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Effect of Lysine Modification on the Activity of the σ Subunit of *Escherichia coli* RNA Polymerase†

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ABSTRACT: The function of lysyl residues of the σ subunit of the RNA polymerase from *Escherichia coli* was investigated by chemical modification with trinitrobenzenesulfonic acid (TNBS). Following reaction with TNBS, analysis of the modified σ indicated that trinitrophenylation was limited to the ϵ-amino groups of lysyl residues. Progressive loss in the activity of σ followed increasing trinitrophenylation as assayed by the ability to stimulate RNA polymerase core enzyme in a reaction directed by T7 DNA. Modification of five lysyl groups resulted in the complete loss of σ activity. Kinetic analysis indicated that one lysyl group is critical for the function of σ. TNP-σ was able to form a holoenzyme complex with a binding affinity comparable to that of σ. Promoter recognition studies were done by using *Hin*dIII fragments from T5 DNA. The TNP-σ core complex was unable to form a tight binary complex with the T5 promoters. Studies on RNA chain initiation were carried out by using $d(A\text{-}T)_n$ and T7 DNA templates. TNP-σ was unable to stimulate RNA chain initiation by core polymerase. Limited proteolytic digests of TNP-σ or σ using *Staphylococcus aureus* V8 protease were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The results suggested a change in the conformation of σ following trinitrophenylation.

The *Escherichia coli* DNA-dependent RNA polymerase consists of a catalytically competent core unit composed of three subunits (α_2, β, β') and a fourth dissociable subunit (σ). The presence of σ in the holoenzyme stimulates the overall rate of transcription by effecting promoter recognition and increasing the rate of RNA chain initiation. The effect of σ on DNA site selection could be due, in part, to a direct interaction of σ in the holoenzyme with the promoter (Simpson, 1979; Kudo & Doi, 1981). Alternatively, binding of σ may induce a specific conformation in the holoenzyme resulting in an increased affinity for promoter sites and a lowered affinity for DNA general sites (Wu et al., 1976). RNA chain initiation at nicks in double-stranded DNA seen with the core

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polymerase is markedly reduced for the holoenzyme (Hinkle et al., 1972).

One approach toward an elucidation of the manner in which σ is able to produce the various effects attributed to it is chemical modification of specific amino acid residues. In this paper, we report on the effect of modification of the ϵ-amino group of lysine residues in σ by trinitrobenzenesulfonic acid (TNBS).[1]

Materials and Methods

Materials. E. coli K_{12} cells (half log phase) were obtained from Grain Processing. Nucleoside triphosphates and $d(A\text{-}T)_n$ were obtained from P-L Biochemicals. The labeled nucleoside triphosphates, [^{3}H]thymidine, and Liquifluor were purchased from New England Nuclear. [^{3}H]TNBS was obtained from Amersham or New England Nuclear. Restriction endonucleases and agarose were from Bethesda Research Laboratories. TNBS obtained from Pierce Chemical Co. was recrystallized prior to use. The concentrations of TNBS solutions were determined by using $E^{1M}_{340nm} = 600$. The reagents used for polyacrylamide gel electrophoresis were products of Bio-Rad Laboratories.

Buffers. The following buffers were used: Tris–borate buffer (80 mM Tris base, 80 mM borate, and 2.5 mM EDTA, pH 8.3); TGED [10 mM Tris-HCl, pH 8, 5% (w/v) glycerol, 0.1 mM EDTA, and 0.1 mM DTT]; TED (TGED minus glycerol); TMS (10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, and 2 mM 2-mercaptoethanol); binding buffer A (10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM NaCl, and 500 μg of BSA/mL); binding buffer B [20 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.1 mM DTT, 10 mM $MgCl_2$, 5% (w/v) glycerol, 40 mM KCl, and 500 μg of BSA/mL]; elution buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, and 0.1% $NaDodSO_4$); WASP [water–saturated ammonium sulfate–2-propanol–0.5 M EDTA (17:80:2:1), pH adjusted to 8 with ammonium hydroxide (Hansen & McClure, 1979)].

σ Preparation. E. coli RNA polymerase was prepared by a modification of the procedure of Burgess & Jendrisak (1975). The σ subunit was isolated by passing the holoenzyme in TGED + 0.1 M NaCl through a 3 × 20 cm Bio-Rex 70 column equilibrated with the same buffer (Lowe et al., 1979). The flow through was then passed through a 1.5 × 10 cm DEAE-Sephacel column (equilibrated with TGED + 0.1 M NaCl) which was developed with a linear gradient of 200 mL of 0.1 and 0.35 M NaCl in TED buffer. The σ-containing fractions (as assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis; Laemmli, 1970) in 0.5 mL of TGED + 0.5 M NaCl were layered on a 1.5 × 80 cm Sepharose ACA 34 (Pharmacia) column equilibrated with TGED + 0.5 M NaCl and developed at a flow rate of 5 mL/h with the same buffer. The σ preparation thus obtained was judged to be over 95% pure.

Phage DNA Preparation. The T phage DNAs were purified according to Thomas & Abelson (1966) by using *E. coli* B *thy*$^-$ as host. T7 DNA was labeled in vivo by adding [^{3}H]-thymidine to the growth medium 1 h before infection. The DNA content, unless otherwise noted, is expressed as the nucleotide content by using a molar extinction coefficient of 6750 at 260 nm. Restriction endonuclease fragments of in vivo labeled T7 [^{3}H]DNA were separated on a 1% agarose gel (low-temperature melting) [run according to McDonnell et al. (1977), by using the Tris–borate buffer system] and the fragments recovered from the gel by using the procedure described by Weislander (1979).

Assay for σ Subunit. For routine purposes, the σ subunit was assayed by using core polymerase and T7 DNA at a DNA to core polymerase ratio of 4 (based on weight). The reaction mix (250 μL) contained 80 mM Tris-HCl, pH 7.6, 40 mM mercaptoethylamine, 20 mM $MgCl_2$, and 1 mM each of ATP, CTP, GTP, and [^{3}H]UTP (5000 cpm/nmol). Following incubation at 37 °C for 10 min, the Cl_3CCOOH-precipitable radioactivity was collected on a glass fiber filter, dried, and counted in 5 mL of Liquifluor–toluene.

The nucleotide products in the abortive initiation reactions were separated from the labeled substrate nucleoside triphosphate, UTP, by using paper chromatography (with Whatman 3 paper) in the WASP solvent system as described by Hansen & McClure (1979). The PP_i exchange assay was carried out as described by Krakow & Fronk (1969).

Filter Binding Studies. DNA binding by RNA polymerase was studied by using the nitrocellulose filter binding assay procedure as described by Hinkle & Chamberlin (1972). *Bcl*I "B" fragment and *Hae*III "A" fragment from T7 [^{3}H]DNA (Rosenberg et al., 1979; Studier et al., 1979) were used.

Promoter Recognition Studies. These were carried out as described by Gabain & Bujard (1977). In 50 μL of binding buffer B, 2 pmol of RNA polymerase and 15 nmol of T5 DNA cut with restriction endonuclease *Hin*dIII were incubated at 37 °C for varying time intervals (preincubation), and 20 μg of single-stranded calf thymus DNA in 10 μL of binding buffer B was added and incubation continued for varying time periods. The reaction mix was then filtered on a nitrocellulose filter at a flow rate of 50 μL/20 s and washed with 200 μL of binding buffer B without KCl. The filter disc was cut into 1-mm^2 fragments and extracted into 0.1 mL of elution buffer. After 2 h at 4 °C, the solution was brought to 10% glycerol and electrophoresed on a 0.7% agarose gel as 12 mA for 18 h. The gel was stained with ethidium bromide (2 μg/mL of H_2O) for 30 min and destained in water for 4 h.

Protein Determination. Protein was determined by using the method of Schaffner & Weissmann (1973) with BSA as the standard ($E^{1\%}_{280nm} = 6.6$). The protein content thus determined agreed well with the following extinction coefficients ($E^{1\%}_{280nm}$): core polymerase = 5.8, holoenzyme = 6.7 (Levine et al., 1980), and σ = 5.6 (Burton et al., 1981).

Modification of σ Subunit with TNBS. The reaction with TNBS plateaus at about 20 min irrespective of the TNBS concentration, and unless otherwise noted, the incubation of σ with TNBS was carried out at 37 °C for 30 min. Following incubation with TNBS, the unreacted reagent was removed by dialysis against 20 mM Tris-HCl, pH 8, and 1 mM DTT. The σ preparations for control experiments were prepared the same way as the experimental samples but without the addition of TNBS.

Reconstitution of σ with Core Polymerase. These experiments were performed by using an ultracentrifugation technique. A 15–35% linear glycerol gradient (total volume 4.4 mL) in TMS buffer with 0.5 M NaCl was layered on 0.5 mL of 35% glycerol in TMS buffer + 0.5 M NaCl in 5-mL cellulose nitrate tubes. The protein solution in 50 μL was layered on the gradient and centrifuged at 45 000 rpm for 20 h at 4 °C in an SW 50 rotor. Fractions of 0.4 mL were collected. Aliquots were removed for the determination of protein and

[1] Abbreviations: TNBS, trinitrobenzenesulfonic acid; TNP, trinitrophenyl; DTT, dithiothreitol; BSA, bovine serum albumin; Cl_3CCOOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; BTP or Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

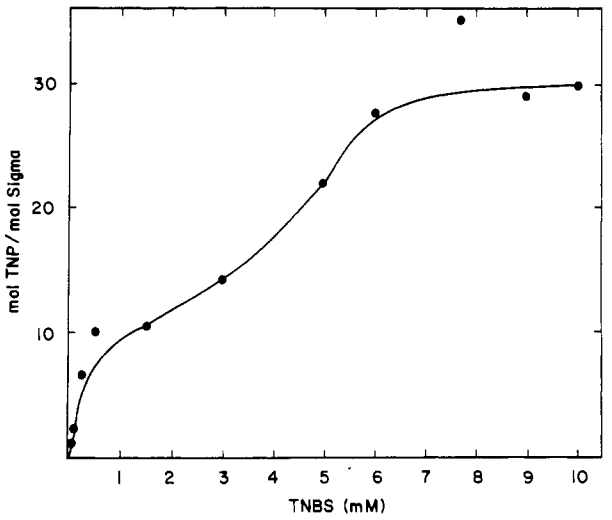

FIGURE 1: Concentration-dependent trinitrophenylation of σ. The reaction mix (50 μL) containing 15 μg of σ, 40 mM BTP buffer (pH 9), and varying concentrations of [^{3}H]TNBS was incubated at 37 °C for 30 min and dialyzed overnight. Following protein determination, an aliquot containing 5 μg of σ was Cl_3CCOOH precipitated, filtered, and counted. The moles of TNP incorporated per mole of σ was calculated by using a molecular weight of 70 263 for σ.

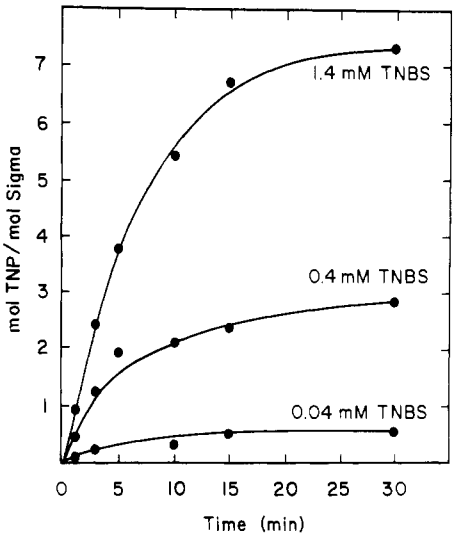

FIGURE 2: Time-dependent trinitrophenylation of σ. The reaction mix (70 μL) containing 70 μg of σ, BTP buffer (pH 9), and varying concentrations of [^{3}H]TNBS was incubated, and 10-μL aliquots were added to 500 μL of 1 M L-lysine in 20 mM BTP buffer (pH 9) in a dialysis bag. Following overnight dialysis, the moles of TNP incorporated per mole of σ was calculated.

radioactivity and analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Results

Modification of σ with TNBS. Modification of σ as a function of TNBS concentration (Figure 1) shows an obvious biphasic response. At TNBS concentrations up to 0.5 mM, approximately seven lysyl groups are modified. Increasing the TNBS concentration to 5 mM results in trinitrophenylation of about 20 lysyl groups. As the concentration is raised to 10 mM TNBS, approximately 30 lysyl groups of σ have been modified. On the basis of a molecular weight of 70 263 (Burton et al., 1981), this represents 90% of the total lysyl residues in σ. The data suggest at least two different classes of lysyl groups with respect to reactivity with TNBS. Following incorporation of eight TNP groups per σ, the adduct shows an absorption spectrum characteristic of trinitrophenyl-ε-aminolysine. Following acid hydrolysis of [^{3}H]-TNP-σ, the hydrolysate was analyzed by paper chromatography. The [^{3}H]TNP cochromatographed with authentic TNP-ε-aminolysine and not with TNP-*S*-cysteine (data not shown).

The time course of the reaction at three concentrations of TNBS (Figure 2) shows an initial rapid rate of lysyl groups modified followed by a slower rate approaching a plateau. Using low concentrations of TNBS, it is possible to limit the reaction to only a few lysyl groups per σ. A plot of the percent residual activity vs. the moles of TNP incorporated per mole of σ extrapolates to a value of five lysyl groups modified per σ for 100% inactivation (Figure 3).

Kinetics of the Inactivation of σ. The inactivation of σ by trinitrophenylation follows pseudo-first-order kinetics at 1.4 or 1 mM TNBS (Figure 4). The reaction of TNBS with σ can be represented by $E + nI \xrightarrow{k''} EI_n$ where k'' is the apparent second-order rate constant, E is the free enzyme, and n is the number of molecules of inhibitor I. Since the inactivation follows pseudo-first-order kinetics, the pseudo-first-order rate constant $k' = k''[I]^n$. The logarithmic conversion of this relation, namely, $\log k' = \log k'' + n \log [I]$, is an equation for a straight line, and a plot of $\log k'$ vs. log [TNBS] should give a straight line with a slope equal to n, the number of

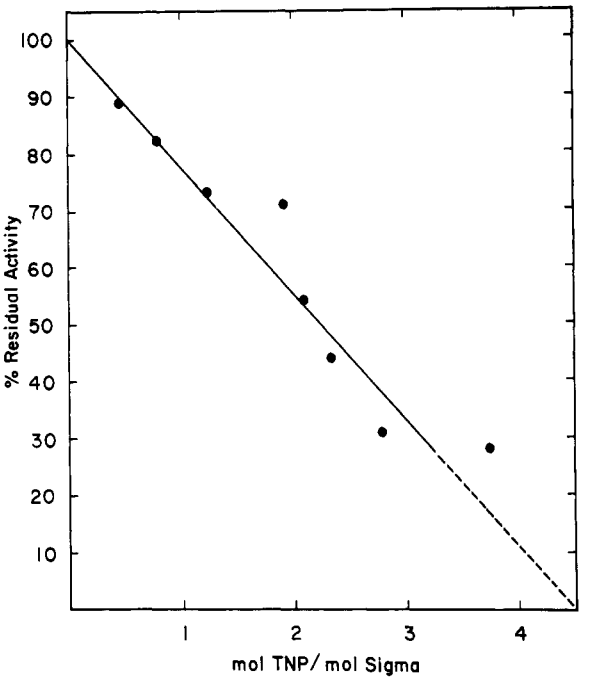

FIGURE 3: Relationship of lysine modification to loss of σ activity. Reactions were run with 10 μg of σ, BTP buffer (pH 9), and varying concentrations of ^{3}H-labeled or unlabeled TNBS. Following dialysis, aliquots containing 2.5 μg of σ from the unlabeled TNBS reaction mix were taken for the determination of activity by using T7 DNA as template and a stoichiometric amount of core polymerase (10 μg). Aliquots from the [^{3}H]TNBS reaction mix were taken to determine the moles of TNP incorporated per mole of σ.

inhibitor molecules needed to inactivate a single σ subunit. Figure 5 shows such a plot with the slope of the straight line equal to 1.2, suggesting that the modification of one lysyl group by TNBS results in inactivation of the σ subunit.

Inactivation as a Function of pH. Figure 6 shows a plot of k'' as a function of pH. The activity of σ remained unchanged following incubation in the absence of TNBS at alkaline pHs as high as 11 and was 30–50% lower at pHs below 6. For this reason, the reactions were studied between pH 6.5 and 11. The k'' values were calculated by taking the ratio of

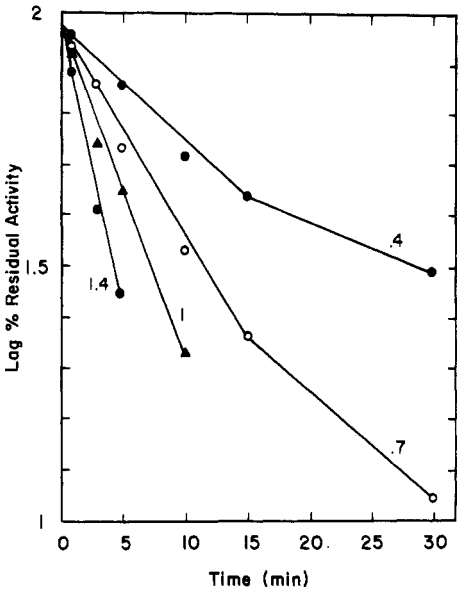

FIGURE 4: Inactivation of σ by TNBS. In a 35-μL reaction mix, 17.5 μg of σ with BTP buffer and varying concentrations of TNBS was incubated. At various time intervals, 5-μL aliquots were removed and added to the RNA polymerase assay system containing 10 μg of core polymerase and T5 DNA. Appropriate controls with no TNBS added and a blank with TNBS (1.5 mM) but no σ added were also run.

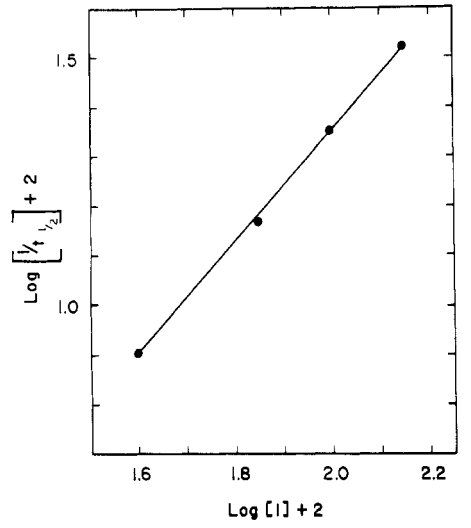

FIGURE 5: Reaction order of σ inactivation with respect to TNBS concentration. Logarithmic values of $1/t_{1/2}$ (log k') were plotted against the respective log [TNBS] values in millimolar units (log [I]). Both values were obtained from Figure 4.

the reciprocal $t_{1/2}$ of the inactivation to the TNBS concentration (Figure 4). Two assay systems were used to follow the activity. The k'' values using the T5 DNA assay system were slightly higher than those using the pApU assay system with $d(A\text{-}T)_n$ as template. The rate constant for inactivation is approximately 50 M^{-1} min^{-1} at pH 6.5 and increases by 10–15-fold at pH 11.

Reconstitution of [^3H]TNP-σ with Core Polymerase. Under the conditions employed in the ultracentrifugation experiments, free σ can be found between fractions 3 and 6 with the peak at fractions 4 and 5 and core polymerase or holoenzyme peaks at fraction 9 or 10. As shown in Figure 7, when core polymerase and TNP-σ are mixed in a molar ratio of 0.25:1 (Figure 7B), 0.5:1 (Figure 7C), and 0.75:1 (Figure 7D), 25%, 50%, and 75%, respectively, of the TNP-σ (six TNP groups per σ) can be titrated out, and TNP-σ can be titrated out completely by using a full equivalent of core polymerase

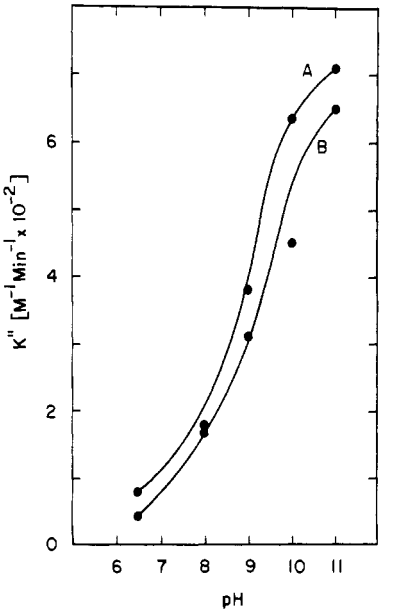

FIGURE 6: Inactivation of σ by TNBS as a function of pH. Reactions were carried out as described in Figure 4 with the pH of the BTP buffer varying from 6.5 to 11. The second-order rate constant, k'', for each of the pH values was calculated from the equation $k'' = k'/[I]^n$. (A) pApU assay system; (B) T5 DNA assay system.

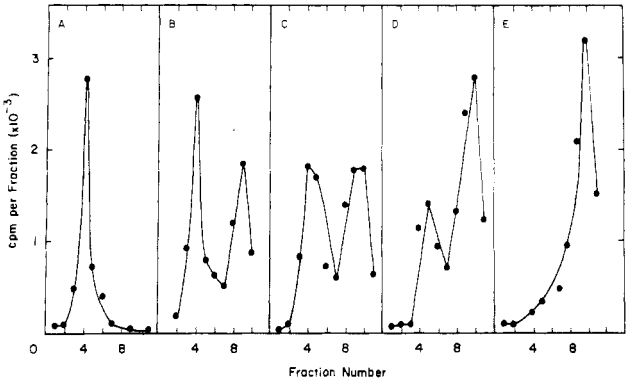

FIGURE 7: Reconstitution of TNP-σ with core polymerase. In 50 μL of TMS buffer containing 0.5 M NaCl, 100 μg of core polymerase was mixed with varying amounts of [^{3}H]TNP-σ (six TNP groups per σ), incubated at 37 °C for 5 min, layered on the gradient, and centrifuged as described under Materials and Methods. Fractions of 0.4 mL were collected, and aliquots were used for determination of protein and radioactivity and analyses by $NaDodSO_4$–polyacrylamide gel electrophoresis. The core polymerase:[^{3}H]TNP-σ ratios are (A) 0:1, (B) 0.25:1, (C) 0.5:1, (D) 0.75:1, and (E) 1:1.

(Figure 7E). $NaDodSO_4$–polyacrylamide gel electrophoresis of the fractions (data not shown) also confirmed the ability of TNP-σ to bind to core polymerase. So that the affinity of TNP-σ for binding to core polymerase could be studied, TNP-σ with six TNP groups bound per σ (in stoichiometric amounts to core polymerase) was mixed with σ in 0.5, 1, or 2 times the amount of TNP-σ and the reconstitution with core polymerase was studied (Figure 8). If the binding of TNP-σ to core polymerase is comparable to that of σ, it is possible to predict the amount of free TNP-σ and TNP-σ bound to core polymerase in each of the three experiments. The values calculated based on the experiments agree well with the predicted ones suggesting that TNP-σ binds to core polymerase with an affinity comparable to that of unmodified σ. It is known that high glycerol concentrations strengthen the binding of σ to core polymerase (Gonzalez et al., 1977; Levine et al., 1980). Since the binding of TNP-σ to core polymerase was carried out by centrifugation in 15–35% glycerol gradients,

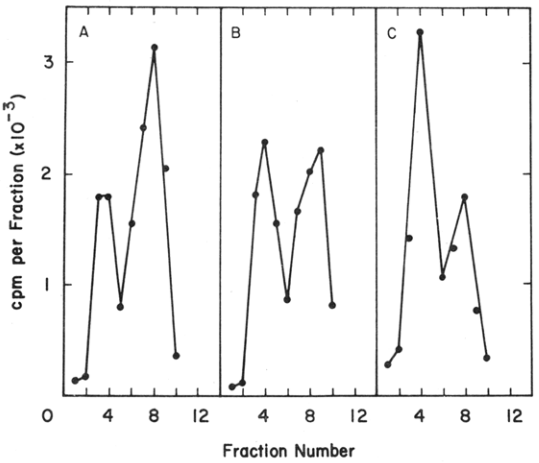

FIGURE 8: Binding affinity of TNP-σ to core polymerase. In 50 μL of TMS buffer containing 0.5 M NaCl, 25 μg of [^{3}H]TNP-σ (six TNP groups per σ) was mixed with 12.5, 25, or 50 μg of σ (curves A, B, or C, respectively) and incubated with 100 μg of core polymerase at 37 °C for 5 min. Following centrifugation, the collected fractions were analyzed as described in Figure 7. The molar ratios of core polymerase:[^{3}H]TNP-σ:σ are (A) 1:1:0.5, (B) 1:1:1, and (C) 1:1:2.

it is possible that the presence of glycerol compensated for the effects resulting from trinitrophenylation of σ. For determination of whether the TNP-σ bound to core polymerase at a low glycerol concentration, chromatography on DNA–cellulose [native calf thymus DNA coupled to cellulose by the method of Litman (1968)] was carried out. Whereas free TNP-σ (five TNP groups per σ) did not bind, the complex of TNP-σ and core polymerase bound to DNA–cellulose in TGED + 0.1 M NaCl containing 5% or 35% glycerol (data not shown).

Possible Conformational Change in σ due to the Modification. Susceptibility to limited proteolytic digestion of σ or modified σ was employed as a probe to detect the possibility of a conformational change following trinitrophenylation. After limited digestion of TNP-σ or σ with *Staphylococcus aureus* V8 protease, the residual amount of acid-insoluble polypeptides was comparable when determined by the method of Schaffner & Weissmann (1973). A comparison of the electrophoretic pattern of the TNP-σ (six TNP groups per σ) and σ digests (Figure 9) suggests that the TNP-σ seems to be more resistant to protease attack than σ. A stable band migrating just below σ found in the σ digest can no longer be found in the case of the TNP-σ digest, and in its place a band of slightly lower molecular weight is seen.

Template Binding Studies. The studies of Hinkle & Chamberlin (1972) showed that the formation of high-affinity complexes between RNA polymerase and promoter involved the holoenzyme. The data presented in Figure 10 show that a 10-fold excess of unlabeled T7 DNA displaced only 20% of the promoter containing the *Bcl*I B fragment of T7 [^{3}H]DNA from the binary holoenzyme–DNA complexes and 85% of the T7 [^{3}H]DNA fragment initially bound by the core polymerase (Figure 10C). The holoenzyme formed from core polymerase plus TNP-σ (six TNP groups per σ) formed a complex in which the T7 [^{3}H]DNA fragment was displaced to an extent comparable to that of the core polymerase–DNA complex. Modification of σ with TNBS resulted in an almost complete inactivation with regard to its ability to support the formation of the high affinity, open binary complex with T7 promoters.

Promoter Recognition Studies. The studies of Gabain et al. (1976) and Gabain & Bujard (1977) indicate that of the 16 *Hin*dIII fragments of T5 DNA, fragments i, l, m, and o lack promoters for *E. coli* RNA polymerase and other fragments contain from one to four promoters and are able to form tight complexes with the holoenzyme. As seen in Figure 11E–H, the holoenzyme bound to fragments i, l, m, and o can be displaced by single-stranded competitor DNA while the holoenzyme bound to the promoter-containing fragments is not dissociated. The core polymerase (Figure 11B–D) in-

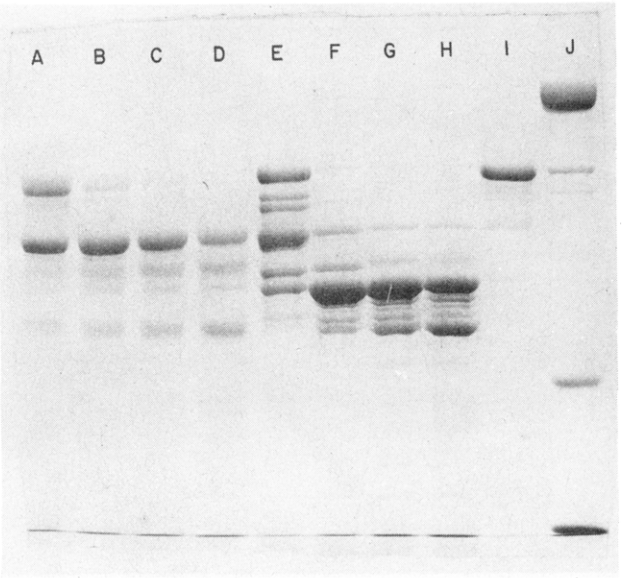

FIGURE 9: Possible conformational change following modification. In a reaction volume of 110 μL, 66 μg of σ or TNP-σ (six TNP groups per σ) in 20 mM Tris-HCl, pH 8.0, and 1 mM DTT was digested with 2.6 μg of *Staphylococcus aureus* V8 protease at 37 °C. Aliquots of 25 μL were taken at intervals of time and electrophoresed on a $NaDodSO_4$–polyacrylamide gel (7.5%). (A–D) TNP-σ digested for 5 (A), 30 (B), 60 (C), and 90 (D) min; (E–H) σ digested for 5 (E), 30 (F), 60 (G), and 90 (H) min; (I) 10 μg of σ digested for 0 min (TNP-σ shows an identical pattern); (J) 10 μg of holoenzyme and 5 μg of cAMP receptor protein (M_r 22 500).

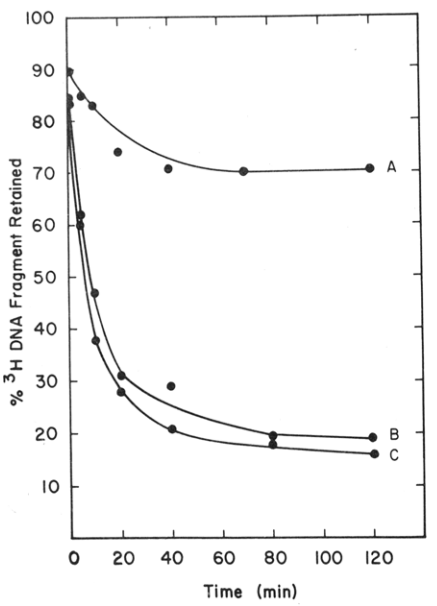

FIGURE 10: Formation of a stable T7 DNA–polymerase complex. In 800 μL of binding buffer A, 25 nmol of *Bcl*I B fragment from T7 DNA (7500 cpm/nmol) was incubated at 37 °C with 2.4 μg of core polymerase (curve C), 1.8 μg of core polymerase + 0.6 μg of σ (curve A), or 1.8 μg of core polymerase + 0.6 μg of TNP-σ (six TNP groups per σ) (curve B). At the end of 5 min (zero time), a 100-μL aliquot was diluted to 1 mL with binding buffer A and filtered on a nitrocellulose filter. To the remaining mix was added 250 nmol of unlabeled T7 DNA, the incubation was continued, and 100-μL aliquots were removed at intervals, diluted with 1 mL of binding buffer A, and filtered.

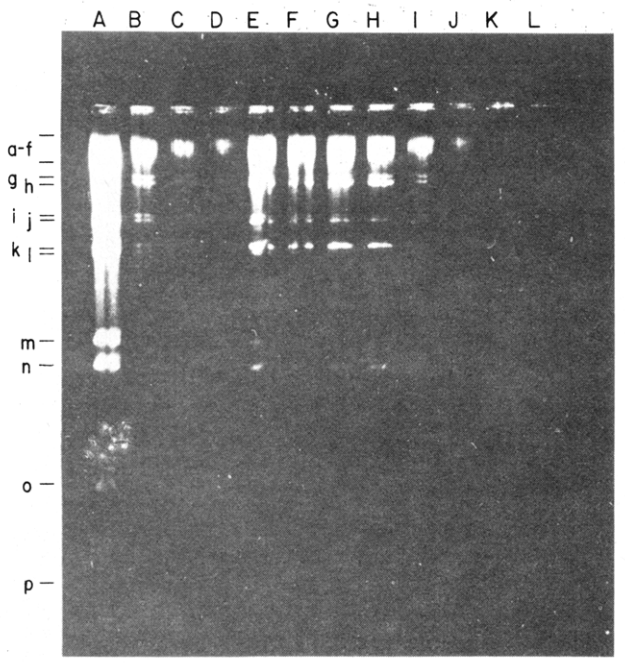

FIGURE 11: Formation of T5 DNA promoter–polymerase complex. T5 DNA (25 nmol) restricted with *Hin*dIII and 1 μg of core polymerase (B–D), 1 μg of core polymerase + 0.25 μg of σ (E–H), or 1 μg of core polymerase + 0.25 μg of TNP-σ (six TNP groups per σ) (I–L) was preincubated at 37 °C for 2 min in 50 μL of binding buffer B and competed with denatured calf thymus DNA (15 μg), incubation was continued for 10 min or 1, 3, or 6 h, and the resulting solution was filtered and electrophoresed as described under Materials and Methods. (A) 9 nmol of T5 DNA fragments; (B–D) core polymerase–DNA fragments competed by calf thymus DNA for 10 min, 1 h and 3 h, respectively; (E–H) core polymerase + σ DNA fragments competed for 10 min, 1 h, 3 h, and 6 h, respectively; (I–L) core polymerase + TNP-σ–DNA fragments competed for 10 min, 1 h, 3 h, and 6 h, respectively.

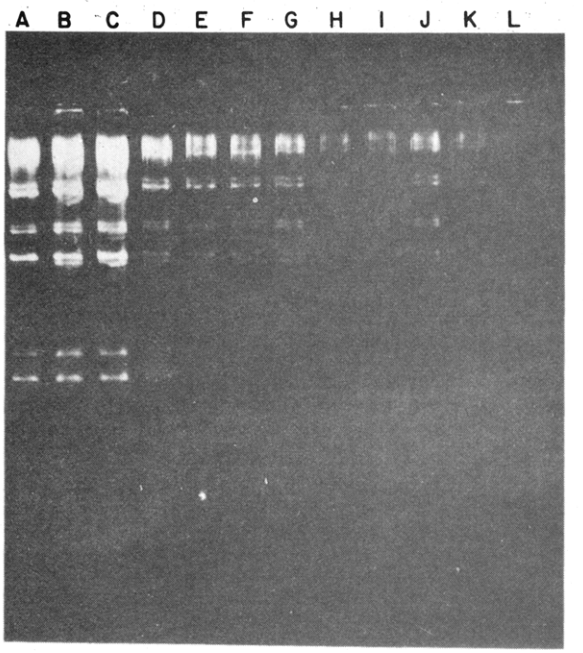

FIGURE 12: Progressive trinitrophenylation of σ and promoter recognition. The study was done as described in Figure 11 by using core polymerase and TNP-σ modified to various degrees. The competition was for 10 min, 1 h, or 5 h. (A–C) One TNP group per σ; (D–F) three TNP groups per σ; (G–I) four TNP groups per σ; (J–L) eight TNP groups per σ.

discriminately binds to both promoter and nonpromoter fragments; the binding is not tight, and the fragments can be displaced from the complex within 3 h after addition of the competitor DNA. This effect of σ seems to be completely abolished upon modification. As shown in Figure 11I–L, the band pattern with core polymerase + TNP-σ (six TNP groups per σ) is similar to that of the core polymerase.

The effect of progressive modification on promoter recognition is shown in Figure 12. With one lysyl group modified, the σ is still able to recognize promoters and can bind to the promoter as tightly as does the unmodified σ with core polymerase. Upon modification of two lysyl groups, the binding of core polymerase + TNP-σ to the fragments seems to be very loose (Figure 12D–F). The fragments can be completely displaced from the binary complex within 6 h of competition in the case of five or more lysyl groups modified (Figure 12G–L).

The ability of σ to lower the affinity of core polymerase for nonpromoter sites was unaffected by the modification. This was supported by filter binding studies using fragment A of in vivo labeled T7 [^{3}H]DNA cut with restriction endonuclease *Hae*III. This fragment does not have any major promoters (Studier et al., 1979). The data from Figure 13 show that 80–90% of the tritium-labeled fragment A bound by holoenzyme core polymerase or core polymerase plus TNP-σ (seven TNP groups per σ) can be displaced by a 6-fold excess of unlabeled denatured T7 DNA with 60 min of competition.

Effect of Modification on Initiation. The effect of RNA chain initiation of σ on core polymerase can be studied in several ways with natural templates such as T7 DNA or synthetic templates such as d(A-T)$_n$. Addition of heparin a few seconds after RNA chain initiation in the T7 DNA directed reaction inhibits the unbound and the loosely bound polymerases. Only RNA polymerase in open promoter and ternary complexes is refractory to heparin attack, and under such conditions, most of the polymerase molecules transcribe the DNA until the termination signal is reached [about 7 min

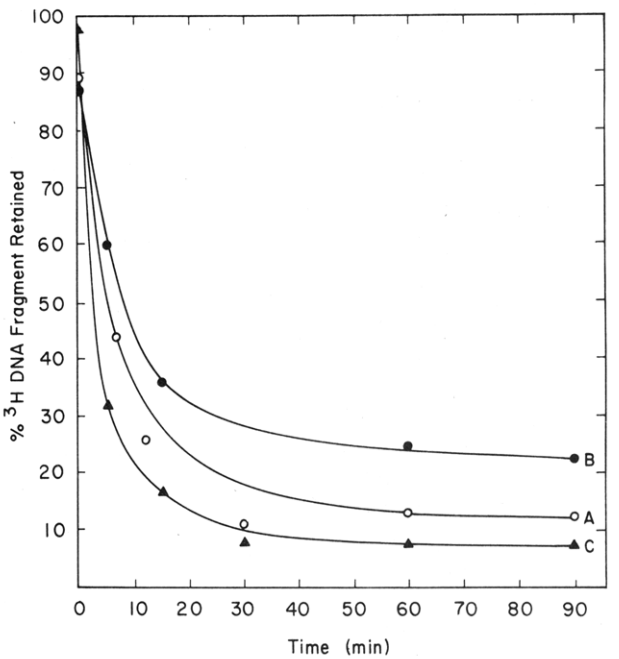

FIGURE 13: Formation of a general DNA–polymerase complex. ^{3}H-Labeled T7 *Hae*III fragment A (Studier et al., 1979) (15 nmol) in 60 μL of binding buffer A was incubated at 37 °C with 1 μg of core polymerase or 1 μg of core polymerase with σ or TNP-σ (seven TNP groups per σ). At the end of 10 min (zero time), 10 μL was diluted into 1 mL of binding buffer A and filtered. To the remainder was added 75 nmol of unlabeled denatured T7 DNA, and 10-μL aliquots were removed at 10, 30, 60, or 90 min, diluted with 1 mL of binding buffer A, and filtered. (A) Core polymerase + σ; (B) core polymerase + TNP-σ; (C) core polymerase.

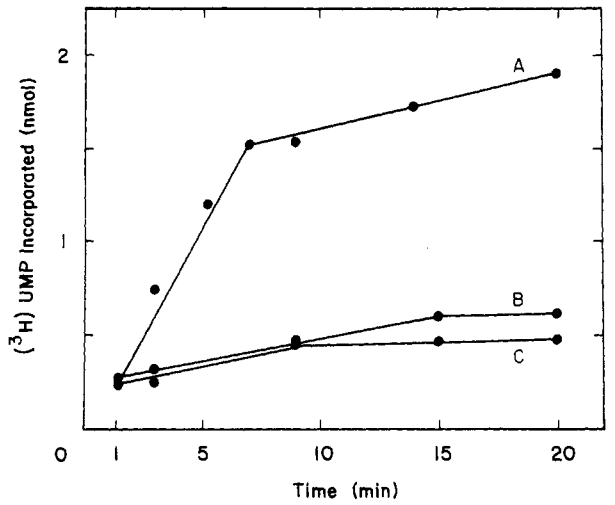

FIGURE 14: Single-round transcription of T7 DNA in the presence of heparin. The reaction mix (1-mL volume) containing 1 mM each of ATP, GTP, CTP, and [^{3}H]UTP (specific activity 15 000–20 000 cpm/nmol), 80 mM Tris-HCl (pH 7.8), 40 mM mercaptoethylamine, 20 mM $MgCl_2$, 0.5 mg of BSA/mL, 100 pmol of core polymerase or core polymerase with 100 pmol of σ or TNP-σ (five TNP groups per σ), and 1.2 μmol of T7 DNA was incubated at 37 °C. At the end of 1 min, a 100-μL aliquot was removed for the determination of [^{3}H]UMP incorporated into acid-precipitable material. To the remainder was added 15 μL of 20 μg/μL heparin. At intervals, 100-μL aliquots were removed for analysis. (A) Core polymerase + σ; (B) core polymerase + TNP-σ; (C) core polymerase.

for T7 DNA; see Chamberlin et al. (1979)] and then are inhibited by heparin. Such an assay involving single round transcription should be very sensitive to the presence of σ. As seen in Figure 14, at the end of the chain termination (about 7 min), there is about a 4-fold increase in the amount of UMP incorporated in the presence of σ (curves A and C), and the trinitrophenylation renders the σ inactive (curve B).

A direct way of studying initiation in natural templates is by transcribing the DNA in the presence of either of the initiation nucleoside triphosphates plus UTP or CTP. Under such conditions, RNA chain elongation is aborted due to the absence of the other two substrates (Johnston & McClure, 1976). The results of such an experiment are shown in Figure 15. A greater than 7-fold difference can be observed between core polymerase and holoenzyme in the synthesis of pppApU and a 2-fold difference in the case of pppGpU. The modification of σ results in complete loss of this function in either of the assay systems.

Several d(A-T)$_n$-directed initiation reactions are also very sensitive to the presence of σ. RNA chain initiation, that is, the formation of the first phosphodiester bond, catalyzed by polymerase, can be reversed in the presence of excess inorganic pyrophosphate. When [^{32}P]PP_i is employed, the label can be exchanged for the PP_i of the elongation nucleoside triphosphate, UTP (Krakow & Fronk, 1969). Such an assay system gives a marked difference in the activity of core polymerase and holoenzyme; a 2–3-fold increase in the amount of PP_i exchanged can be observed with holoenzyme (Figure 16, curve A) compared to core polymerase (curve C) or core polymerase + TNP-σ (six TNP groups per σ) (curve B). Under the conditions employed, the rate of PP_i exchange is approximately 25 pmol/min for core polymerase, and this rate can be stimulated to approximately 60 pmol/min by σ but not by TNP-σ.

Nucleotides such as AMP or UpA can be used to support initiation but not chain elongation in the d(A-T)$_n$-directed assay, when only UTP is present (Hansen & McClure, 1979). As seen in Figure 17, a gradual increase in the amount of

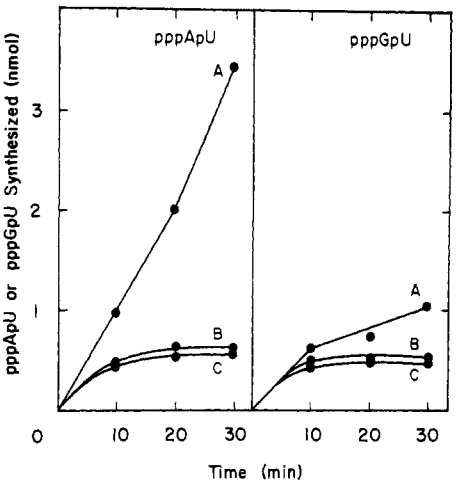

FIGURE 15: Abortive initiation reaction directed by T7 DNA. The reaction mix contained 20 pmol of core polymerase or core polymerase with 20 pmol of σ or TNP-σ (six TNP groups per σ), 10 mM $MgCl_2$, 1 mM DTT, 50 mM Tris-HCl (pH 8), and 200 nmol of T7 DNA. Following a preincubation of 2 min at 37 °C, the reaction mix was brought to 1 mM ATP or GTP and [^{3}H]UTP (5000–8000 cpm/nmol). The final volume was 200 μL, and 50-μL aliquots were removed at intervals, and the reaction was terminated with 10 μL of 0.5 M EDTA (pH 7.6) and chromatographed by using the WASP system. (A) Core polymerase + σ; (B) core polymerase + TNP-σ; (C) core polymerase.

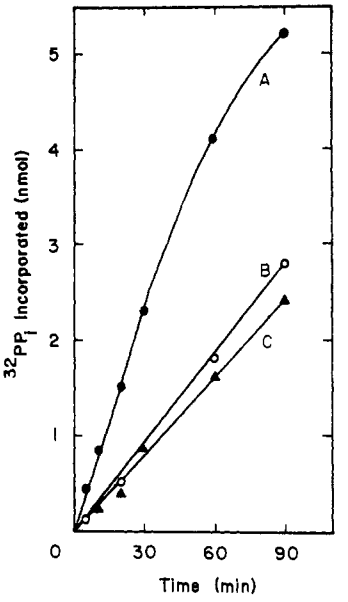

FIGURE 16: Pyrophosphate exchange assay directed by d(A-T)$_n$. The reaction mix containing 1.5 nmol of d(A-T)$_n$, 10 pmol of core polymerase or core polymerase with σ or TNP-σ (six TNP groups per σ), 80 mM Tris-HCl (pH 8), 40 mM mercaptoethylamine, and 4 mM $MgCl_2$ was incubated for 10 min at 37 °C. The mix was brought to 1 mM [^{32}P]PP_i (5000–8000 cpm/nmol), 4 μM ATP, and 400 μM UTP (the final volume of the mix was 800 μL), and 250-μL aliquots were removed at intervals, the reaction was terminated with unlabeled pyrophosphate, and the amount of [^{32}P]PP_i incorporated into the charcoal-adsorbable form was determined as described by Krakow & Fronk (1969). (A) Core polymerase + σ; (B) core polymerase + TNP-σ; (C) core polymerase.

UpApU formed can be observed with increasing amounts of σ in the presence of 10 mM Mg^{2+} (left panel, curve A). A greater than 3-fold increase in the amount of UpApU formed can be seen upon addition of a full equivalent of σ to core polymerase. The progressive addition of TNP-σ (five TNP groups per σ) has no stimulatory effect on core polymerase (left panel, curve B). In presence of Mn^{2+}, although the core polymerase synthesizes about the same amount of UpApU as it does in presence of Mg^{2+}, addition of σ inhibits the synthesis

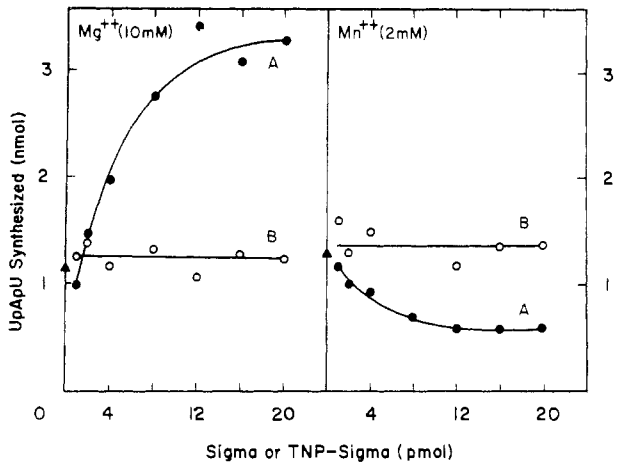

FIGURE 17: Abortive initiation reaction directed by d(A-T)$_n$ with UpA as the initiation dinucleoside monophosphate. Core polymerase (10 pmol) with varying amounts of σ or TNP-σ (five TNP groups per σ) and 10 nmol of d(A-T)$_n$ were incubated at 37 °C for 10 min in a reaction mix containing 50 mM Tris-HCl (pH 8), 1 mM DTT, 2 mM Mn^{2+}, or 10 mM Mg^{2+}. The mix was brought to 1 mM [^{3}H]UTP (5000 cpm/nmol) and 1 mM UpA (50 μL final volume of the mix) and further incubated for 10 min. The reaction was terminated and chromatographed. (A) Core polymerase + σ; (B) core polymerase + TNP-σ; (▲) core polymerase.

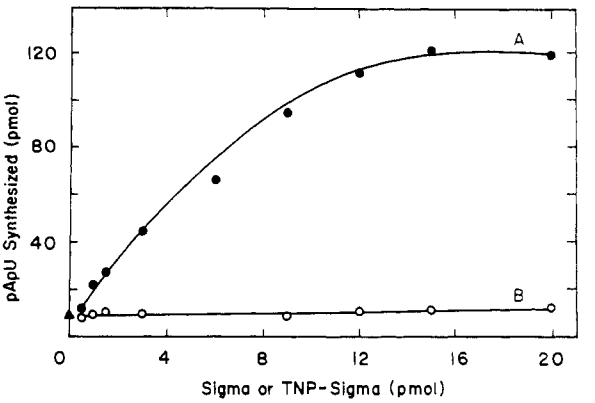

FIGURE 18: d(A-T)$_n$-directed abortive initiation reaction with AMP as the inititation nucleoside monophosphate. Core polymerase (10 pmol) and d(A-T)$_n$ (10 nmol) with varying amounts of σ or TNP-σ (five TNP groups per σ) were incubated with 10 mM Tris-HCl (pH 8), 80 mM KCl, 1 mM DTT, and 10 mM $MgCl_2$ for 10 min at 37 °C. The mix (final volume, 50 μL) was brought to 2 mM AMP and 200 μM [^{3}H]UTP (150 cpm/pmol) and incubation continued for 10 min. The reaction was stopped and chromatographed. (A) Core polymerase + σ; (B) core polymerase + TNP-σ; (▲) core polymerase.

of UpApU by core polymerase. At saturating amounts of σ, greater than 80% inhibition of UpApU synthesis can be observed (right panel, curve A) compared to core polymerase. The modification of σ again fails to support this inhibition of the UpApU synthesis by core polymerase (right panel, curve B).

The most striking difference between core polymerase and holoenzyme can be seen in their ability to synthesize pApU in the presence of AMP and UTP directed by d(A-T)$_n$. The presence of σ enhances the synthesis of pApU by core polymerase by greater than 12-fold (Figure 18, curve A), and TNP-σ (five TNP groups per σ) has no stimulatory effect (curve B).

Discussion

Under the conditions used for modification of σ by TNBS, trinitrophenylation was limited to lysyl residues. This was demonstrated by chromatography of acid-hydrolyzed TNP-σ; the only modified amino acid was TNP-lysine. The absorption spectrum of TNP-σ was consonant with that of a TNP-ϵ-aminolysine derivative. Even after extensive trinitrophenylation (28 TNP groups per σ), modification of cysteine or methionine was not found. The lysyl groups of σ are present in different TNBS-reactive classes, the most reactive group containing the lysine which is critical for σ activity. σ contains 34 lysyl residues (Burton et al., 1981), and trinitrophenylation of 5 lysyl residues results in the loss of σ activity. Kinetic analysis (Hollenberg et al., 1971; Marcus et al., 1976; Marschel & Bodley, 1979) indicated that the incorporation of one trinitrophenyl group per σ resulted in inactivation of σ activity. It would appear that there are approximately five lysyl groups of comparable reactivity with TNBS, one of which is critical for σ function.

In order for σ to affect transcription, it must first bind to the RNA polymerase core enzyme. The complex of σ and core polymerase has properties distinct from those of core polymerase. The holoenzyme shows a lower affinity for DNA general sites and is able to form the closed and open promoter complexes. The formation of the open complex is required for the initiation of transcription at a promoter. The binary open complex is characterized by its high degree of stability. The data presented demonstrate that reaction of σ with TNBS results in the modification of the ϵ-amino group of lysine residues. The trinitrophenylation of the lysine side chain does not affect the ability of TNP-σ to bind to the core polymerase. However, modification of five lysyl groups results in the loss of the capacity of the TNP-σ–core polymerase complex to form an open promoter complex. This was shown by the inability of the TNP-σ holoenzyme to form stable complexes with restriction fragments from T5 DNA known to contain promoter sequences. Filter binding studies showed that the unmodified holoenzyme formed stable complexes with T7 [^{3}H]DNA. The holoenzyme containing TNP-σ (six TNP groups per σ) formed complexes with T7 DNA which were dissociated by competitor DNA as readily as the complex formed between T7 DNA and core polymerase. Filter binding studies with T7 *Hae*III A fragment containing no major promoters showed that the holoenzyme containing TNP-σ (seven TNP groups per σ) formed a binary complex with an affinity comparable to that of the holoenzyme.

The effect of σ in initiation of RNA synthesis by RNA polymerase can be demonstrated with several different procedures. These include heparin challenge, abortive initiation in the T7 DNA directed reaction, single-step synthesis of pApU or UpApU, and pyrophosphate exchange in the d(A-T)$_n$-directed reaction. For each of these, the data indicate that the TNP-σ holoenzyme is unable to effectively engage in the steps required for initiation. The TNP-σ–core complex behaved in a manner comparable to that of the core polymerase alone when assayed by using the aforementioned procedures. In one system, the d(A-T)$_n$-directed synthesis of UpApU, in the presence of Mn^{2+}, the holoenzyme was less active than the core polymerase. In the presence of Mg^{2+}, the holoenzyme synthesized UpApU at a more rapid rate than the core polymerase. With either Mn^{2+} or Mg^{2+} as the divalent cation, the TNP-σ holoenzyme behaved like the core polymerase.

It is evident that the effect of σ is not merely a consequence of the binding to the core polymerase. σ is the most acidic of the RNA polymerase subunits. There is an unusually high concentration of acidic amino acid residues present in the amino-terminal third of the polypeptide (Burton et al., 1981). Interaction of σ with the core polymerase may involve the clustered glutamic and aspartic residues. A synthetic copolymer comprised of glutamic acid and tyrosine (Glu_9, Tyr_1)

binds to RNA polymerase (Krakow, 1974) perhaps in a manner similar to σ. The trinitrophenylation of σ does not inhibit σ activity by blocking its binding to the core polymerase. Limited digestion of σ with *Staphylococcus aureus* V8 protease, an enzyme specific for glutamyl and aspartyl residues, results in a reproducible polypeptide pattern. Following lysine modification with TNBS, the TNP-σ shows an altered polypeptide pattern after digestion by the protease. While not conclusive, the data are consonant with the imposition of an altered conformation for the TNP-σ. This could be a consequence of the incorporation of the bulky trinitrophenyl groups or be due to a possible blocking of the interaction between the modified lysyl groups with glutamic or aspartic acid carboxyl groups in σ.

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