100 to 200 residues of RNA between the transcription complex and the boxB site coded by the *nut* sequence) is just what was estimated on a totally different basis using RNA looping calculations (Van Gilst *et al.*, 1997; see also Rees *et al.*, 1996). In contrast, for the less efficient $tR2$ terminator, the additional stabilization required is somewhat less and the observed TE effect can be attributed in larger measure to the rate enhancement of elongation (Figure 6(c)), although an additional ~ 20 -fold stabilization of the complex (decrease in the complex dissociation rate) is also required to explain the level of N-dependent antitermination observed at terminator $tR2$.

† Note that for values of $f_{\text{total}} < 1$, the theoretical plots corresponding to Figure 6(c) will curve upwards, since TE will be increased with increasing regulator concentrations. These general issues will be discussed further elsewhere (M. R. Van Gilst & P.H. von Hippel, unpublished results).

‡ Since TE in the kinetic competition model (von Hippel & Yager, 1991) can be defined directly in terms of the rates of release and elongation as:

$$TE = k_{\text{release}} / (k_{\text{release}} + f_{\text{total}} \cdot k_{\text{elong}})$$

(with f_{total} being unity in the absence of N), it is also possible to calculate f_{total} for a given value of ΔTE directly from this mathematically simpler relationship. On the other hand, in our view there is a conceptual advantage in getting to Figure 6(c) through Figure 6(b), since Figure 6(b) shows directly the free energy of activation change ($\Delta\Delta G^\ddagger$) that a given value of ΔTE represents, making the partition of f_{total} into f_{rate} and $f_{\text{stability}}$ more physically meaningful.

These results and others (Rees *et al.*, 1996; Van Gilst *et al.*, 1997) indicate that both elongation rate increases (lowering the free energy of activation barrier to elongation) and stability increases (raising the effective activation barrier to termination) are involved in regulation of the N-dependent anti-termination system. Of course, the same formalism can be employed to describe any regulatory change (up or down) in TE that occurs within the range of $\Delta\Delta G^\ddagger$ that is subject to regulation (-2 to $+2$ kcal/mol; see Figure 6(b)).

In general terms we can therefore rewrite equation (2) as:

$$\Delta TE = (1 + f_{\text{total}} \exp\{-\Delta\Delta G_{\text{N-0}}^\ddagger / RT\})^{-1} \quad (3)$$

where ΔTE is any change observed in the termination efficiency as a consequence of the regulatory change and:

$$f_{\text{total}} = f_{\text{rate}} \cdot f_{\text{stability}} \quad (4)$$

Here f_{total} is the net rate change that corresponds to the product of the factor by which the elongation rate (f_{rate}) is increased or decreased and the factor by which the stability (reflected as a change in the dissociation rate) of the transcription complex ($f_{\text{stability}}$) is increased or decreased. Thus, f_{rate} and $f_{\text{stability}}$ (and f_{total}) can all have values greater than or less than unity.

Operationally, we can use plots like Figure 6(c) to determine f_{total} for any given set of regulatory conditions. Thus, for a given ("ground-state") situation (here termination efficiency under standard conditions in the absence of N) one can use the measured value of TE_0 to calculate the ground state free energy of activation difference factor ($\Delta\Delta G^\ddagger / RT$). TE_0 then becomes the y -axis intercept of theoretical plots of the sort shown in Figure 6(c), and the value of f_{total} corresponding to any value of TE measured under regulatory conditions can simply be read-off the x -axis at the appropriate point.† Further independent measurements, such as those of the N-dependent rate enhancement effects shown here, will generally be required to dissociate f_{total} into its constituent components of f_{rate} and $f_{\text{stability}}$ under each set of experimental conditions.‡

Significance of multiple template positions for RNA release within an intrinsic terminator

As predicted theoretically (Yager & von Hippel, 1991) and shown experimentally (Wilson & von Hippel, 1994, 1995; this study), intrinsic terminators (defined as the "zone of opportunity" for termination along the template) appear to be about 6 bp in length. However, significant termination tends to occur at only two or three positions within these terminator zones. Which positions these are, and how effective termination is at each, will depend on the actual elongation and dissociation rates at each position and can be manipulated by changing the concentrations of the appropriate NTPs (Table 1), as well as by other potential (and

more physiological?) regulatory mechanisms. The length of the termination zone (i.e. the "length" of the intrinsic terminator) is controlled by the mechanism of termination, and as proposed elsewhere (von Hippel & Yager, 1991) may reflect the passage of the transcription complex through a particularly destabilizing sequence of the RNA-DNA hybrid (see discussion of this point by Wilson & von Hippel, 1994, 1995). We note also that this definition of the length of the terminator appears (within limits) to be independent of the length or stability of the terminator hairpin (Wilson & von Hippel, 1994; this work), as predicted by a modified Yager-von Hippel termination model (Wilson & von Hippel, 1995).

The fact that there are several template positions for termination within an intrinsic terminator may also have functional advantages, since this allows several relatively inefficient individual termination positions to work together to form an efficient terminator by sequential iteration. The overall efficiency of a terminator containing several termination positions can be written:

$$TE_{\text{total}} = TE_1 + TE_2(X_2) + \dots + TE_n(X_n) \quad (5)$$

where $TE_1, TE_2 \dots TE_n$ are the termination efficiencies at template positions 1, 2, ... n within the terminator, and $X_2 \dots X_n$ represent the fractions of the transcription complexes that initially arrive at position 1 and then at each subsequent termination position. As an example, equation (5) may be used to show that if TE for every termination position within the terminator is a relatively modest 0.3, the overall efficiency of the entire terminator is still $0.3 + 0.21 + 0.16 + 0.10 + 0.07 + 0.05 = 0.89$. Thus, although TE at individual termination positions may be low, the overall terminator can still be very effective.

We note also that even at this relatively low TE per template position (0.3), ~75% of the chains terminate at the first three positions within the terminator. The major termination positions within tR' (this work) and $tR2$ (Wilson & von Hippel, 1994) typically manifest higher termination efficiencies, and thus discernable termination is effectively complete over two to three termination positions within these typical intrinsic terminators. This tends to be less true at rho-dependent terminators (see Lau *et al.*, 1982; Morgan *et al.*, 1983; Galloway & Platt, 1988; A.Q. Zhu & P.H. von Hippel, unpublished results), where the size of the termination zone itself may be largely determined by kinetic coupling between rho and polymerase (Jin *et al.*, 1992) and by the sequence-dependent destabilization that rho can exert at each template position (A.Q. Zhu & P.H. von Hippel, unpublished results). At these rho-dependent terminators RNA may be released over 100 bp along the template.

From a regulatory point of view, the fact that termination occurs over several template positions may protect an intrinsic terminator against severe damage or destruction by mutation at a single

nucleotide position, and regulatory flexibility is provided by the availability of more than one position at which control mechanisms for termination can operate. Thus, the principle of reiteration that seems to be built into intrinsic terminators by their structure again means that relatively minor changes in elongation rate or complex stability can be amplified by repetition to produce a qualitatively definitive biological effect, as required for "regulatory switches" of this type (see von Hippel *et al.*, 1996).

Conclusions

These mechanistic findings lend support to the idea that biological regulation must be treated as a quantitative problem, with both thermodynamic and kinetic components. Clearly changes in stability and rate can be utilized to design biological circuits that regulate by kinetic competition between alternative reaction pathways to form switches that are both definitive and specific (see von Hippel *et al.*, 1996). It seems likely that such simple principles, in various combinations, will be found to underlie the more complex and multi-component switches of eukaryotic systems as well. Molecular aspects of how N actually functions in controlling some of the regulatory processes of N-dependent antitermination are considered elsewhere (Van Gilst *et al.*, 1997; Van Gilst & von Hippel, 1997).

Experimental Procedures

Plasmids and transcription templates

Oligonucleotides were purchased from the Midland Certified Reagent Co. (Odessa, TX). DNA cleavage was performed using enzymes purchased from New England Biolabs or Boehringer-Mannheim Biochemicals. Clones were screened by restriction analysis of plasmids prepared by anion exchange chromatography (Qiagen), following alkaline-SDS lysis. Sequences of the newly constructed regions of the plasmids used herein were confirmed by DNA sequencing using a dideoxynucleotide method with a modified T7 DNA polymerase (US Biochemicals). PCR reactions were performed essentially as described by Krummel (1990), using Vent DNA polymerase (New England Biolabs). All the templates used in this study are shown in Figure 1.

Plasmid pRB2 was cloned in two steps. The *EcoRI-PstI* fragment from pDL11 carrying the tR' terminator (Lazinski *et al.*, 1989) was cloned between the *EcoRI-PstI* sites in the plasmid pSK+ (Stratagene), creating pRB1. pRB2 was engineered by replacing the *EcoO109-EcoRI* fragment from pRB1 with the *pL-nutL* containing *EcoO109-EcoRI* fragment from pWW10 (Whalen *et al.*, 1988). pRB5 was created by replacing the *EcoRI-tR'-XbaI* fragment from pRB2 with an *EcoRI-XbaI* fragment containing terminator $tR2$. The *EcoRI-tR2-XbaI* fragment was generated by PCR from phage λ DNA using two oligonucleotide primers (22 bases each) that created *EcoRI* and *XbaI* restriction sites by introducing the appropriate mutations at lambda coordinates 40559 and 40562 with primer 1 and at coordinates 40674 and 40675 with primer 2.

pRB15 was derived from pRB2 by site-directed mutagenesis, using a 24-residue oligonucleotide that introduced an A-to-C mutation in the *tR'* terminator at lambda coordinate 44782 (Kunkel, 1985). pRB35 and pRB37 were derived from pRB2 and pRB15, respectively, by a site-directed deletion-mutagenesis procedure that deleted 162 bp containing *nutL* from the sequence between *pL* and *tR'*, using a 40 bp oligonucleotide that is complementary to 17 bp of sequence on either side of the ultimately deleted sequence (Kunkel, 1985). Template fragments for *in vitro* transcription were prepared as described elsewhere (Whalen & Das, 1990).

Proteins

E. coli RNA polymerase was purified as described by Burgess & Jendrisak (1975) and by Lowe *et al.* (1979). The holoenzyme was further purified as described by Gonzalez *et al.* (1977) or was purchased from Eppicentre Technologies (Madison, WI). NusA was purified from an overproducer strain as described by Schmidt & Chamberlin (1984) with an additional degree of purification added by chromatography over a MonoQ FPLC column (Pharmacia). λ N protein was overexpressed from a T7-promoter-containing vector and purified as described by Rees *et al.* (1996). The concentrations of RNA polymerase, NusA, and λ N were determined spectrophotometrically using calculated molar extinction coefficients (Gill & von Hippel, 1989).

In vitro transcription reactions

Transcription reactions were carried out essentially as described by Whalen & Das (1990). Stalled transcription elongation complexes were formed initially, and then supplemented with proteins and substrate NTPs to permit continued RNA chain elongation. This design provides for relatively synchronous, single-round transcription elongation reactions. By radioactively labeling the RNA (using α -labeled NTPs) only initially during stalled complex formation, the total cpm measured for an RNA band on a gel are directly proportional to the number of transcripts of that size, and not to the length of the transcript.

In a typical reaction, 150 μ M ApU (Sigma), 5 μ M ATP, 5 μ M CTP, 5 μ M GTP, 25 nM *E. coli* RNA polymerase, 20 to 50 nM template (in units of template molecules), and [α -³²P]ATP or CTP (at 1 to 2 Ci/mol), were incubated in transcription buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM EDTA, 5% (v/v) glycerol, and 1 mM DTT, at 30°C for five to ten minutes (Whalen & Das, 1990). Due to the sequence of the template region initially transcribed from the *pL* promoter, transcription on the pRB2, pRB5 and pRB15 DNA templates with only three of the four required NTPs yields stalled complexes containing nascent RNA transcripts that are 15 nt in length and stalled at a 3'-CMP residue (C15 complexes; Levin & Chamberlin, 1987). This same procedure yielded A18 complexes from the pRB35 and pRB37 templates.

Transcription elongation reactions were performed at either 30°C or 37°C in transcription buffer containing 100 or 300 mM potassium acetate, 10 μ g/ml rifampicin, and NTP concentrations, as indicated in the Figure legends, together with 90 nM N protein and 120 nM NusA, when used. In the experiments that defined the dependence of the termination efficiency on the concentration of the required next nucleotide, the (final) concentrations of three of the NTPs were always held constant at 1.0 mM

each, while the concentration of the fourth was varied as indicated in the Figure legends. Stalled transcription complexes labeled with [α -³²P]ATP or [α -³²P]CTP were employed in transcription elongation reactions containing less than saturating concentrations of CTP or ATP. This yielded transcripts labeled only within the first 15 (or 18) nt at the 5' end, and also resulted in significant labeling only of transcripts made in single-round transcription reactions.

In the reactions in which the net rate of transcription elongation was limited by the reduced concentrations of one of the four substrate NTPs, C15 complexes on the pRB2 DNA template were permitted to resume elongation at 22°C on the addition of three NTPs at 1 mM concentration, a variable concentration of the fourth NTP, and N and NusA as indicated. Elongation reactions performed with 1 mM NTPs at 2°C were conducted in a 2°C water bath placed in an 8°C cold room. At the times indicated in the Figure legends, reactions were quenched with EDTA to a final concentration of 20 mM, processed as described above, and analyzed on 6% (w/v) polyacrylamide-urea gels cast with 0.4 to 1.2 mm exponential wedge spacers. The mean position of the elongation complexes at each time point was determined by quantitation with the AMBIS Radioanalytic Imaging System and accompanying software (San Diego, CA), and mean transcript lengths were estimated using DNA size markers that had been end-labeled with [α -³²P]dCTP using *E. coli* DNA polymerase I (Klenow fragment).

Net efficiencies of termination (*TE*) were calculated by dividing the amount of radioactivity in each terminated band by the total present in the terminated and run-off bands. The *TE* at each position within the *tR'* terminator was calculated by dividing the amount of radioactivity in a particular terminated band by the amount present in that and all longer (but not shorter) bands, as described (Wilson & von Hippel, 1994). Since the terminated transcript bands could not always be completely resolved to the baseline, overlapping peaks were deconvoluted into a series of Gaussians or Lorentzians to assess the absolute amounts of each size class present.

Acknowledgements

This work was submitted (by W. A. R.) to the Graduate School of the University of Oregon in partial fulfillment of the requirements for the PhD. in Chemistry. It was supported in part by NIH research grants GM-15792 and GM-29158 (to P. H. v. H.), by NIH research grant GM28946 (to A. D.), and by a grant from the Lucille P. Markey Charitable Trust to the Institute of Molecular Biology. W. A. R. was a pre-doctoral trainee on USPHS institutional training grant GM-07759. P. H. v. H. is an American Cancer Society Research Professor of Chemistry. We are grateful to William Whalen, Joe DeVito, Marc Van Gilst, Mark Young, and other colleagues in both the Oregon and the Connecticut laboratories for many helpful and stimulating discussions.

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Edited by K. Yamamoto

(Received 5 February 1997; received in revised form 24 July 1997; accepted 27 July 1997)