Amplification and Isolation of *Escherichia coli nus*A Protein and Studies of Its Effects on in Vitro RNA Chain Elongation†

Martin C. Schmidt and Michael J. Chamberlin*

ABSTRACT: The *Escherichia coli nus*A gene product is an RNA polymerase binding protein which has been implicated in a variety of cellular and viral termination and antitermination processes. To facilitate large-scale preparation and biochemical studies of the *nus*A protein, we have cloned the *nus*A gene into a λ P_L-derived overexpression vector. *E. coli* strains bearing the resulting plasmid (pMS7) produce large amounts of *nus*A protein when induced, and the protein is easily purified to homogeneity. Biochemical studies of *nus*A protein reveal that it inhibits in vitro RNA chain elongation by *E. coli* RNA polymerase with a variety of templates. Two modes of inhibition are found. Inhibition of elongation with poly[d(A-T)] template is completely competitive with nucleoside triphosphates and shows an inhibitory constant (K_i) of 3×10^{-7} M. In contrast, inhibition of elongation with T7 DNA as template is mixed. One component of the inhibition is competitive with nucleoside triphosphate substrates and is reversed at elevated substrate concentrations. A second inhibitory component remains even at saturating substrate concentrations; this sequence-dependent mode of inhibition shows a much lower K_i of 2×10^{-8} M. The existence of two different modes of inhibition might be explained if two molecules of *nus*A protein can bind to each RNA polymerase complex. The interaction of *nus*A protein with elongating RNA polymerase molecules is not processive but appears to be characterized by rapid association and dissociation. Under proper conditions, a σ–*nus*A cycle [Greenblatt, J., & Li, J. (1981) *Cell* (*Cambridge, Mass.*) *24*, 421–428] can be demonstrated in vitro in which each polymerase goes through multiple rounds of transcription involving successive interactions with σ and the *nus*A protein.

In 1975, Kung, Spears, and Weissbach (Kung et al., 1975) reported the isolation of a protein (L factor) from *Escherichia coli* which was required for coupled transcription–translation of active β-galactosidase from *lac*Z DNA. They suggested that L factor might act to reduce ρ-dependent termination of transcription within *lac*Z and showed that L factor was able to bind directly to *E. coli* RNA polymerase (Kung & Weissbach, 1980). In subsequent studies, it was discovered that L factor is the product of the *E. coli nus*A gene, which is required for function of the phage λ antitermination protein N (Greenblatt & Li, 1981b). These results, linking *nus*A protein with antitermination functions, led us to test whether this factor could suppress transcriptional pausing, a phenomenon which accounts for slower rates of transcriptional elongation in vitro compared to in vivo (Kassavetis & Chamberlin, 1981). Surprisingly, *nus*A protein reduces the rate of transcriptional elongation about 2-fold and enhances transcriptional pausing at specific sites (Kassavetis & Chamberlin, 1981). This is seen with a variety of templates (Greenblatt et al., 1981; Kingston & Chamberlin, 1981; Fisher & Yanofsky, 1983; Lau et al., 1983) and accounts for the inhibition of overall transcription reported originally by Kung et al. (1975). In fact, *nus*A protein can enhance transcriptional termination at some sequences in vitro (Greenblatt et al., 1981), and the *nus*A1 mutation gives some relief of polarity in vivo (Ward & Gottesman, 1981).

Greenblatt & Li (1981a) have shown that *nus*A protein binds specifically to *E. coli* core RNA polymerase and have suggested that in vivo it may be generally associated with elongating RNA polymerase molecules. The apparently contradictory properties of *nus*A protein, in which it can enhance both termination and antitermination, together with its possible role as a general transcription factor in *E. coli*, have led us to explore the isolation and biochemical properties of this protein in more detail.

Materials and Methods

Materials

RNA polymerase holoenzyme was purified from *E. coli* K12 by the method of Gonzalez et al. (1977). Except where noted otherwise, the holoenzyme preparation used in this study contained 40% active molecules as determined by the quantitative RNA polymerase assay described by Chamberlin et al. (1979). T7 D111 DNA (Thomas & Abelson, 1961), poly[d(A-T)] (Schachman et al., 1960), and [α-^{32}P]CTP (Symons, 1980) were prepared as referenced. $H_3{}^{32}PO_4$ and [α-^{32}P]UTP were purchased from New England Nuclear. [^{14}C]ATP was purchased from Schwarz/Mann. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals and were used without further purification.

Methods

Protein concentrations were determined by the method of Lowry et al. (1951) after precipitation of the samples with trichloroacetic acid to remove interfering substances. Bovine serum albumin was used as a protein standard. Protein concentrations of *E. coli* RNA polymerase in terms of weight of amino acids were determined by ultraviolet absorption using an extinction coefficient of $\epsilon^{1\%}_{280nm} = 6.5$. DNA concentrations were determined by ultraviolet absorption using an extinction coefficient of $\epsilon^{1\%}_{260nm} = 200$.

Construction of a Plasmid for Amplification of the nusA Protein. General procedures employed were those described by Maniatis et al. (1982). Plasmids pGW7 and pBA2 (Plumbridge & Springer, 1983) were the generous gifts of William Konigsberg and Jacqueline Plumbridge, respectively (Figure 1). Plasmid pGW7 was constructed by Geoff Wilson and William Konigsberg at Yale University, New Haven, CT.

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received July 25, 1983.* This investigation was supported by Research Grant GM12010 from the Institute of General Medical Sciences, U.S. Public Health Service, and by National Research Award Training Grant GM07232.

0006-2960/84/0423-0197$01.50/0

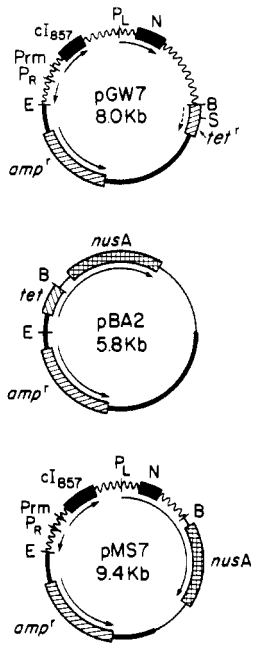

FIGURE 1: Schematic representation of plasmids pGW7, pBA2, and pMS7. All restriction sites shown are unique for each plasmid (E) = *Eco*RI; B = *Bam*HI; S = *Sal*I). Transcription units are indicated with arrows, showing the direction of transcription but not transcript size. λ promoters, P_L, P_R, and P_{rm}, and λ genes, cI_{857} and N, are indicated (Szybalski & Szybalski, 1979): (thin solid line) *E. coli* DNA; (squiggly line) λ DNA; (thick solid line) pBR322 DNA.

They will publish details of its construction and properties later. Plasmid pMS7 was constructed by ligating pBA2 which had been cleaved with *Eco*RI and *Bam*HI with a 5-fold molar excess of pGW7 which had been cleaved with *Eco*RI, *Bam*HI, and *Sal*I. Ampicillin-resistant transformants were screened for plasmid size and restriction sites as described by Holmes & Quigley (1981). To prepare *nus*A protein, *E. coli* 294 transformed with pMS7 (294/pMS7) were grown in M9 medium (Miller, 1972) containing 1% glucose and 25 μg/mL ampicillin at 32 °C, to an OD_{550} of 1 (about 5×10^8 cells/mL). The suspension was then shifted to 42 °C, and aeration was continued for 4 h, after which the cells were chilled to 4 °C and were harvested by centrifugation.

Purification of nusA Protein from pMS7-Containing Cells. The procedure employed was a modification of the method of Kung et al. (1975). All steps were performed at 4 °C. The presence of *nus*A protein in different fractions was determined by subjecting samples to sodium dodecyl sulfate (SDS)[1]–polyacrylamide gel electrophoresis and following the protein band which represents the *nus*A protein. Twenty-five grams of heat-induced 294/pMS7 cells was suspended in 80 mL of a buffer solution (50 mM Tris-HCl, pH 7.9, 5% glycerol, 2 mM EDTA, 0.1 mM DTT, 1 mM β-ME, 0.3 M KCl, and 25 μg/mL PMSF) and disrupted by sonication (maximum temperature of 12 °C). The extract was cleared by centrifugation at 8000*g* for 20 min, and the supernatant fraction was then centrifuged at 100000*g* for 3 h. The 100K supernatant fraction (Table I) was diluted with 4 volumes of distilled water before being loaded onto a 5 × 5 cm DEAE-cellulose column (DE-52) equilibrated with 20 mM potassium phosphate, pH 7.5, 5% glycerol, and 0.1 mM DTT (RB 7.5). The column was rinsed with 200 mL of RB 7.5, followed by 500 mL of 0.25 M potassium phosphate, pH 6.5, 5% glycerol, and 0.1 mM DTT. Concentrated phosphate was removed from the column by rinsing with a solution containing 20 mM potassium phosphate, pH 6.5, 5% glycerol, and 0.1 mM DTT (RB 6.5) before the *nus*A protein was eluted with RB 6.5 containing 0.6 M $(NH_4)_2SO_4$. Fractions containing significant absorbance at 280 nm were pooled (DEAE I pool) and precipitated by the addition of a saturated solution of $(NH_4)_2SO_4$ (pH 6.5) to a final concentration of 70%. The precipitate was dissolved in 20 mL of RB 7.5, was dialyzed against 2 L of RB 7.5, and was then loaded onto a 1.5 × 25 cm DEAE-Sephadex column previously equilibrated with RB 7.5. The column was washed first with a solution containing 0.25 M potassium phosphate (pH 6.5), 5% glycerol, and 0.1 mM DTT, and the *nus*A protein was then eluted with a 200-mL linear gradient of RB 6.5 containing 0.25–1.0 M potassium phosphate. Fractions containing *nus*A protein were dialyzed against storage buffer (10 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.1 mM DTT, 0.1 M NaCl, and 50% glycerol) and were stored at −20 °C.

Table I: Purification of the *nus*A Protein[a]

fraction	volume (mL)	protein (mg)	*nus*A (%)
8K supernatant	112	1340	2
100K supernatant	98	906	3
DEAE I pool	220	114	30
ammonium sulfate precipitate	30	78	40
DEAE II pool	38	24	100

[a] Fractions are identified under Methods, and aliquots of each were analyzed by SDS-polyacrylamide gel electrophoresis (Figure 2). The ammonium sulfate precipitate fraction was assayed after dialysis. Protein concentrations were determined as described under Methods. The *nus*A protein content in each fraction was estimated from Coomassie-stained SDS-polyacrylamide gels.

Preparation of Poly[d(A-T)] Ternary Complexes. Poly[d(A-T)] ternary complexes (Rhodes & Chamberlin, 1974) were prepared in a reaction containing 44 mM Tris-HCl, pH 8, 14 mM β-ME, 14 mM $MgCl_2$, 14 mM NaCl, 40 μg/mL acetylated bovine serum albumin, 0.04 mM EDTA, 2% glycerol, 1 mM ATP, 1 mM UTP, 0.15 mM poly[d(A-T)], and 0.16 mg/mL RNA polymerase. After incubation at 37 °C for 1 min, one-fourth volume of glycerol was added, and the entire reaction (200 μL) was loaded onto a 1 × 13 cm Sepharose 6B column equilibrated with 20 mM Tris-HCl (pH 8), 1 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, and 5% glycerol at room temperature. One-milliliter fractions were collected, and the absorbance at 260 nm was monitored. Ternary complexes elute in the excluded volume [6.7×10^4 poly[(A-T)] elongation units; Rhodes & Chamberlin, 1974] and can be stored at room temperature for up to 48 h without loss of activity.

Transcription Assays with Poly[d(A-T)] Ternary Complexes. Transcription with poly[d(A-T)] ternary complexes was carried out at 37 °C in a reaction mixture (0.25 mL) containing 44 mM Tris-HCl (pH 8), 14 mM β-ME, 14 mM $MgCl_2$, 20 mM NaCl, 2% glycerol, 40 μg/mL acetylated bovine serum albumin, and 10 μg/mL rifampin. ATP and [α-^{32}P]UTP (500 cpm/pmol) were included at 0.2 mM unless indicated otherwise. Transcription was initiated by the addition of ternary complexes (75–150 elongation units; Rhodes & Chamberlin, 1974) to reaction mixtures prewarmed to 37 °C. Aliquots were removed, and the RNA was precipitated and measured as described elsewhere (Chamberlin et al., 1979).

Quantitative Transcription Assays with T7 D111 DNA Template. The quantitative assay follows the procedures

[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; β-ME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

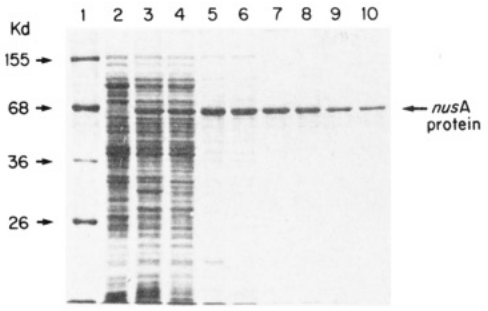

FIGURE 2: SDS–polyacrylamide gel analysis of *nus*A protein amplification and purification. Protein samples were analyzed on a 10% polyacrylamide–SDS gel as described by Laemmli (1970). (Lane 1) Molecular weight markers: *E. coli* core RNA polymerase [155, 150, and 36 kilodaltons (Kd)], bovine serum albumin (68 kilodaltons), and rabbit triosephosphate isomerase (26 kilodaltons). (Lane 2) Extract from *E. coli* 294 (20 μg of protein); (lane 3) extract from *E. coli* 294/pMS7 induced as described under Methods (20 μg); (lane 4) 100K supernatant fraction (18 μg); (lane 5) pool from DEAE-cellulose column (4 μg); (lane 6) ammonium sulfate precipitate fraction (5 μg); (lanes 7, 8, 9, and 10) fractions 64, 68, 72, 76 from the DEAE-Sephadex column, containing 1.8, 1.4, 0.9, and 0.5 μg of total protein, respectively.

described by Chamberlin et al. (1979) as modified by Chamberlin et al. (1983). Transcription reactions with T7 D111 DNA template were carried out at 30 °C in a reaction (0.2 mL) containing 40 mM Tris-HCl (pH 8), 10 mM β-ME, 4 mM $MgCl_2$, 120 mM KCl, 4 mM spermidine, 0.81 pmol of T7 D111 DNA molecules, and 2.0 pmol of RNA polymerase holoenzyme (1.0 μg of total protein). Nucleoside triphosphates were included at 2.7 mM ATP, 1.4 mM UTP, 1.1 mM GTP, and 0.7 mM [α-^{32}P]CTP (25–100 cpm/pmol), unless otherwise indicated. Reactions were initiated by warming to 30 °C, and rifampin was added to a final concentration of 10 μg/mL at 1.5 min after initiation. Aliquots were removed, and the RNA was precipitated and measured as described (Chamberlin et al., 1979).

Results

Cloning of the nusA Gene into an Overexpression Vector. Although procedures have been described for the purification of *E. coli nus*A protein to homogeneity (Kung et al., 1975; Greenblatt et al., 1981), these methods yield small amounts of protein and make extensive studies of its properties difficult. The cloning and mapping of the *E. coli* genetic region bearing the *nus*A gene by Plumbridge & Springer (1983) provided a potential source of that gene for genetic manipulation. Plasmid pMS7 was constructed (Figure 1) to put the *nus*A gene under the transcriptional control of the λ P_L promoter by inserting a P_L-containing segment from the overexpression vector pGW7, developed by Konigsberg and Wilson, into plasmid pBA2 of Plumbridge & Springer (1983). Transcription from P_L in pGW7 is under the control of the temperature-sensitive cI857 repressor gene and also the λ N antitermination protein gene, both of which are carried on the vector. At 32 °C, strains carrying pMS7 make only normal amounts of *nus*A protein, as shown by SDS–polyacrylamide gel electrophoresis of extracts (data not shown). However, after 4 h at 42 °C, a protein of molecular weight 65 000 becomes the predominant species (Figure 2).[2] Confirmation that this is the *nus*A protein was obtained by probing nitrocellulose blots of such gels with antibody to authentic L factor provided by Dr. H. F. Kung (Roche Institute, Nutley, NJ) using the method of Kane & Linn (1981), and subsequently by purification and characterization of the protein.

Purification of nusA Protein from pMS7-Containing Cells. The *nus*A protein was purified from thermally induced cultures carrying pMS7 by a procedure similar to that employed by Kung et al. (1975). Partitioning of the *nus*A protein was followed through different fractionation steps by SDS gel electrophoresis.

The *nus*A protein was eluted from the first DEAE column with ammonium sulfate instead of potassium phosphate buffer because concentrated potassium phosphate interferes with the subsequent ammonium sulfate precipitation step. The *nus*A protein which elutes from the second DEAE column is homogeneous as judged by polyacrylamide gel electrophoresis and can be stored in a 50% glycerol storage buffer at −20 °C without loss of activity in transcriptional inhibition assays. The yield from such a preparation is about 1 mg of *nus*A protein/g of cells (Table I), about 100-fold greater than the yield from wild-type cells (Greenblatt et al., 1981).

The protein obtained with cells transformed with pMS7 is authentic *nus*A protein as judged by the following criteria: (1) the *nus*A gene used for constructing pMS7 complements the *nus*A1 mutation (Plumbridge & Springer, 1983); (2) *nus*A protein from recombinant cells comigrates on SDS–polyacrylamide gels with *nus*A protein isolated from wild-type cells by the procedure of Kung et al. (1975); (3) *nus*A protein from recombinant cells reacts strongly with antisera raised against authentic L factor by Dr. H. F. Kung when tested with the Western blotting procedure of Kane & Linn (1981) (data not shown); (4) the protein inhibits T7 RNA synthesis at concentrations comparable to those found previously with authentic L factor (Kassavetis & Chamberlin, 1981).

Preparations of *nus*A protein obtained by this procedure were assayed for the presence of several contaminating enzymatic activities that might affect transcriptional studies. There was no detectable hydrolysis of [γ-^{32}P]ATP to Norite nonadsorbable material (Goldmark & Linn, 1972); 25 μg/mL *nus*A protein hydrolyzed 5 μM ATP at a rate less than 2 nM/min. There was no detectable conversion of supercoiled pGW7 DNA to a relaxed circular or linear form; 25 μg/mL *nus*A protein cleaved 3.2 nM pGW7 DNA molecules at a rate less than 0.8 pM/min. There was no detectable hydrolysis of RNA in a solution containing 25 μg/mL *nus*A protein; T7 RNA (1.7 μM acid-insoluble [α-^{32}P]CMP) was converted to an acid-soluble form at a rate less than 0.02 nM/min.

Effects of nusA Protein on in Vitro RNA Chain Elongation. (i) Transcription with Poly[d(A-T)] Template. Previous studies of *nus*A protein have shown that it inhibits in vitro transcription from 30% to 50% at concentrations in the range of 5–10 μg/mL (Kung et al., 1975; Kassavetis & Chamberlin, 1981; Greenblatt et al., 1981). This inhibition is at the level of RNA chain elongation (Kassavetis & Chamberlin, 1981; Kingston & Chamberlin, 1981). However, the ability of *nus*A to enhance pausing or even termination at specific sites suggested that the *nus*A protein might act in a sequence-specific manner. In fact, Olson et al. (1982) have identified a decanucleotide sequence from studies of *nut* sequences in several phages that they suggest may be generally involved in *nus*A function.

For these reasons, it was unexpected to find that *nus*A protein is an effective inhibitor of transcription elongation with poly[d(A-T)] templates (Figure 3). In these experiments,

[2] Previous studies (Kung et al., 1975; Greenblatt & Li, 1981b) have proposed a molecular weight for the *nus*A protein of 69 000, and we have used that value for all calculations in this paper. An accurate molecular weight for the *nus*A protein awaits the DNA sequence analysis of the *nus*A gene.

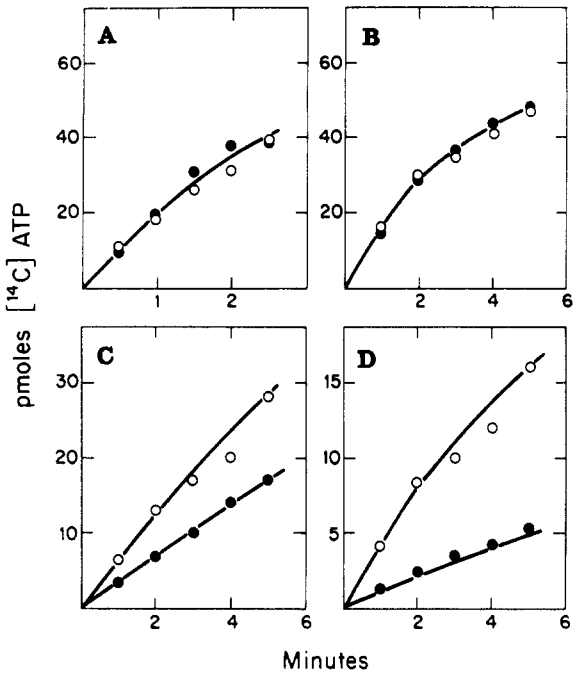

FIGURE 3: Effect of *nus*A protein on poly[r(A-U)] chain elongation. Transcription was initiated by the addition of poly[d(A-T)] ternary complexes to reaction mixtures (0.25 mL), and 50-μL aliquots were removed at the indicated times and assayed for poly[r(A-U)]. UTP and [^{14}C]ATP (9 cpm/pmol) were present at (A) 100, (B) 50, (C) 10, or (D) 5 μM. (○) No *nus*A protein; (●) 45 μg/mL *nus*A protein.

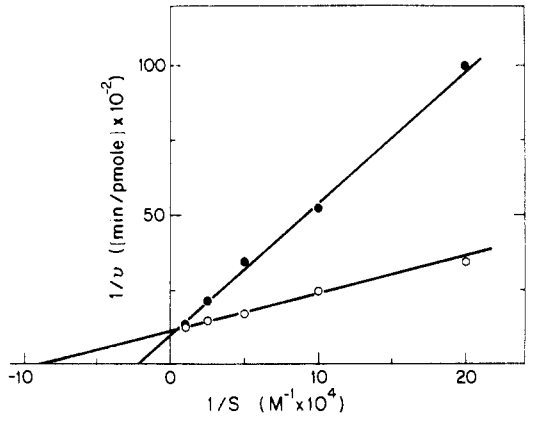

FIGURE 4: Analysis of the kinetics of elongation with poly[d(A-T)] template. Ternary complexes were added at time zero, and 50-μL aliquots were removed at various times as in Figure 3 to determine the initial reaction velocity (v), shown on the y axis in terms of picomoles of [α-^{32}P]UTP incorporated into RNA per minute. Reactions contained 100 μM [α-^{32}P]UTP (500 cpm/pmol) and either no added *nus*A protein (○) or 21 μg/mL *nus*A protein (●). The concentration of ATP, S, was 100, 40, 20, 10, and 5 μM as indicated on the x axis.

transcription has been restricted to the elongation phase of synthesis by isolating ternary RNA polymerase–poly[d(A-T)]–poly[r(A-U)] complexes by the method of Rhodes & Chamberlin (1974) and following subsequent elongation in the presence of 10 μg/mL rifampin to block RNA chain initiation. The presence of the *nus*A protein (45 μg/mL) inhibits the reaction markedly at 5 μM substrate concentrations (Figure 3D), but this inhibition is completely reversed as the substrate concentrations are increased to 100 μM (Figure 3A). Thus, the inhibition of poly[r(A-U)] synthesis appears to be completely competitive with nucleoside triphosphate substrates, and this impression is confirmed by representing the data on a double-reciprocal plot (Figure 4). Using the kinetic treatment of Rhodes & Chamberlin (1974) and assuming

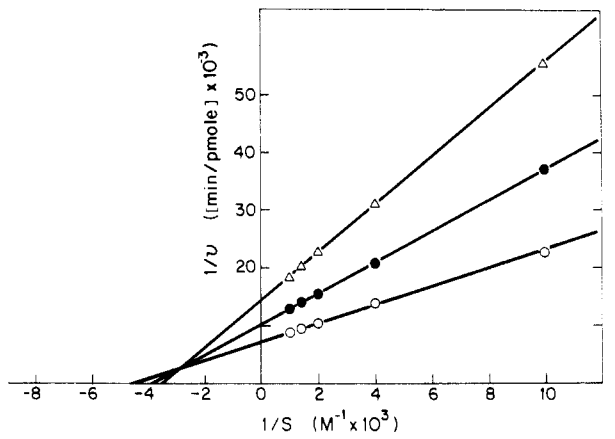

FIGURE 5: Analysis of the kinetics of elongation with T7 D111 DNA template. The rate of elongation of T7 D111 DNA template was measured by the method of Chamberlin et al. (1979). The initial reaction velocity, v, is shown on the y axis in terms of picomoles of [α-^{32}P]CTP incorporated into RNA per minute. RNA polymerase was included at 4.8 μg/mL (10 nM). Nucleoside triphosphates were included at the concentrations described under Methods except UTP concentration was varied over the range of 1, 0.7, 0.5, 0.25, and 0.1 mM as indicated on the x axis. (○) Absence of *nus*A protein; (●) 2 μg/mL *nus*A protein; (△) 15 μg/mL *nus*A protein.

purely competitive inhibition, we can calculate the inhibitor dissociation constant, K_i, by using the equation

$$K_i = \frac{i}{(K_s'/K_s) - 1}$$

where i is the concentration of inhibitor, K_s is the substrate dissociation constant, and K_s' is the apparent K_s in the presence of the inhibitor at concentration i. The K_i for the *nus*A protein in such an experiment is 3×10^{-7} M (about 20 μg/mL). It should be noted that, as expected from the purely repeating nucleotide sequence, transcription elongation with poly[d(A-T)] templates is very rapid and shows no evidence of pausing at specific sites [J. Levin and M. Chamberlin, unpublished results; also see Axelrod et al. (1978)].

(ii) Transcription with T7 DNA as Template. Bacteriophage T7 DNA is an ideal template for studying the effects of various factors on RNA chain elongation, since at low molar enzyme to DNA ratios transcription can be restricted to the early T7 transcription unit. Since promoter binding and RNA chain initiation are rapid with this template, it is easy to follow the kinetics of subsequent RNA chain elongation through the 7000 base pair early region in the absence of termination or repeated chain initiation (Chamberlin et al., 1979). Using this assay, we have previously shown that *nus*A protein inhibits RNA chain elongation (Kassavetis & Chamberlin, 1981). Since these experiments were carried out at 400 μM substrate concentrations, it seemed unlikely that the inhibition by *nus*A in these studies was primarily due to competitive inhibition of substrate binding. However, the K_s values for RNA chain elongation with T7 DNA templates (100–550 μM; Kingston & Chamberlin, 1981) are much higher than those obtained for simple alternating templates such as poly[d(A-T)] (7–20 μM; Rhodes & Chamberlin, 1974). Hence, we studied the kinetics of *nus*A protein inhibition of RNA chain elongation with DNA from the T7 deletion D111 in more detail. As in the case of the poly[d(A-T)] template, inhibition is reduced as the concentration of substrates is increased, indicating a competitive component to the *nus*A effect (Figure 5). However, unlike the situation with poly[d(A-T)], *nus*A protein inhibits T7 RNA chain elongation even at saturating substrate concentrations. Thus, there appear to be two components to the *nus*A effect with natural DNA templates, one of which

is not competitive with substrates. This latter type of inhibition is not detected with the simple alternating template; hence, specific DNA sequences are implicated in the effect.

The K_i for *nus*A protein inhibition for this noncompetitive mode of inhibition can be estimated from the kinetic data represented in Figure 5 by calculating V_{max} at several different *nus*A protein concentrations and extrapolating to $-K_i$ at $1/V_{max} = 0$. A value of 2×10^{-8} M (about 1.4 μg/mL) is obtained which is over 1 order of magnitude lower than that found for the competitive mode of inhibition.

Evidence that this is a true K_i and not simply a titration of all active elongating polymerase complexes was obtained by studying the dependence of the apparent K_i on polymerase concentration. In a separate experiment, the value of $K_i = 2.7 \times 10^{-8}$ M was obtained at 12 nM RNA polymerase. At 2.4 nM RNA polymerase, a value $K_i = 1.7 \times 10^{-8}$ M was obtained. These values of the apparent K_i must be reduced by subtracting a factor $^1/_2([E]_n)$ to give the true K_i value, where [E] is the enzyme concentration and n is the number of moles of I bound in the complex. This is a small correction. If n is 1, values of $K_i = 1.6 \times 10^{-8}$ and 2.1×10^{-8} M are obtained for the lower and higher polymerase concentrations, respectively. If n is 2, K_i is 1.5×10^{-8} M for both concentrations of enzyme.

The existence of two distinct modes of inhibition with characteristically different K_i values is consistent with the possibility that 2 mol of *nus*A protein can bind per mol of elongating RNA polymerase. However, it is also possible that the much lower K_i for inhibition of T7 transcription is simply caused by enhanced binding of *nus*A protein to the polymerase brought about by specific T7 DNA or RNA sequences and is a K_i characteristic of both noncompetitive and competitive modes of *nus*A protein binding. We have not measured K_i separately for the competitive binding mode with T7 DNA because of experimental factors which make accurate measurement of that parameter difficult.

(*iii*) *nusA Protein Is Not Bound Processively during in Vitro RNA Chain Elongation.* A simple model for the ability of *nus*A protein to alter the interaction of RNA polymerase with DNA sequences or RNA structures during elongation would involve tight binding of *nus*A to the polymerase to form a complex (Greenblatt & Li, 1981a) that remains intact during the course of synthesis of that transcript. Such a processive model for binding is consistent with the ability of *nus*A to induce long pauses, up to 30 min, during transcription elongation (Kingston & Chamberlin, 1981; Fisher & Yanofsky, 1983). However, even the lower of the two K_i values we obtain (2×10^{-8} M) suggests a fairly weak interaction, and hence, we set out to test this question directly. If *nus*A binding to an elongating polymerase complex is essentially irreversible, then titration of a population of elongating polymerases with increasing amounts of *nus*A protein should produce two populations. Polymerases with *nus*A bound will elongate slowly; those without it will elongate quickly. In contrast, if binding is rapidly reversible, a single population, elongating more slowly, will be found.

Such an experiment is shown in Figure 6 in which terminally labeled T7 RNA chains are initiated synchronously at the strong T7 A1 promoter and samples are then presented with increasing levels of *nus*A protein during elongation. After 3 min of elongation, the size distribution of the growing chains is measured by RNA gel electrophoresis. The experiment is carried out at high levels of nucleoside triphosphates so that only the sequence-dependent, tighter binding effect of *nus*A protein will be seen. In the absence of *nus*A protein, elongating

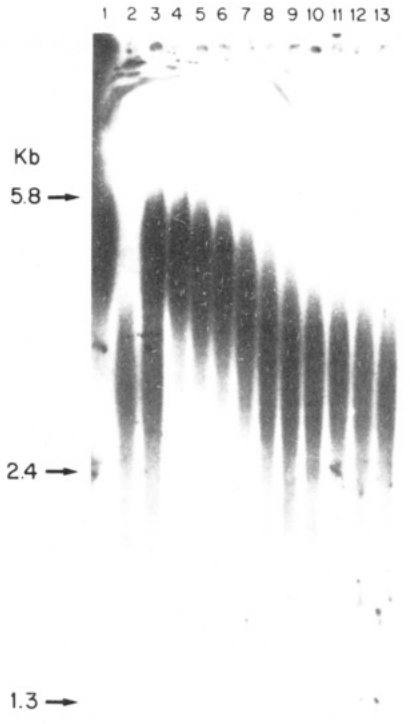

FIGURE 6: Titration of elongating RNA polymerase molecules with *nus*A protein. Reaction conditions were those described under Methods for reactions with T7 templates except that 11 μg/mL RNA polymerase holoenzyme was incubated at 30 °C with 0.41 mM T7 D111 DNA (nucleotide concentration) in the presence of 10 μM each of GTP, UTP, CTP, and [γ-^{32}P]ATP (10^5 cpm/pmol). After 5 min of incubation, either *nus*A protein or storage buffer was added along with enough unlabeled nucleoside triphosphates to give the final concentrations described under Methods for reactions with T7 DNA templates. After 3 min of elongation with high concentrations of triphosphates, reactions were stopped by the addition of a buffered detergent solution, and RNA products were resolved on a 0.5% agarose–2.25% acrylamide composite gel as described (Kassavetis & Chamberlin, 1979). (Tracks 1 and 4) No *nus*A protein; (tracks 2 and 13) 54 μg/mL *nus*A protein; (track 3) an equal volume of the reactions in tracks 1 and 2; (track 5) 0.54 μg/mL *nus*A protein; (track 6) 1.1 μg/mL *nus*A protein; (track 7) 2.2 μg/mL *nus*A protein; (track 8) 5.4 μg/mL *nus*A protein; (track 9) 11 μg/mL *nus*A protein; (track 10) 22 μg/mL *nus*A protein; (track 11) 32 μg/mL *nus*A protein; (track 12) 49 μg/mL *nus*A protein. Each track contains approximately 30 fmol of T7 RNA molecules (3000 cpm).

chains have nearly reached the T7 D111 early terminator at 5.8 kilobases (kb) in 3 min (track 4). In the presence of saturating *nus*A protein, a much slower rate is seen (tracks 2 and 13). A mixture of these two latter samples after synthesis clearly shows two size populations (track 3). This mixture should be compared to track 7, the distribution of chains formed when the *nus*A protein concentration is 50% of that needed for full inhibition. Although the resolution is not sharp, due to the wide distribution of chain lengths brought about by in vitro pausing (Kassavetis & Chamberlin, 1981), it is evident that track 7 does not display a bimodal distribution and, in particular, lacks the fastest and slowest migrating RNA chains, characteristic of fully inhibited and fully uninhibited growing complexes. Thus, the effect of half-saturating *nus*A protein is to reduce the growth rate of all chains by about half, which is inconsistent with a fully processive mode of binding.

Operation of the σ–nusA Cycle in Vitro. Greenblatt & Li (1981a) have shown that the *nus*A protein can bind to core RNA polymerase but not to holoenzyme. They have proposed that *nus*A protein is a normal subunit of elongating RNA polymerase in vivo that replaces σ subunit after the early stages

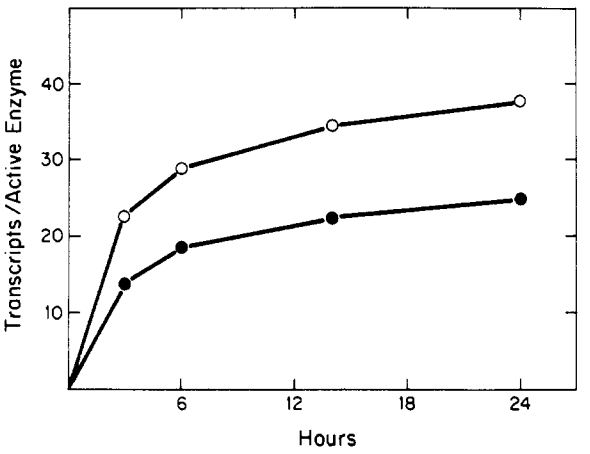

FIGURE 7: Extensive transcription of T7 D111 DNA. Optimal reaction conditions for the extensive synthesis of T7 A1 RNA have been described by Chamberlin et al. (1983). 100-μL aliquots were removed at the indicated times from 600-μL reactions containing 16 μg/mL RNA polymerase holoenzyme, 80 μg/mL T7 D111 DNA, and either no *nus*A protein (○) or 48 μg/mL *nus*A protein (●). T7 A1 RNA was labeled with [α-^{32}P]CTP (0.8 cpm/pmol), and its synthesis is plotted on the *y* axis as full-length transcripts per active enzyme. The preparation of holoenzyme used in this study contained 25% active molecules as determined by the quantitative RNA polymerase assay described by Chamberlin et al. (1979).

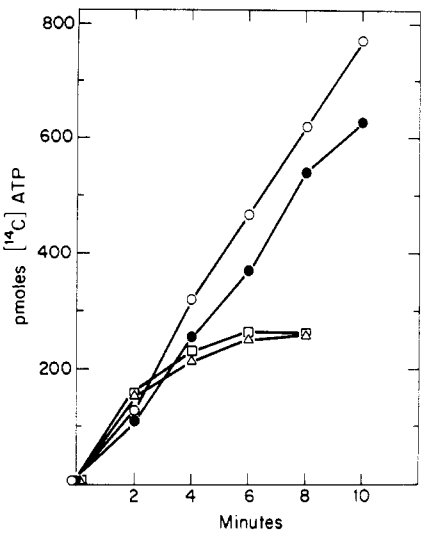

FIGURE 8: Extensive transcription of poly[d(A-T)] template. Reaction mixtures were as described under Methods for ternary complexes except that RNA polymerase and poly[d(A-T)] were added to 9 μg/mL and 0.1 mM, respectively, and rifampin was not present in the initial reaction. Reactions (250 μL) were initiated by warming to 37 °C, and 50-μL samples were removed at the indicated times. Rifampin was added to a final concentration of 10 μg/mL at t = 0.5 min (□, △) or was not added (○, ●). (○, □) No *nus*A protein; (●, △) 45 μg/mL *nus*A protein.

of chain elongation. Such a σ–*nus*A cycle requires efficient replacement of the *nus*A protein on the core polymerase by σ after chain termination to allow reconstitution of the holoenzyme needed for promoter binding and chain initiation. To test whether such a cycle could be demonstrated to occur in vitro, we took advantage of the fact that *E. coli* RNA polymerase will go through many transcriptional cycles in vitro. This extensive recycling requires optimal reaction conditions and a template which has a strong promoter such as T7 D111 DNA (Chamberlin et al., 1983). With an *E. coli* RNA polymerase concentration of 16 μg/mL, transcription of T7 D111 DNA continues for about 20 h (Figure 7) and ultimately converts 60% of the substrates to the T7 A1 transcript as shown by RNA gel analysis (data not shown). This involves the production of about 40 RNA chains for each active RNA polymerase molecule added to the reaction (only 25% of the added protein in this preparation was active as defined by quantitative assays; Chamberlin et al., 1979). Hence, this extensive synthesis involves continuous and repeated cycles of initiation, elongation, termination, and reconstitution of holoenzyme. Addition of an excess of *nus*A protein to such a reaction reduces the rate of RNA accumulation and also reduces the overall yield somewhat. However, there are still about 25 RNA chains produced per active polymerase; hence, the σ–*nus*A cycle can operate effectively in vitro even at very low concentrations of σ and an excess of *nus*A protein.

The reduced rate of RNA synthesis is easily explained by the ability of *nus*A to reduce the rate of RNA chain elongation. However, the reduction in overall yield is not explained by this effect, since a reduced rate of elongation would simply increase the time needed to reach a limit. Instead, we suspect that this effect of *nus*A is due to inhibition of the rate with which RNA polymerase holoenzyme can be reconstituted after it is released from the terminator and that such free RNA polymerase molecules are inactivated at a significant rate.

Evidence for this idea comes from studies of extensive synthesis of poly[r(A-U)] which can also continue for long periods. At elevated triphosphate concentrations (100 μM), this reaction involves repeated termination, reconstitution, and reinitiation, as shown by the sharp curtailment of the reaction brought about by the addition of rifampin (Figure 8). The presence of the *nus*A protein with this template and these substrate concentrations has no effect on chain elongation as shown previously (see Figure 3A), but there is a significant reduction in the rate of transcription. This result is consistent with the idea that the *nus*A protein slows the recycling reaction.

In control experiments, the *nus*A protein was shown to have no direct effect on the rate of the initiation reaction carried out by holoenzyme itself either by the assay of Nierman & Chamberlin (1979) with T7 D111 DNA and the A1 promoter or with the *lac*UV5 promoter following the formation of abortive initiation products by the method of Carpousis & Gralla (1980).

Discussion

The *nus*A protein appears to play an important role in termination and antitermination processes in *E. coli*. Its ability to inhibit elongating RNA polymerase molecules and to bind to core polymerase suggests that it may serve in vivo as a subunit of RNA polymerase specific to the elongation–termination phase of transcription (Greenblatt & Li, 1981a). While the amount of *nus*A protein in normal *E. coli* has not been measured carefully, even the amount recovered after purification would give cellular concentrations of over 10^{-7} M. This would saturate elongating RNA polymerases with *nus*A protein if the K_i for binding in vivo is comparable to that which we find in vitro and if *nus*A protein is free to bind to polymerase. Thus, a model for transcription in which *nus*A protein alternates with σ in binding to core polymerase, creating a "σ–*nus*A cycle" (Greenblatt & Li, 1981a), is attractive, and we have shown that such a cycle can continue for some time even in vitro.

Nevertheless, there are many aspects of the *nus*A protein–polymerase interaction that remain unknown or puzzling. First, the stoichiometry of the interaction is not yet known. The existence of two distinct modes of inhibition of transcription elongation by *nus*A protein, each with a quite different K_i, suggests that 2 mol of *nus*A protein may bind separately to RNA polymerase and may have quite different

effects. But these kinetic studies do not rule out a single binding site, and a direct demonstration of the stoichiometry of binding is needed.

Second, there is good evidence that *nus*A function in vivo may depend on a short RNA or DNA sequence (CGCTCTTT) termed "box A" by Friedman and his collaborators (Olson et al., 1982). Similar sequences are found near all of the known strong in vitro pause sites or terminators for which *nus*A function is implicated. There are several close fits to this sequence in the early region of T7 including a perfect fit beginning at nucleotide 812 [sequence numbering is that of Dunn & Studier (1983)]. The T7 box A sequence at 812 is deleted in T7 D111 (deletion from 532 to 1662), but in vitro *nus*A inhibition of transcription with this template does not differ significantly from that with wild-type T7 DNA as template. Certainly, *nus*A protein binding to the polymerase does not depend on the box A sequence since it can occur even with poly[d(A-T)]-elongating complexes. Furthermore, *nus*A protein binding is not processive in vitro but appears to involve repeated rapid association–dissociation from the complex. This fits well with the modest K_i value (2×10^{-8} M) we find which would correspond to a *nus*A–polymerase complex lifetime of a few seconds or less if a reasonable value for the association rate constant of about 10^7–10^8 M^{-1} s^{-1} is assumed. Hence, it is hard to see how the presence of a box A sequence in a transcript could have more than a transient effect on the *nus*A–polymerase interaction. Perhaps in the presence of a box A sequence and some associated RNA or DNA structure, *nus*A protein can bind more tightly than usual to the RNA polymerase complex, and this brings about a pause in transcription. If so, the binding of *nus*A protein to paused RNA polymerase complexes at loci such as the *rrn*B leader region or the *E. coli trp* attenuator should be quite stable.

A final and major uncertainty in in vitro studies of the properties of the *nus*A protein lies in the fact that the *nus*A protein may be only one of several proteins that bind to polymerase in normal transcription in vivo. In the presence of the *nus*A protein especially, transcription elongation in vitro is much slower than the process in vivo; hence, additional factors which enhance the rate of elongation are required (Kassavetis & Chamberlin, 1981). These may substantially change the interaction of *nus*A protein with polymerase or with specific DNA or RNA sequences.

Acknowledgments

We thank William Konigsberg for providing the overexpression vector pGW7 and Jacqueline Plumbridge for generously providing us with the cloned *nus*A gene and its characterization. We appreciate the exchange of ideas and findings with Charles Yanofsky and co-workers.

Registry No. RNA polymerase, 9014-24-8; poly[d(A-T)], 26966-61-0.

References

Axelrod, V., Vartikyan, R., Aivazashvili, V., & Beabealashvili, R. (1978) *Nucleic Acids Res. 5*, 3549–3563.

Carpousis, A. J., & Gralla, J. D. (1980) *Biochemistry 19*, 3245–3253.

Chamberlin, M. J., Nierman, W. E., Wiggs, J. L., & Neff, N. (1979) *J. Biol. Chem. 254*, 10061–10069.

Chamberlin, M. J., Kingston, R. E., Gilman, M. Z., & deVera, A. (1983) *Methods Enzymol. 101*, 540–568.

Dunn, J. J., & Studier, F. W. (1983) *J. Mol. Biol. 166*, 477–535.

Fisher, R., Yanofsky, C. (1983) *J. Biol. Chem. 258*, 9208–9212.

Goldmark, P., & Linn, S. (1972) *J. Biol. Chem. 247*, 1849–1860.

Gonzalez, N., Wiggs, J., & Chamberlin, M. J. (1977) *Arch. Biochem. Biophys. 182*, 404–408.

Greenblatt, J., & Li, J. (1981a) *Cell (Cambridge, Mass.) 24*, 421–428.

Greenblatt, J., & Li, J. (1981b) *J. Mol. Biol. 147*, 11–23.

Greenblatt, J., McLimont, M., & Hanly, S. (1981) *Nature (London) 292*, 215–220.

Holmes, D. S., & Quigley, M. (1981) *Anal. Biochem. 114*, 193–197.

Kane, C. M., & Linn, S. (1981) *J. Biol. Chem. 256*, 3405–3414.

Kassavetis, G. A., & Chamberlin, M. J. (1981) *J. Biol. Chem. 256*, 2777–2786.

Kingston, R. E., & Chamberlin, M. J. (1981) *Cell (Cambridge, Mass.) 27*, 523–531.

Kingston, R. E., Nierman, W. C., & Chamberlin, M. J. (1981) *J. Biol. Chem. 256*, 2787–2797.

Kung, H., & Weissbach, H. (1980) *Arch. Biochem. Biophys. 201*, 544–560.

Kung, H., Spears, C., & Weissbach, H. (1975) *J. Biol. Chem. 250*, 1556–1562.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lau, L. F., Roberts, J. W., & Wu, R. (1983) *J. Biol. Chem. 258*, 9391–9397.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Nierman, W. C., & Chamberlin, M. J. (1979) *J. Biol. Chem. 254*, 7921–7926.

Olson, E. R., Flamm, E. L., & Friedman, D. I. (1982) *Cell (Cambridge, Mass.) 31*, 61–70.

Plumbridge, J. A., & Springer, M. (1983) *J. Mol. Biol. 167*, 227–243.

Rhodes, G., & Chamberlin, M. J. (1974) *J. Biol. Chem. 249*, 6675–6683.

Schachman, H. K., Adler, J., Radding, C. M., Lehman, I. R., & Kornberg, A. (1960) *J. Biol. Chem. 235*, 3242–3249.

Szybalski, E., & Szybalski, W. (1979) *Gene 7*, 217–270.

Symons, R. (1977) *Nucleic Acids Res. 4*, 4347–4355.

Thomas, C., & Abelson, J. (1966) *Procedures in Nucleic Acids Research* (Cantoni, G., & Davies, D. R., Eds.) p 553, Harper and Row, New York.

Ward, D. F., & Gottesman, M. E. (1981) *Nature (London) 292*, 212–215.