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Transcription by T7 RNA Polymerase Is Not Zinc-Dependent and Is Abolished on Amidomethylation of Cysteine-347†

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ABSTRACT: T7 RNA polymerase has been purified to homogeneity from an overproducing clone of *Escherichia coli* containing pAR1219. Preparations have a zinc content as low as 0.01 mol/mol of enzyme and a high specific activity, 300 000–500 000 units/mg. There are no intrinsic zinc sites. Furthermore, extrinsic Zn^{2+} does not function as an activator. Supplementation of the assay mix with up to 5 mM ethylenediaminetetraacetic acid has little effect on activity while added Zn^{2+} is strongly inhibitory at concentrations above 10 μM. This monomeric RNA polymerase is not a zinc metalloenzyme, unlike its multimeric bacterial counterparts. Titration of the urea-denatured protein with 5,5′-dithiobis(2-nitrobenzoic acid) reveals that all 12 Cys residues are present in the free sulfhydryl form, 5 of which are readily accessible to reagent in the native enzyme. More preferential labeling of the sulfhydryls can be achieved with low concentrations of [^{14}C]iodoacetamide, where inactivation of the enzyme proceeds with incorporation of approximately 1.2 mol of [^{14}C]iodoacetamide/mol of polymerase. Amidomethylation primarily occurs at Cys-347, with lesser reaction at Cys-723 and Cys-839. Cys-347 and Cys-723 are in segments of the primary sequence containing numerous basic residues. These same segments have previously been implicated in promoter binding, suggesting that both residues are located within or near the active site region.

While considerable progress has been made toward understanding the molecular basis of transcription (von Hippel et al., 1984), the identities of the RNA polymerase functional groups involved in promoter recognition and catalysis remain unknown. Zinc has been postulated to play a role in catalysis, since all RNA polymerases analyzed to date have been reported to contain at least 1 mol of zinc/mol of enzyme (Mildvan & Loeb, 1979; Coleman, 1983). Amongst the simplest of RNA polymerases is the monomeric protein encoded by gene 1 of the T7 bacteriophage (M_r 98 856) and isolated from T7-infected *Escherichia coli* (Chamberlin et al., 1970; Niles et al., 1974; Moffatt et al., 1984). Significant amounts of zinc were reported to be associated with extensively purified preparations of this enzyme (Coleman, 1974). Recently, the study of T7 RNA polymerase has taken a large advance with the successful cloning and expression of gene 1 by J. J. Dunn, F. W. Studier, and colleagues (Davanloo et al., 1984). It became clear during our own isolation of the overproduced protein that Zn was not present in sufficient concentration to satisfy even a 1:1 stoichiometry. This finding prompted a reinvestigation of the relationship between Zn and the enzyme activity.

T7 RNA polymerase is known to be inhibited by sulfhydryl-selective reagents (Chamberlin & Ring, 1973; Oakley et al., 1975). A previous study from our laboratory has reported that reaction with a large excess of [^{14}C]iodoacetamide resulted in the incorporation of 1 mol of reagent/mol of enzyme, causing complete inactivation of the protein (Oakley et al., 1975). The selectively amidomethylated polymerase was found to interact with DNA fragments containing a T7 promoter, but in an aberrant manner and without initiation of RNA synthesis (Oakley et al., 1975, 1979; Strothkamp et al., 1980). These observations are consistent with, but not sufficient proof of, the presence of a sulfhydryl group within the active site of the enzyme. As a first step toward clarifying this issue, we have identified the site(s) of amidomethylation by peptide analysis after reaction with [^{14}C]iodoacetamide. The results of earlier studies of sulfhydryl content and reactivity are updated in the light of new, more reliable data obtained for the overproduced protein.

Materials and Methods

T7 RNA polymerase was prepared from *E. coli* strain BL 21 containing plasmid pAR1219 (Davanloo et al., 1984),

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kindly supplied by William Studier and John Dunn. This host is deficient in a protease activity that was found to cause nicking of T7 polymerase in the original HMS 174/pAR1219 system (Davanloo et al., 1984). Protein was purified by two methods. A preliminary low-zinc preparation was obtained by the modification of the method of Niles et al. (1974) published in Oakley et al. (1975). In this procedure, the cell lysate is first fractionated with Polymin P and/or ammonium sulfate and then purified by chromatography on phosphocellulose, hydroxylapatite, and DEAE-Sephadex. Subsequent preparations used the recent method from the laboratory of F. W. Studier in which the Polymin P concentration is kept below 1.25%. The enzyme is subsequently precipitated from the supernatant with ammonium sulfate and chromatographed on SP-Trisacryl (LKB), TSK CM-Fractogel (EM Science), and TSK DEAE-Fractogel (EM Science) columns. A complete description of this variation will be published elsewhere (J. J. Dunn et al., unpublished results). All experiments described here were performed on protein that was pure as judged by lack of extra bands on SDS–PAGE of 5-μg samples.

Amino acid analyses on duplicate samples of measured absorbance were used to determine a molar extinction coefficient $\epsilon_{280} = (1.4 \pm 0.1) \times 10^5$ M^{-1} cm^{-1} for pure T7 polymerase. This value is considerably higher than the ϵ_{280} of 0.74×10^5 M^{-1} cm^{-1} originally reported (Niles et al., 1974) but in agreement with that estimated from the amended gene 1 DNA sequence (Moffatt et al., 1984) and extinction coefficients for the aromatic amino acids (Cantor & Schimmel, 1980). Good agreement was obtained between experimental and predicted (Moffatt et al., 1984) amino acid compositions for most residues (±3%), with Ala and Arg being more variant (±10%).

Activity assays used a standard assay mix (67.5 μL), which after addition of protein contained 40 mM Tris-HCl,[1] pH 7.7, 30 mM $MgCl_2$, 1 mM mercaptoethanol, 50 μg/mL T7 DNA, and 30 nmol of NTPs, including [^{3}H]ATP of final specific activity 11.1 μCi/mol. Rifamycin (1295 μg/mL) was present in some reaction mixtures as was added EDTA or Zn where appropriate. Enzyme (0.75 pmol) was added in 7.5-μL aliquots. Incorporation of [^{3}H]AMP into RNA was measured as previously described (Oakley et al., 1975). Quench correction was achieved by counting an aliquot from a 1-h reaction with 50–300 pmol of protein as a 100% incorporation blank. With this method, absolute counting efficiencies of 27% for [^{3}H]ATP in Hydrofluor (National Diagnostics) and 18% for [^{3}H]RNA on Whatman 3MM filter paper were obtained. One unit of specific activity is defined as nanomoles of AMP incorporated per hour at 37 °C in the standard assay mix (Chamberlin et al., 1970; Chamberlin & Ring, 1973).

Metal analyses were performed by atomic absorption on an IL 157 spectrophotometer. Both liquid aspiration and graphite furnace methods were employed.

Amidomethylation of T7 polymerase for peptide analysis was carried out at 4 °C with a 10–20-fold excess of [^{14}C]-iodoacetamide (New England Nuclear) over protein (1–10 μM) in 100 mM NH_4HCO_3, 1 mM EDTA, or 20 mM sodium phosphate, 100 mM NaCl, and 1 mM EDTA, pH 7.7. Protein was reacted to 50–100% inactivation, followed by quenching of unreacted [^{14}C]iodoacetamide with mercaptoethanol and dialysis. Other reactions with iodoacetamide or Ellman's reagent (DTNB) were carried out by literature methods (Torchinsky, 1981). [^{14}C]Iodoacetamide incorporation was measured as described previously (Oakley et al., 1975).

Peptide analyses were performed after the ^{14}C-labeled protein was digested with a 1/25 w/w quantity of TPCK-trypsin (Worthington) for 24 h at 37 °C in 2 M urea, 100 mM NH_4HCO_3, and 1 mM EDTA. The tryptic digest was initially fractionated by reverse-phase HPLC on a Vydac C-4 column, eluting with a programmed gradient of buffer A (0.05% TFA) and buffer B (0.05% TFA, 80% CH_3CN) at pH 2: 0–90 min (0–37.5% B), 90–138 min (37.5–75% B), and 135–150 min (75–100% B). Fractions containing significant radioactivity were dried with a Speedvac apparatus, dissolved in 6 M guanidinium chloride, and rechromatographed with a programmed gradient as above with 10 mM potassium phosphate as buffer A and 20% 10 mM potassium phosphate–80% CH_3CN as buffer B at pH 6.0. Purified peptides and protein were hydrolyzed in 6 N HCl/0.1% phenol at 115 °C for 16 h and applied to a Beckman 121M amino acid analyzer.

Table I: Specific Activity and Zn Content of T7 RNA Polymerase Preparations[a]

prepn	EDTA	sp act. (units/mg)[b]	[protein] (μM)	[Zn] (μM)	Zn content (mol/mol)
I	+	300 000	3.3	0.5	0.15
II	+	450 000	22.9	0.2	0.01
III	–	500 000	26.4	0.1	<0.01

[a] Analytical data refer to material from the elution profile apex of the final DEAE-Fractogel column. [b] Estimated error ±10–15%.

Results

Polymerase Activity and Effect of Zinc. When cells are lyzed and subsequent columns run in the presence of 1–2 mM EDTA, T7 RNA polymerase elutes from the final DEAE-Fractogel column as a single peak with an average Zn:protein ratio of less than 0.2 mol/mol (Table I). There is no significant difference between the concentration of Zn^{2+} associated with the protein-containing peak and the Zn background of the buffer (~0.5 μM). Protein from this peak is highly active in the standard assay, with a routine rifamycin-insensitive specific activity in the range 300 000–500 000 units/mg (Table I). These values compare favorably with the maximum obtainable specific activity of 400 000–600 000 units/mg suggested by previous studies (Chamberlin & Ring, 1973; Coleman, 1974).

Cell lysis in a Zn-supplemented buffer followed by the use of EDTA-free buffers during protein purification results in a preparation with a very low Zn content and high specific activity (Table I). The low zinc content of protein exposed to EDTA during preparation cannot be due to leaching of the metal from a relatively weak binding site. This result is independent of the purification scheme employed, since a preliminary preparation by the method of Oakley et al. (1975) also possessed a low zinc content and high specific activity. Consistent with the above, rifamycin-insensitive activity in cell lysates prepared from bacteria grown on a Chelex-treated medium is very similar to the activity obtained when a Zn-supplemented growth medium is used. These results clearly demonstrate the T7 RNA polymerase does not require intrinsic Zn atoms for activity or, by implication, for structure.

Atomic absorption measurements show the standard assay mix to be contaminated by Zn at a concentration of 4–5 μM. The possibility that the reaction may utilize Zn as a loosely associated activator must therefore be examined. Since it is

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

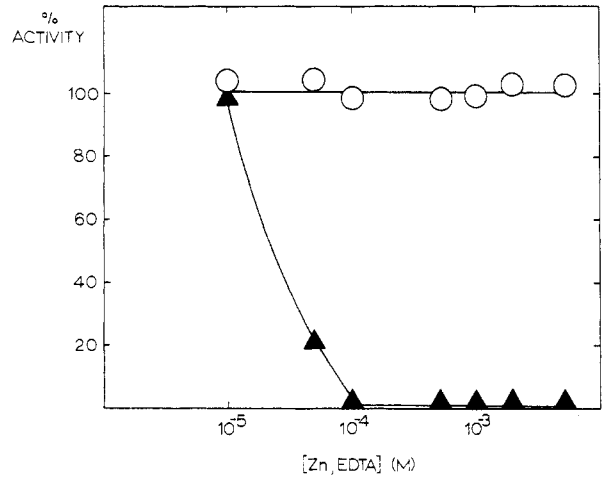

FIGURE 1: Instantaneous effect of Zn^{2+} and EDTA on T7 RNA polymerase activity. Zn (▲) and EDTA (○) were added to the standard assay mix. Full activity for this sample is 450000 units/mg.

not feasible to reduce the Zn content of the assay medium to levels below that of the protein (10 nM) by Chelex treatment, we have investigated the instantaneous effect of added Zn and complexing agents on specific activity. As shown in Figure 1, supplementation with EDTA has no inhibitory effect up to a concentration of 5 mM. Calculations using the stability constants of $ZnEDTA^{2-}$ and $MgEDTA^{2-}$ indicate that EDTA concentrations of 5 μM to 5 mM are sufficient to reduce the free Zn^{2+} in the standard assay mix to levels below 20 pM. Gradual inhibition is observed at EDTA concentrations of 5–30 mM, caused by diminution of the relatively high concentrations of free Mg^{2+} required for full activity. Conversely, added Zn (as $ZnCl_2$) is found to be strongly inhibitory at much lower concentrations, i.e., <100 μM. This inhibition is quantitatively similar to effects reported in an earlier study (Coleman, 1983), which demonstrated that several other divalent metal ions are also inhibitory in this concentration range. It is clear from the above experiments that Zn^{2+} has no functional role in the catalytic mechanism of this polymerase.

Identification of [^{14}C]Iodoacetamide-Labeled Cysteine. The amended gene 1 sequence predicts T7 RNA polymerase to contain 12 Cys residues (Moffatt et al., 1984). When freshly prepared protein is titrated with DTNB in the presence of 4–8 M urea or 3–6 M guanidinium chloride, a reproducible yield of 11.7 ± 0.8 free sulfhydryls per enzyme is obtained. The observed absence of disulfide bonds is considered typical of proteins that function in bacterial cytoplasm. Addition of a 20-fold excess of DTNB to the native enzyme results in the rapid reaction of five to six sulfhydryls, followed by slow reaction of the remainder over a period of hours. Mercaptoethanol-free assays performed in concert with DTNB titrations yield linear plots of activity vs. equivalents of reagent that reach complete inactivation at 3.7 ± 0.5 mol of DTNB/mol of enzyme. Plots of $a^{1/i}$ vs. m (Tsou, 1962) are linear when i = 1 and 2, suggesting that reaction of one or two of the five available sulfhydryls is responsible for the observed activity loss. Protein that has been stored for a long period in the absence of reducing agents frequently yields a lower number of free sulfhydryls on titration with DTNB, presumably due to partial unfolding accompanied by disulfide formation. The loss of titrable sulfhydryl groups on storage and the smaller original extinction coefficient may explain the substantially lower number of free sulfhydryl groups reported in an earlier study (Oakley et al., 1975).

As reported previously (Oakley et al., 1975), the T7 polymerase is slowly inactivated by [^{14}C]iodoacetamide. In the

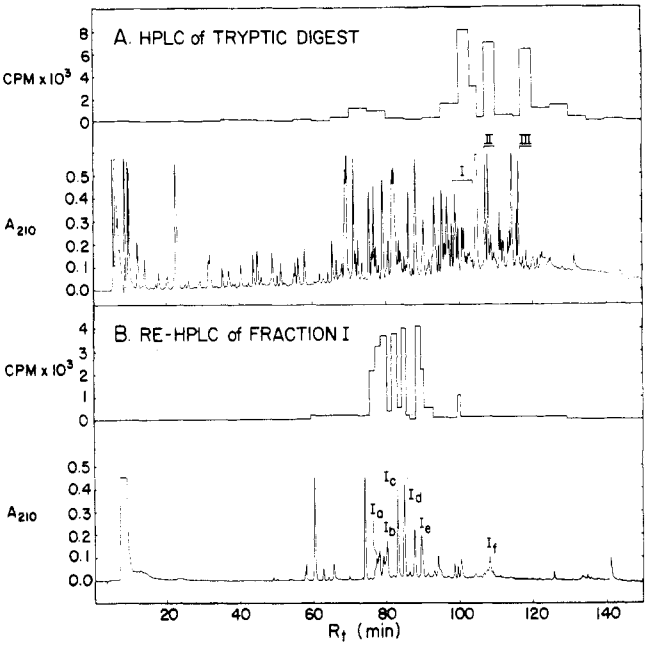

FIGURE 2: Reverse-phase HPLC on tryptic digests of [^{14}C]amidomethylated T7 RNA polymerase. (A) Fractionation at pH 2. Peaks containing significant radioactivity are designated I–III. (B) Refractionation of peak I at pH 6.0. Six ^{14}C-containing peptides are separated from contaminants.

presence of a 10–20-fold excess of reagent, complete inactivation is achieved after 48–72 h with the incorporation of 1.2 ± 0.2 mol of ^{14}C/mol of enzyme. Reactions performed over several days yielded a stoichiometry of ~2 mol of ^{14}C/mol of enzyme, a value not significantly changed by addition of fresh reagent and further incubation. To identify the modified residues, several samples with ^{14}C contents of 0.65–1.3 mol/mol were subjected to tryptic hydrolysis and HPLC. A representative experiment is shown in Figure 2A. The HPLC elution profile is very complex due to the high Lys/Arg content of the protein. Column losses were found to be relatively minor, retention of 10–25% of the radioactivity applied to the column was typically observed. In all cases, significant eluted radioactivity was found to be associated with three crude fractions designated I–III (see Figure 2A). Together, these fractions contained 77–90% of the eluted radioactivity, with 46 ± 10% in fraction I, 20 ± 5% in fraction II, and 13 ± 3% in fraction III. Within the relatively large uncertainty of the measurements, the populations of fractions I–III were found to be similar at different ^{14}C/enzyme stoichiometries in the above range. The remaining radioactivity was widely distributed in the chromatogram, with no single fraction containing over 5% of the eluted radioactive material. One sample with a ^{14}C/enzyme ratio of 2.2 that had been reacted for a period of 14 days was hydrolyzed with trypsin to determine the population distribution at "maximum" stoichiometry. Fractions I–III were observed to contain approximately 30, 15, and 15% of the eluted radioactivity, respectively, with other fractions containing less than 5%.

Isolated crude fractions were subjected to HPLC at pH 6 to obtain pure ^{14}C-containing peptides. The result of one such purification is shown in Figure 2B, where six radioactive peptides are resolved from a particular fraction I isolate. After purification, isolates of fractions II and III yielded one and two radioactive peptides, respectively. Amino acid analyses of the resulting pure radioactive peptides are presented in Table II. When the amino acid compositions of these peptides are compared with the known sequence of T7 RNA polymerase,

Table II: Amino Acid Compositions of ^{14}C-Labeled Peptides

peptide	I_a	346–378	I_b	346–379	I_c	344–378	I_d	344–379	II	723–740	III_a	830–860	III_b	830–867
Ala[b]	3.1	3	3.2	3	3.2	3	3.3	3	1.4	1	2.3	2	4.1	3
Arg	0.9	1	2.0	2	1.4	1	1.8	2	0.1	0	0.0	0	0.0	0
Asx	4.1	4	4.1	4	4.1	4	4.1	4	1.2	1	6.1	6	5.9	6
Cys	*a*	1	*a*	1	*a*	*a*	*a*	1	*a*	1	*a*	1	*a*	1
Glx	6.0	6	6.1	6	6.0	6	6.2	6	2.2	2	6.3	6	6.1	6
Gly[b]	0.5	0	0.5	0	0.6	0	0.4	0	1.1	1	0.3	0	0.2	0
His	0.9	1	0.9	1	0.9	1	0.9	1	0.8	1	1.0	1	1.0	1
Ile	2.8	3	2.9	3	2.8	3	2.9	3	0.3	0	0.1	0	0.0	0
Leu	2.1	2	2.2	2	2.2	2	2.3	2	0.4	0	3.1	3	4.0	4
Lys	2.1	2	2.0	2	2.8	3	2.9	3	1.1	1	1.1	1	2.1	2
Met[c]	1.7	2	1.3	2	1.4	2	1.8	2	0.0	0	0.8	1	1.8	2
Phe	0.0	0	0.0	0	0.0	0	0.2	0	0.9	1	2.0	2	2.0	2
Pro	4.5	5	4.7	5	5.1	5	4.9	5	1.9	2	0.0	0	2.2	2
Ser[b]	0.3	0	0.2	0	0.3	0	0.3	0	0.2	0	1.9	2	1.8	2
Thr	1.0	1	1.0	1	1.0	1	1.0	1	1.0	1	2.0	2	1.9	2
Trp	*a*	1	*a*	1	*a*	2	*a*	2	*a*	2	*a*	0	*a*	0
Tyr	0.0	0	0.0	0	0.0	0	0.0	0	0.9	1	2.0	2	2.0	2
Val	1.1	1	1.0	1	1.1	1	1.1	1	2.6	3	1.9	2	2.0	2

[a] Not determined. [b] Contamination by Ala, Ser, and Gly was observed for some samples. [c] Some samples showed partial destruction of Met.

fractions I_{a-d}, II, and $III_{a,b}$ are found to be derived from peptides containing Cys-347, -723, and -839, respectively. The presence of partial hydrolysis in parent peptides I and III helps to confirm their identification. Peptide I_e, which accounts for 12% of the eluted radioactivity, could not be identified due to unresolvable peptide overlap. Peptide I_f was present at levels two low for accurate amino acid analysis. For the case of a ^{14}C/enzyme stoichiometry of 0.65, ~70% of the eluted radioactivity has been positively assigned to individual peptides, including ~35% from modification of Cys-347, 20% and Cys-723, and 13% from Cys-839.

Discussion

Results presented in this paper show unequivocally that T7 RNA polymerase is neither Zn^{2+}-containing nor Zn^{2+}-activated. The only previous study on this subject reported a variable Zn:protein stoichiometry (Coleman, 1974). That observation in itself was a cause for some concern and may be explained by adventitious binding of Zn to a protein containing many sulfhydryl groups in combination with the relatively low enzyme concentrations obtainable from phage-infected cells. In the earlier study, long-term dialysis against several complexing agents was reported to cause substantial inhibition (Coleman, 1974). It has become apparent that some of those results were probably caused by destabilization of the enzyme, which is relatively fragile. We have, however, observed instantaneous inhibition by anions such as SH^- in the course of the present work. At present, the mechanisms by which some chelating agents cause inactivation are unknown. Similarly, the previously reported activation of some preparations by exogenous Zn^{2+} (Coleman, 1974) may have been caused by poorly understood processes such as nonspecific structural stabilization or removal of anionic inhibitors.

On the basis of their homology with the T7 enzyme, the absence of a functional role for Zn may be extended to the other monomeric phage RNA polymerases. In contrast, a recent review of the literature shows clear agreement that the multisubunit RNA polymerases from *E. coli* and *Bacillus subtilis* contain zinc in a well-defined stoichiometry of 2.0 mol/mol (Coleman, 1983). Workers in the laboratories of Wu and Wu have extensively studied the function of Zn in the *E. coli* enzyme by metal substitution and reconstitution procedures (Speckhard et al., 1977; Chatterji & Wu, 1982a,b; Chatterji et al., 1984; Solaiman & Wu, 1984). There is persuasive evidence that one Zn atom is involved in subunit interactions (Solaiman & Wu, 1984). Other evidence has suggested that one Zn atom is involved in a nucleotide binding role (Speckhard et al., 1977; Chatterji & Wu, 1982a; Chatterji et al., 1984). If the phage and bacterial RNA polymerases can be considered to share a common catalytic mechanism, then our current results for the T7 enzyme indicate that zinc need not perform a catalytic role in the bacterial proteins. In fact, recent work performed in this laboratory shows that one Zn can be removed from the *E. coli* enzyme without loss of transcriptional activity from the class A promoters of T7 DNA (D. P. Giedroc and J. E. Coleman, unpublished data).

Iodoacetamide is a selective inactivator of T7 RNA polymerase, reacting significantly with only 3 of the 12 Cys residues, with an order of reactivity Cys-347 > Cys-723 > Cys-839. Some care should be taken when attempting to assign functional roles on the basis of relative populations derived from the HPLC analyses. There are several experimental variables that can conspire to produce misleading conclusions. Nevertheless, it is apparent from its dominant presence in the ^{14}C-containing peptides that reaction of Cys-347 causes a loss of activity. The presence of two other significantly amidomethylated residues, in conjunction with an inactivation stoichiometry of 1.2 ± 0.2 mol of ^{14}C/mol of enzyme, suggests that modification of one of these two causes inactivation, while the other may not. It is also possible that amidomethylation of Cys-347 and Cys-723 is a mutually exclusive process, and reaction of either one inactivates the enzyme. Such a situation may account for the complete loss of activity on incorporation of 1.2 mol of ^{14}C/mol of enzyme. The molecular basis of the amidomethylation pattern is clearly difficult to interpret and must await further information.

A comparison of the T7 enzyme with the identically sized T3 RNA polymerase may provide clues on the possible functional significance of the three iodoacetamide-reactive Cys residues. The two proteins share 82% amino acid sequence homology (Moffatt et al., 1984; McAllister et al., 1983), although neither will appreciably transcribe from the other's promoter sites (Ryan & McConnell, 1982). Both Cys-347 and -723 are conserved between species. Cys-839 is replaced by Asn in the T3 enzyme, which suggests that the latter residue can have no essential role in the specific functions common to both polymerases, such as catalysis. Experiments involving the genetic construction of hybrid T7/T3 polymerases suggest that the transcriptional selectivity determinants, and therefore at least some DNA binding loci, lie in two regions at 25–59% and 80–100% of the gene 1 length (Ryan & McConnell, 1982). Cys-347 and -723 lie at 39.3% and 86.2% of gene 1, respec-

tively, which places them within these regions.

The amino acid sequences immediately around Cys-347 and -723 in T7 RNA polymerase are

-Lys-Trp-Lys-His-Cys^{347}-Pro-Val-

and

-Arg-Lys-Arg-Cys^{723}-Ala-Val-His-Trp-

respectively (Moffatt et al., 1984). The presence of Arg, Lys, His, and Trp side chains, all with potential for interacting with DNA, also suggests that these Cys residues may lie within DNA binding loci. This is consistent with the results of single-strand endonuclease digestion of protein–promoter complexes, which showed that amidomethylation of T7 RNA polymerase causes altered interaction with promoter-containing DNA fragments (Strothkamp et al., 1980). At this point, it remains unclear whether the sulfhydryl groups of Cys-347 and Cys-723 have a specific functional role in transcription or whether their modification results in subtle conformational changes[2] or steric hindrance that causes indirect inactivation. In order to address this issue, we are now using site-directed mutagenesis to remove the sulfhydryl functionality of these residues with a minimum of structural perturbation.

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Registry No. Zn, 7440-66-6; L-Cys, 52-90-4; RNA polymerase, 9014-24-8.

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[2] Circular dichroism of the amidomethylated polymerase from 350 to 200 nm shows that there is no significant change in gross secondary structure on modification of the protein.