Fine Mapping of DNA Single-Stranded Regions Using Base-Specific Chemical Probes: Study of an Open Complex Formed between RNA Polymerase and the *lac* UV_5 Promoter†

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ABSTRACT: We have used diethyl pyrocarbonate (DEP), which carbethoxylates adenine bases, and dimethyl sulfate (DMS), which methylates guanine residues and single-stranded cytosines, to probe bases in open complexes between RNA polymerase and the *lac* UV_5 promoter in vitro. We compared the kinetics of reactivity between bases in an open complex and those in a single-stranded 35-mer fragment corresponding to the lower template strand of *lac* UV_5 in the region −25 to +10 relative to the transcription start site. We observed that cytosine and adenine residues in the 35-mer fragment reacted according to a second-order process with DMS and DEP, respectively, at sufficiently low concentrations of the reagents and that the degree of reactivity was base position independent. In an open complex in the absence of substrates, we observed reactivity with DEP in adenines from −12 to +4 as well as +21 on the template strand and methylation by dimethyl sulfate of cytosines −6, −4, −2, and −1. No hyperreactivity was observed on the nontemplate strand. The degree of reactivity of bases between −12 and +4 was position dependent, maximum reactivity being displayed by bases in the middle of the region. The reaction was first order within the range of reagent concentration investigated. It was confirmed that in the presence of ApA and UTP cytosine +5, as well as cytosines −6, −4, −2, and −1, in an open complex became reactive to DMS. With regard to DEP the extent of reactivity of the adenine at position +3 was increased markedly, adenine +4 was brought into the single-stranded region, and the overall reactivity of adenine −10 decreased. The general shift in the reactivity profile was taken as an indication that the primary determinant of the gradient of reactivity found in the single-stranded area was the position of the base. Our data challenge a too simplistic interpretation of most results obtained by chemical probing of nucleoprotein complexes. They suggest that, within unstacked and unwound regions of double-stranded DNA in open complexes, DMS and DEP reactions are limited by a base-specific structural rearrangement.

Prior to initiation of transcription, RNA polymerase interacts with promoter sequences to form a kinetically competent binary complex. Studies with various strong promoters indicate that the open complexes between RNA polymerase and different promoters share several homologies [for a recent review, see Yager and Von Hippel (1987)]. An open complex is topologically unwound (Wang et al., 1977; Gamper & Hearst, 1982; Amouyal & Buc, 1987). It contains a well-defined region (12–16 base pairs in length) which becomes accessible to reagents that probe single-stranded bases (Siebenlist & Gilbert, 1980; Kirkegaard et al., 1983; Spassky, 1986). The addition of RNA polymerase induces a significant hyperchromicity in the UV spectrum of the promoter DNA consistent with an unstacking of the corresponding bases (Reisbig et al., 1979). It is not at all certain that the different techniques listed above report exactly the same type of deformation of the DNA double helix. Differential hyperchromicity results from a static and permanent unstacking; topological unwinding can correspond to various changes in writhing or in untwisting of the template during the sequential process leading to open complex formation (Amouyal & Buc, 1987).

It is important to characterize this locally melted region in an open complex; following the kinetics of formation of this entity provides information about the strength of the promoter under study; following the translocation of this region as substrates are added furnishes details concerning the mechanism of promoter escape (Spassky et al., 1985; Straney & Crothers, 1987). It is furthermore essential to determine the order of the reaction with respect to the bases and specific reagent in single-stranded DNA, duplex DNA, and duplex DNA containing locally melted regions.

The promoter *lac* UV_5 is a strong promoter containing mutations which allow efficient transcription in the absence of the complex between cyclic AMP and its receptor protein (CRP). It is one of the best cases studied with respect to the fine positioning of RNA polymerase on the DNA molecule (Johnsrud, 1978; Spassky et al., 1985). RNA polymerase occupies a region of more than 50 base pairs in length on *lac* UV_5. In the final open complex the extent of strand separation and the fine geometry of the local structure are not precisely characterized but are thought to extend over approximately 12 base pairs (Siebenlist & Gilbert, 1980). It has been shown that on the lower template strand cytosines −6, −4, −2, and −1 react with DMS[1] and are single stranded. In the presence of ApA and UTP, RNA polymerase enters cycles of abortive initiation, and cytosine at position +5 becomes reactive to DMS, showing that the region of locally separated DNA has in fact extended downstream (Spassky et al., 1985). Monitoring the hyperreactivity of specific bases to the artificial nuclease copper–*o*-phenanthroline, A. Spassky deduced that

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[1] Abbreviations: DMS, dimethyl sulfate; DEP, diethyl pyrocarbonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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the translocation corresponded to two base pairs (Spassky, 1986). This displacement was not observed when ATP and UTP were the added substrates (Carpousis & Gralla, 1985). The total amount of topological unwinding was not appreciably modified when this translocation was taking place (Amouyal & Buc, 1987).

Diethyl pyrocarbonate (DEP) reacts with purine ribonucleotides to produce ring-opened products (Leonard et al., 1971; Vincze et al., 1973). The major site of attack is the nitrogen at position 7 accessed via the major groove of B DNA. DEP is however a sterically bulky molecule, and its degree of reactivity is severely restricted by the accessibility of the target molecules. At sufficiently high concentrations, DEP produces a purine ladder in reactions with B DNA (Herr et al., 1982). However when DNA was in the Z form stabilized by negative supercoiling, a large enhancement in reactivity to DEP was observed (Nordheim, 1984; Herr, 1985; Johnston & Rich, 1985; Runkel & Nordheim, 1986). It was demonstrated by Furlong and Lilley (1986) and Scholten and Nordheim (1986) that adenine bases in the unpaired loop region of cruciform structures were also hyperreactive to DEP. Studies on antibiotic binding induced structural changes on DNA showed a DEP hyperreactivity to Hoogsteen base-paired adenines within a double helix (Mendel & Dervan, 1987).

We have attempted to characterize the reactivity of bases such as cytosines and adenines in a locally melted region of DNA with base-specific reagents. It was of interest to see if this reactivity was analogous to that of bases in a single-stranded fragment or exhibited a pattern peculiar to bases in an open complex and thus to deduce information concerning the exposure and spatial disposition of such bases. We used DMS to examine the kinetics of reactivity of cytosines in single-stranded fragments or open complexes. Furthermore, we developed a methodology to utilize DEP to look at reactive adenines under the same conditions.

Materials and Methods

DNA. Fragments of *lac* UV_5 were isolated from plasmids as described in Schaeffer et al. (1982). The 5′ labeling with [γ-^{32}P]ATP was carried out on dephosphorylated *lac* UV_5 fragments by use of T4 polynucleotide kinase and 3′ labeling with [α-^{32}P]dATP by use of Klenow fragment (Maxam & Gilbert, 1980). Uniquely end-labeled fragments were generated by select cleavage of the labeled DNA with *Pvu*II. RNA polymerase was isolated according to the technique described by Burgess and Jendrisak (1975), modified as indicated in Lowe et al. (1979). The 35-mer single-stranded fragment corresponding to the lower template strand of *lac* UV_5 from −25 to +10 was synthesized on a gel synthesizer (Pharmacia) and was labeled at the 5′ end by use of T4 polynucleotide kinase as described above.

Open Complex Formation. Labeled duplex DNA (4 nM) was incubated at 37 °C for 15 min with RNA polymerase (200–250 nM) in a buffer containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM HEPES (pH 8.4), 1 mM 2-mercaptoethanol, 3% glycerol, 100 μg/mL BSA, and sonicated calf thymus DNA (25 μM).

Abortive Initiation. To the open complex formed as above was added a mixture of ApA (500 μM final concentration) and [α-^{32}P]UTP (50 μM final concentration) or ATP (500 μM final concentration) and [α-^{32}P]UTP (50 μM final concentration). At predetermined time intervals aliquots of 15 μL were removed to Whatman paper pretreated with 10 mM EDTA. When the rapid-mixing apparatus was used (cf. legend to Figure 7), aliquots of 10–15 μL were removed at regular intervals from the reaction chamber and quenched with 10 mM EDTA prior to spotting onto Whatman paper. In all cases the products of abortive initiation were separated from [α-^{32}P]UTP by ascending chromatography as described in McClure (1980). Inactivation of transcription by DEP was followed in the rapid-mixing apparatus. To an already actively transcribing mixture was added a solution of DEP, and aliquots were removed at 1-s intervals for analysis.

DEP Reactions. To the complexes formed above was added a freshly prepared aqueous solution of DEP. For visualization of reactive DNA bases, the final concentration was usually 18 mM DEP. The solution was stirred vigorously with an Eppendorf tip but not vortexed as this tends to disrupt the DNA–RNA polymerase complex. Aliquots (10 μL) were removed at predetermined time intervals into aqueous solutions (40 μL) of imidazole (10 mM), which competes for the reagent; then, they were extracted with phenol to remove proteins, and the DNA was precipitated with ethanol. After a second ethanol precipitation the DNA was dissolved in 100 μL of 1 M piperidine and heated at 90 °C for 30 min prior to extensive lyophilization.

Methylation of Unpaired Cytosines. Dimethyl sulfate methylation of free and complexed DNA was carried out by the addition of a freshly prepared aqueous suspension of DMS to the preformed open complex to a final concentration of 2 mM DMS (unless otherwise specified). At specific time intervals aliquots were removed to a stop solution containing 100 μg/mL tRNA and 0.3 M ammonium acetate and extracted with phenol. Subsequent hydrazine and piperidine treatment was carried out as described in Kirkegaard et al. (1983).

Qualitative and Quantitative Analysis of Modification Reactions. Sequencing reactions were carried out according to Maxam and Gilbert (1980). Gels were run on 8% acrylamide containing 7 M urea and were dried onto 3MM Whatman paper and autoradiographed on Fuji or Kodak X-ray films with Ilford fast-tungstate intensifier screens. Nondenaturing gel electrophoresis was carried out essentially as described by Straney and Crothers (1985).

Densitometric traces of developed films were obtained with a Bio-Rad densitometer. The observed increase in reactivity for a particular adenine base toward DEP or guanine and cytosine residues toward DMS was determined from the relative optical density of its corresponding band on a gel expressed as a percentage of the total radioactivity. In order to quantitate as accurately as possible the proportion of radioactivity in a band as deduced from its optical density, gels were scanned after various times of exposure such that at no point did the optical density exceed 1.5. The application of correcting factors pertaining to single-hit kinetics conditions, such as those described in Lutter (1978) and Drew and Travers (1984), did not alter the calculated kinetics.

Results

Accessibility and Reactivity of Cytosine Residues in Single-Stranded DNA and in Open Complexes. Dimethyl sulfate was used to methylate a 35 base pair stretch of the template strand of *lac* UV_5 containing cytosines at positions −6, −4, −2, and −1. The rates of reaction of the various bases were determined by the quantitative techniques described under Materials and Methods. Although the accuracy is not better than 15–20%, within this error limit, for any given base the initial rate of the reaction was found to increase with the concentration of dimethyl sulfate (Figure 1). At any given concentration of DMS all the cytosines in the 35-mer fragment exhibited the same extent of reactivity, indicating that the mechanism of the chemical attack was base position independent.

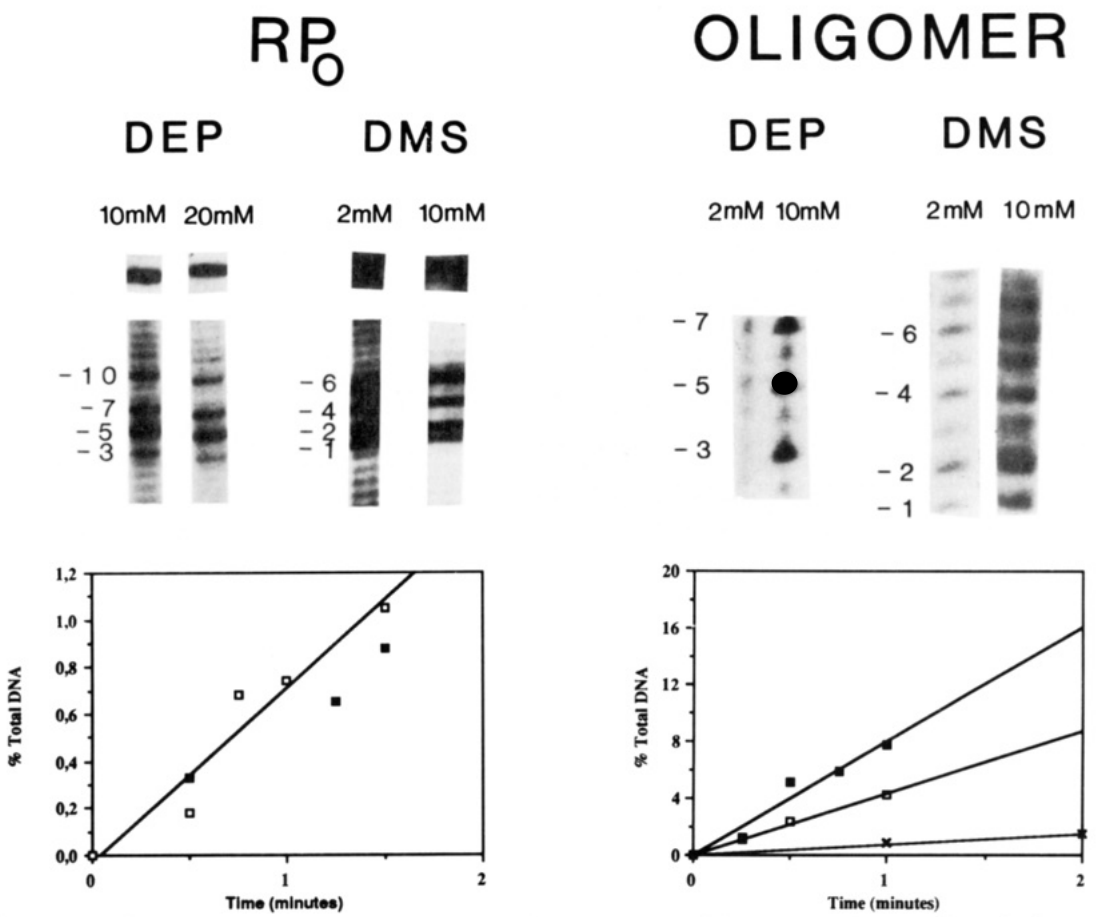

FIGURE 1: Comparison of the reactivity of adenines to DEP and of cytosines to DMS in open complexes and in a single-stranded 35-mer oligonucleotide. The gels show typical profiles for the reactivity of adenines or cytosines with DEP and DMS, respectively. The top bands represent the same experiment but at a shorter time of exposure in order to illustrate the differences between the lanes in terms of overall intensity. The lower left plot shows the reactivity of DMS with cytosines −4 (■) and −2 (□) in an open complex and that on the lower right the reactivity of cytosine −4 in a oligonucleotide at concentrations of DMS of 10 (■), 2 (□), or 0.1 mM (×). Both curves were obtained by densitometric scanning of gels of the type shown above at different times of attack.

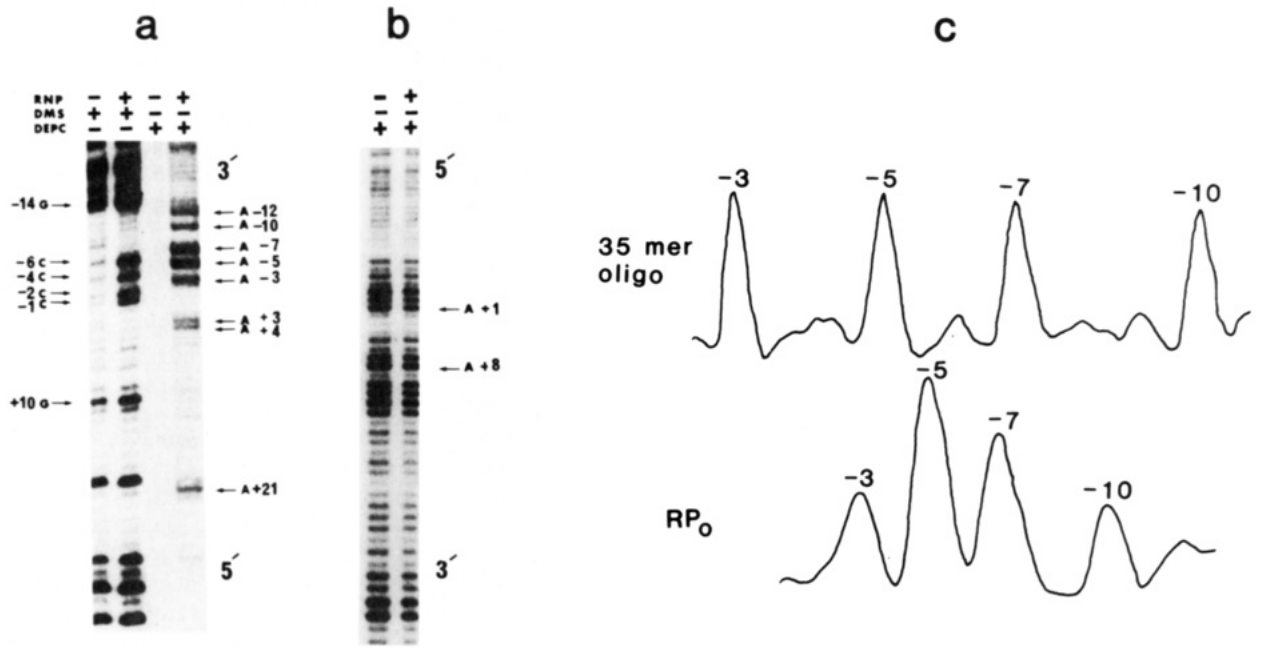

FIGURE 2: DMS and DEP reactivity on the *lac* UV_5 promoter in the presence and absence of RNA polymerase. DNA labeled uniquely at either the 5′ end of the lower template strand or the 3′ end of the upper nontemplate strand was reacted with DMS or DEP in the presence or absence of RNA polymerase as described under Materials and Methods. (a) DNA corresponding to the lower template strand. (b) DNA corresponding to the upper nontemplate strand. Note this film is overexposed in order to show nonspecific activity. (c) Densitometric scans of adenines in the 35-mer oligo fragment *lac* UV_5 and in RP_0 after attack by DEP (20 mM) for 2 min.

Table I: Calculated Rate Constants for the Attack by DEP and DMS on Adenine and Cytosine Residues in an Open Complex between RNA Polymerase and the *lac* UV_5 Promoter[a]

	open complex		open complex + ApA + UTP	
base	λ_i ($min^{-1} \times 10^2$)	μ_i (min^{-1})	λ_i ($min^{-1} \times 10^2$)	μ_i (min^{-1})
A −12	(<0.1)		(<0.10)	
A −10	0.34	0.43	(<0.1)	
A −7	0.44	0.36	0.40	0.38
C −6	1.10		0.5	
A −5	1.26	0.87	1.10	0.53
C −4	0.43		0.43	
A −3	0.55	0.38	0.52	0.31
C −2	0.50		0.9	
C −1	0.38		1.0	
A +3	(<0.1)		0.18	0.19
A +4			0.13	0.19
C +5			0.5	

[a] The rate constants λ_i and μ_i were calculated from plots of the type shown in Figure 4 with the expression $f_i = [\lambda_i/(\mu_0 + \sum\lambda_i)][1 - e^{-(\mu_0+\sum\lambda_i)t}]$, where f_i indicates the fraction of base i which has reacted at time t, λ_i refers to the first-order rate constant for the postulated conformational change for a given base i occurring prior to chemical modification, and μ_0 to the rate of dissociation of a complex subsequent to modification of either the polymerase or the DNA. The rate of dissociation was however found to be base position dependent and is hence described here as being μ_i (see Results and Discussion).

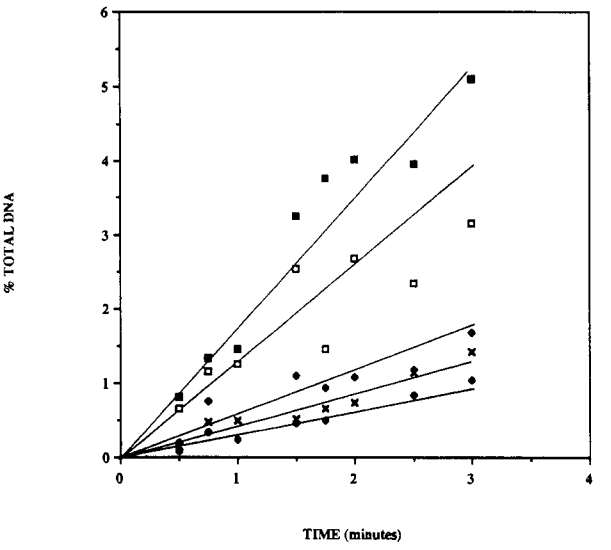

FIGURE 3: Kinetics of reactivity of guanine −14 (■) and cytosines −6 (□), −4 (◆), −2 (●), and −1 (×) in an open complex to DMS. Open complexes were treated with DMS as described under Materials and Methods for various time periods. The resulting gels of the type shown in Figures 1 and 2 were scanned with a Bio-Rad densitometer, and the relative proportions of methylated bases were plotted as a function of total DNA at specific periods.

The same procedure was used to probe the reactive cytosines present in an open complex between RNA polymerase and *lac* UV_5, [cf. Kirkegaard et al. (1983), Spassky et al. (1985), and Figure 2). We first noted that, in contrast to the situation for the 35-mer fragment, the extent of methylation for a particular cytosine depended upon its position in the sequence. When the rate of formation of the modified base was monitored, it was seen (Figure 1) that the rate was considerably lower than that in the case of the single-stranded 35-mer fragment ($<7 \times 10^{-3}$ min^{-1} at 10 mM DMS instead of 8×10^{-2} min^{-1}). Furthermore, this rate was independent of the concentration of DMS when the reagent was present in the millimolar range. These findings suggest that in this open complex a base-specific structural rearrangement was required to expose the template strand to the reagent and that this step was sufficiently slow to limit the rate of the subsequent chemical process.

For each cytosine residue in an open complex (at positions −6, −4, −2, and −1) the apparent first-order rate constant (λ_i) corresponding to the postulated structural rearrangement step was measured (Figure 3 and Table I, column 2). There is a clear hierarchy observed in the magnitude of the calculated rate constants with the order of reactivity being −6 > −4 > −2 > −1. In the presence of ApA and UTP a downstream shift in this reactivity profile was observed (Table I) with cytosine +5 becoming highly reactive although its rate of methylation was still independent of the DMS concentration. In the presence of ATP and UTP no differences in the values for λ_i from those obtained for an open complex were observed, cytosine +5 being totally masked.

Reactivity of Adenine Bases to DEP in a Single-Stranded DNA and in an Open Complex. The same single-stranded 35-mer DNA fragment was exposed to DEP. The reactivity of adenine bases increased appreciably when the DEP concentration was increased (Figure 1), and the degree of reactivity was seen to be of the same order of magnitude as that found for the reactivity of cytosines toward DMS. At concentrations of DEP below 2 mM, the signal/noise ratio was too low to allow a clear distinction of specific reactivity. At a concentration of DEP greater than 10 mM, adenines exhibited the same extent of reactivity independent of their position in the sequence (Figure 2) in a manner analogous to that seen for the reactivity toward DMS.

The incubation for 10 min of a preformed complex of *lac* UV_5 promoter and RNA polymerase with DEP (18 mM final concentration) at 37 °C, followed by alkaline piperidine treatment, produced selected cleavage at specific adenine residues (Figures 1 and 2). Adenines −12, −10, −7, −5, −3, +3, and +4 were hyperreactive in the lower strand (3′ → 5′), but no hyperreactivity of adenines in the upper strand (5′ → 3′) was observed. The bases −12 to +4 displayed a range of differential reactivity toward DEP which was related to the position of the residue in the sequence (Figure 2). Under the conditions used and in the absence of RNA polymerase, none of the bases on the template strand were reactive. On the template strand a hyperreactivity of adenine +21 was observed in an open complex (Figure 2). This base does not lie within a putative single-stranded region, but its reactivity suggests it being in an unusual conformation induced by the presence of polymerase.

The reactivity of adenine residues toward DEP in the open complex was independent of DEP concentration within the range 10–20 mM. This suggests that, as seen for the reactivity of cytosines in an open complex toward DMS, the initial rate of reaction of a reactive adenine was limited by a base-specific structural rearrangement. However contrary to what was observed with DMS, we found that the overall kinetics of DEP modification were quite complex (Figure 4). The reaction was not linear with respect to time, and a plateau of reactivity was reached after about 4 min. Adenine residues could be classified into two categories according to the reaction profile. In the first class are grouped adenines −10, −7, −5, −3, and +3 for which the reaction was exponential. The reaction pattern appeared to be sigmoidal for adenines −12 and +4 (Figure 5), the initial velocity being negligible at all concentrations of reagents tested. These two residues are therefore placed in a second class.

Lifetime of the Open Complex: Characterization of the Enzyme following DEP Attack. We concluded that, as a result of the action of DEP, the lifetime of the open complex was rapidly shortened, reducing considerably the time of ex-

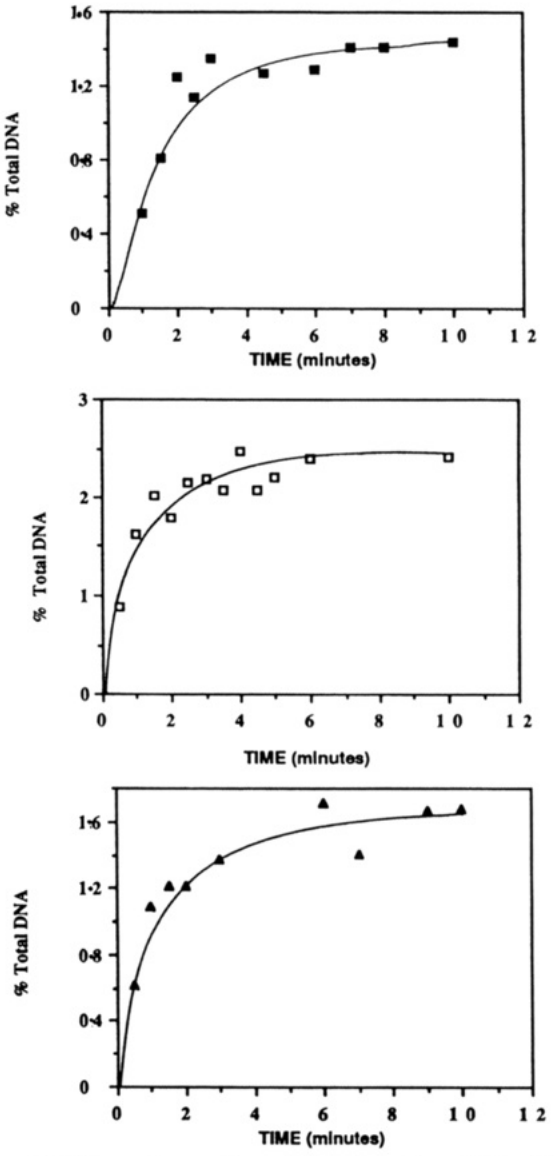

FIGURE 4: Differential reactivity to DEP of adenines −7 (■), −5 (□), and −3 (▲) in an open complex. RNA polymerase–DNA complexes formed as described under Materials and Methods were reacted with DEP for various time periods. Densitometric scanning of gels of the type shown in Figure 1 allowed calculation of the relative proportion of ethoxyformylated adenines with respect to total DNA. These values were plotted as a function of the time of the reaction with DEP.

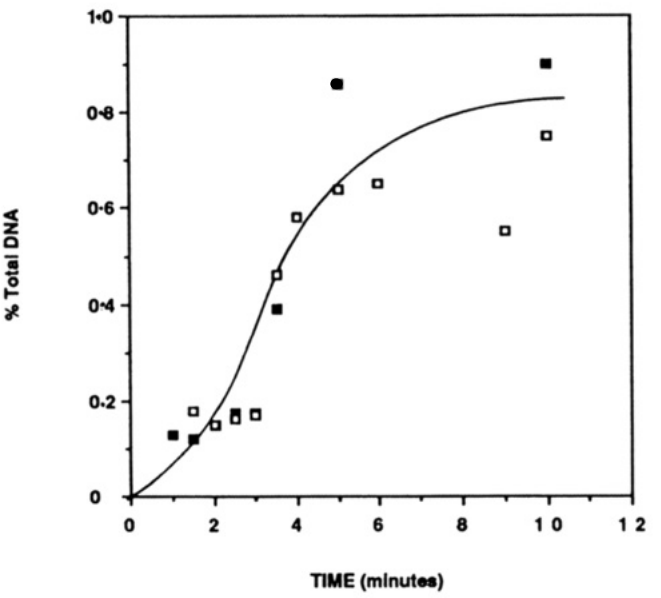

FIGURE 5: Differential reactivity of adenines +4 (□) and −12 (■) in an open complex in the absence of ApA and UTP. Experimental conditions were as for Figure 4. A hyperbolic curve gave an R factor of 0.84 compared with an average value of 0.96 for adenines −7, −5, and −3.

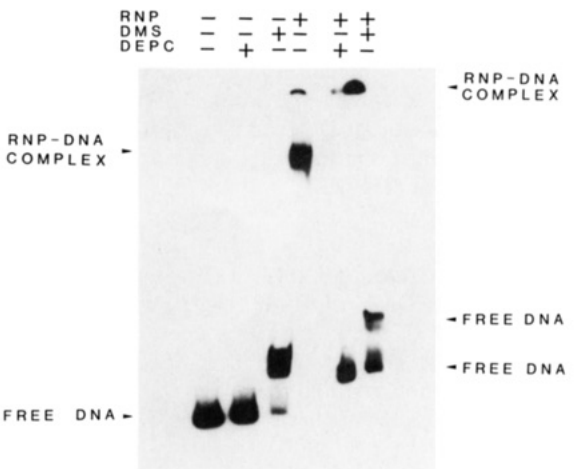

FIGURE 6: Gel retardation assay for RNA polymerase–DNA complex formation. RNA polymerase–DNA complexes were formed at 37 °C as described under Materials and Methods and reacted with either DEP (18 mM) or DMS (2 mM) for 2 min prior to loading onto a 4% polyacrylamide nondenaturing gel. Bands were visualized by autoradiography.

posure of the single-stranded region to the reagent. Conditions of analysis were strictly single hit with respect to DNA cleavage, but DEP was suspected to ethoxyformylate imidazole groups of the protein. This hypothesis was checked at two concentrations of DEP: 18 mM, the conditions required to visualize reactive adenine residues, and 0.3 mM, a concentration that allows inactivation of a single crucial histidine residue and destruction of the catalytic center of the enzyme (Abdulwajid & Wu, 1986). We first compared the stability of open complexes after treatment with DEP or DMS at our concentrations. RNA polymerase (200 nM) was preincubated with DEP (18 mM) for 1 min prior to the addition of DNA (4 nM). Excess DEP was competed with 100 mM imidazole (which in itself did not inhibit subsequent complex formation). As determined by gel retardation assays, no complex between pretreated RNA polymerase and DNA was formed. In a control experiment, pretreatment of RNA polymerase with DMS did not prevent formation of an open complex. Preformed complexes were then treated with DEP or DMS for 1 min and run on nondenaturing gels, a process which took approximately 3 min. DNA and DNA–protein complexes were visualized by autoradiography. A gel is shown in Figure 6. In the period of 3 min following DEP treatment the complex was completely destroyed, and no retardation of DNA was observed. Preincubation of DNA with DEP for 1 min did not alter the mobility of free DNA on the gel. As predicted, DEP modification greatly increased the rate of dissociation of the open complex.

We wondered then whether the inactivation of the catalytic center of the enzyme was responsible for this phenomenon. An abortive initiation assay was used in conjunction with a rapid-mixing apparatus in order to accurately determine a rate constant for the rate of inhibition of abortive initiation of an open complex between RNA polymerase (200 nM) and *lac* UV_5. The rate of incorporation of radioactive UMP into the tetranucleotide ApApUpU (approximately 4–6% total

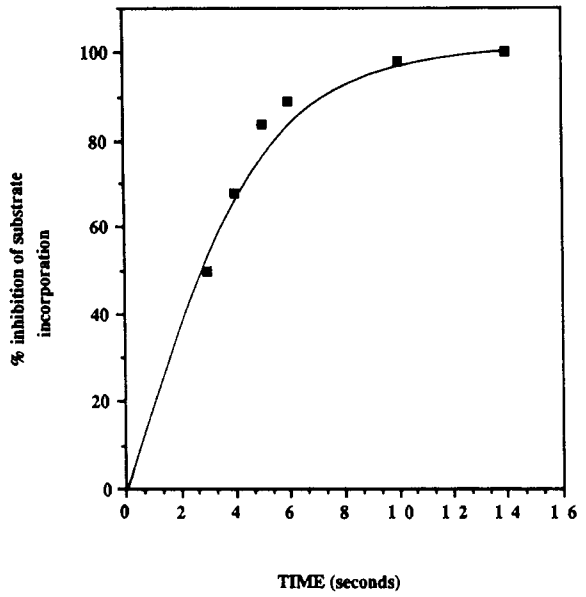

FIGURE 7: Abortive initiation assay in the presence of DEP. Open complexes between RNA polymerase and DNA were incubated in a reaction loop contained within a rapid-mixing apparatus at 37 °C. At zero time the contents of this syringe were mixed with a solution containing substrates to give a final concentration of ApA (500 μM) and [α-^{32}P]UTP (50 μM). At regular intervals 10-μL aliquots were mechanically removed to a quench solution of 10 mM EDTA. Products were separated by ascending chromatography on Whatman paper as described in McClure (1980), and the degree of incorporation of [α-^{32}P]UTP in the ApApUpU transcript was determined by scintillation counting of Cerenkov radiation. Inhibition by DEP was demonstrated under similar conditions except that at zero time the ApA/UTP mixture was added to the same reaction loop as the open complex. At t = 7 min the contents of the reaction loop were rapidly mixed with a solution containing DEP (18 mM), and aliquots were removed after short time intervals to EDTA quench solution and Whatman paper as above. The degree of decreased incorporation of radioactive UMP as compared by that of the same experiment in the absence of DEP was then calculated and plotted as a function of time of incubation of the transcribing mixture with DEP.

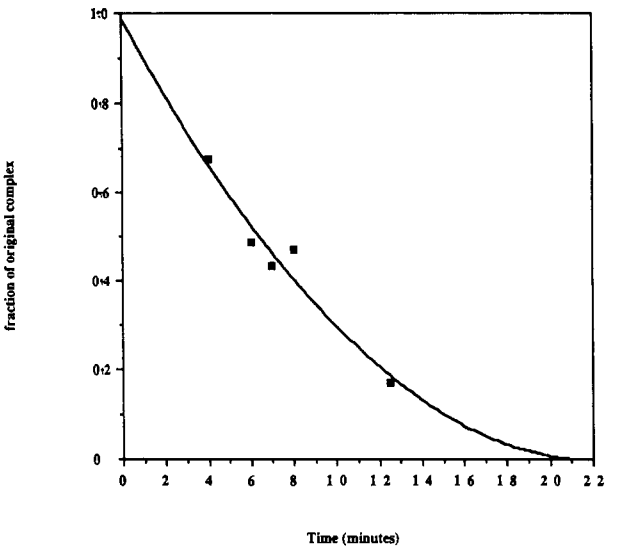

FIGURE 8: Stability of an open complex between DEP-modified RNA polymerase and DNA. To an open complex between RNA polymerase and DNA was added DEP to a final concentration of 0.3 mM. After 1 min at 37 °C the reaction was quenched with imidazole (10 mM final concentration), and aliquots were loaded at regular intervals onto 4% nondenaturing polyacrylamide gels. The resulting gels were autoradiographed and densitometrically scanned to determine the proportion of complex present at given time periods after DEP modification. The fraction of the original complex remaining was plotted as a function of the total time after the addition of the quenched mixture to the gel.

UTP/min) was found to be linear with respect to time (R factor = 0.99) in the period up to 80% incorporation. Upon addition of DEP (18 mM), there was extremely rapid inhibition of this incorporation (Figure 7). The inhibition process showed an apparent first-order rate constant of 15 min^{-1}. Such a rapid event is in agreement with the results obtained at a lower concentration of DEP (0.5 mM) by Abdulwajid and Wu (1986) on a nonspecific template. This rapid inhibition of UTP incorporation was also observed at low (0.3 mM) concentrations of DEP. We decided to investigate also the effect of low (0.3 mM) DEP concentrations on complex formation between RNA polymerase and the *lac* UV_5 promoter. Incubation of an open complex between RNA polymerase (200 nM) and DNA with DEP (0.3 mM final concentration) for 1 min at 37 °C and subsequent analysis on gel retardation assays showed a progressive destruction of the complex, illustrated in Figure 8. From these data a semilog plot of complex remaining as a function of time gave a negative linear slope (R factor = 0.97) which allowed the calculation of a first-order rate constant for the dissociation of a complex between DNA and modified RNA polymerase of around 0.13 min^{-1}.

At this point it was of interest to show that the catalytically inactive enzyme was still able to form a single-stranded region in the correct position on the promoter, but was unable to propagate the single-stranded region when suitable substrates were added. Open complexes composed of RNA polymerase modified by treatment with DEP (0.3 mM) for 1 min were treated with either DMS or an artificial nuclease, the *o*-phenanthroline–cuprous complex [$(OP)_2Cu^+$], in the absence or presence of substrates (ApA and UTP). In an open complex cytosines at −6, −4, −2, and −1 reacted with DMS, and bases at positions −6, −5, and −4 were hyperreactive to $(OP)_2Cu^+$ (Figure 9). In the presence of substrates ApA and UTP, bases at positions −3 and −2 show hyperreactivity to $(OP)_2Cu^+$ in a normal open complex undergoing abortive initiation (Spassky, 1986), and cytosine +5 is methylated by DMS. However after 1 min of premodification of the polymerase, the hyperreactivity of bases at positions −2 and −3 to $(OP)_2Cu^+$ decreased (Figure 9), and a proportion of reactive cytosine +5 was no longer methylated, showing that the modified polymerase was capable of maintaining a single-stranded region yet unable to progress into abortive initiation. At such low DEP concentrations we already showed that the catalytic activity of the modified polymerase is already completely abolished (Figure 7). It appears that the relaxation of the extended open region back to a preabortive initiation complex is a slower process than inactivation of the catalytic site.

Quantitative Analysis of Base Modification. (*a*) *DMS.* Within the open complex each base i is considered to undergo exposure with a first-order rate constant λ_i while the complex dissociates at a rate k_{off}. We assume that the encounter between the reagent and subsequent product formation are fast compared to λ_i.

The process under single-hit conditions may be simply represented as a series of independent reactions for each reactive base i:

$$R + P \underset{k_{off}}{\rightleftharpoons} RP_o \xrightarrow{\lambda_i} RP_i \xrightarrow{\nu_i(DMS)} RP_i^* \qquad (I)$$

P_i indicates the base exposed to the reagent, and P_i* indicates that base i is modified. The dissociation of RP_0 into R + P for *lac* UV_5 (k_{off}) is known to be very slow (Buc & McClure, 1985). Since the overall rate of reaction is seen to be independent of the concentration of the reagent, we assume that the last step (ν_i) is fast and therefore the fraction of base i

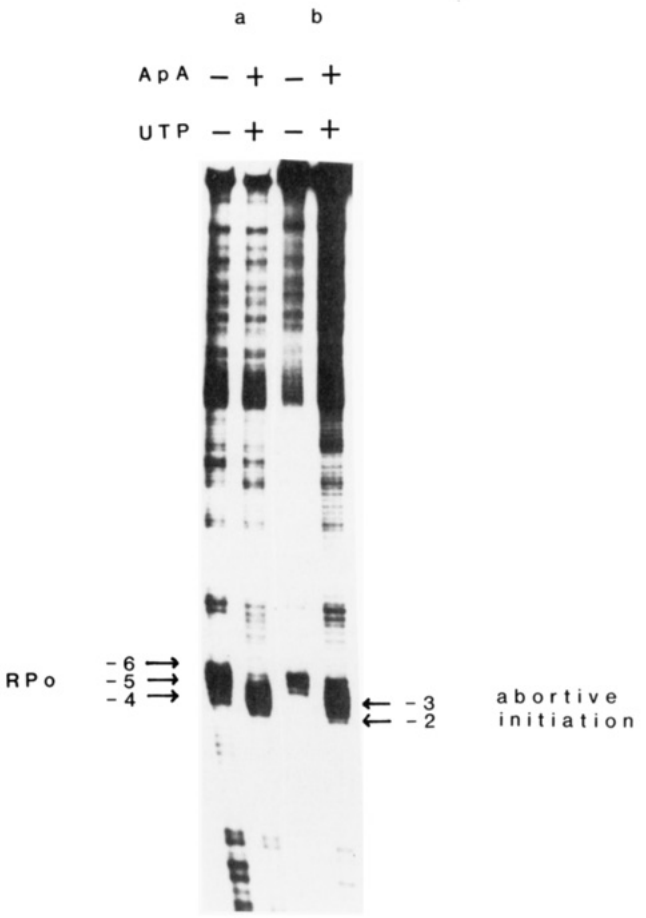

FIGURE 9: Incapacity of a modified RNA polymerase to enter into abortive initiation while still in an open complex as judged from $(OP)_2Cu^+$ hyperreactivity. Open complexes between RNA polymerase in the presence and absence of the substrates ApA and UTP were treated with the artificial nuclease $(OP)_2Cu^+$. An open complex is characterized by hyperreactive bands at positions −6, −5, and −4. In the presence of substrates ApA and UTP the hyperreactivity pattern shifts down to encompass bases −3 and −2. (a) Nonmodified RNA polymerase. (b) RNA polymerase treated for 1 min with 0.3 mM DEP and then quenched by the addition of imidazole (10 mM final concentration) prior to ApA and UTP addition and subsequent $(OP)_2Cu^+$ attack. Under conditions of modification for greater than 10 min, bases −3 and −2 no longer react with $(OP)_2Cu^+$ in RP_0 + ApA + UTP, although bases −6, −5, and −4 remain reactive.

which has reacted at time t (f_i) will be governed by the simple expression

$$RP_0(t) = RP_0(e^{-\sum\lambda_i t})$$

from whence

$$f_i = \frac{\lambda_i(1 - e^{-\sum\lambda_i t})}{\sum\lambda_i}$$

which for conditions of single-hit kinetics may be simply expressed as

$$f_i = \lambda_i t$$

(*b*) *DEP.* Here we have to consider that the residence time of RP_0 during modification of the enzyme by DEP is drastically reduced from k_{off} to μ_0. Otherwise, the pathway is a priori identical:

$$R^* + P \underset{\mu_0}{\rightleftharpoons} R^*P_o \xrightarrow{\lambda_i} R^*P_i \xrightarrow{\nu_i(DEP)} R^*P^*_i \quad (II)$$

Thus f_i becomes

$$f_i = \frac{(R^*P^*_i)_t}{(R^*P_0)_{t=0}} = X[1 - e^{-(\mu_0+\sum\lambda_i)t}]$$

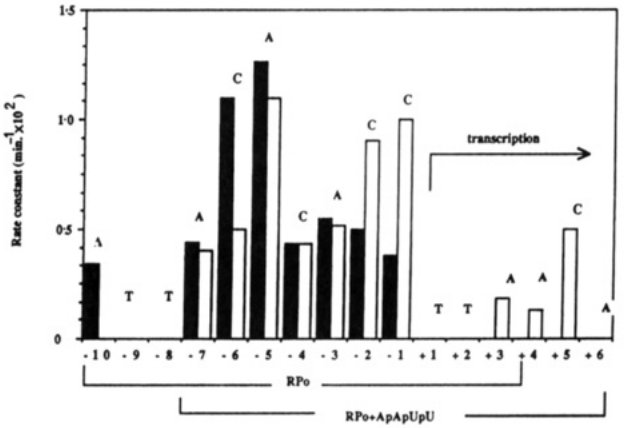

FIGURE 10: Comparison of calculated rate constants for modification by DMS or DEP of bases within either an open complex or a complex undergoing abortive initiation. The overall first-order rate constant (λ_i) for methylation by DMS was calculated from curves of the type shown in Figure 3. Similar values for DEP ethoxyformylation of adenine residues were obtained from curves of the type shown in Figure 4, by measuring $\lambda_i/\mu_i + \sum\lambda_i$ and $\mu_i + \sum\lambda_i$ and assuming $\mu_i \gg \lambda_i$. These values were plotted against the base sequence in the presence (ApA + UTP) and absence (RP_0) of substrates. Results obtained with a thymine-specific reagent (in cooperation with D. M. J. Lilley) are in good agreement with the assignment of the open region in the three cases (RP_0, RP_0 + ApA + UTP, and RP_0 + ATP + UTP).

where $X = \lambda_i/(\mu_0 + \sum\lambda_i)$ and represents the attainable plateau of modification for a given adenine residue. The initial rate of the reaction is still given by $f_i = \lambda_i t$ and therefore yields λ_i which may be calculated from plots of the type shown in Figure 4. Semilog plots of ln $[[(R^*P^*_i)_\infty - (R^*P^*_i)_t]/(R^*P^*_i)_\infty]$ against time for adenines −5, −7, and −3 (R factors of 1.0, 0.92, and 0.96, respectively) allowed calculations of the overall rate constant $\mu_0 + \sum\lambda_i$. μ_0 is fast compared to all the measured values of λ_i, and therefore, the slopes of such plots should give independent estimates for μ_0. In effect, values of μ (given in Table I as μ_i) depend upon the base considered. An empirical correlation is found when the values shown in Table I, λ_i and μ_i, are plotted against each other: a roughly linear relationship (R factor = 0.96) with a positive slope is found that extrapolates for $\lambda i = 0$ to a value for μ_0 of 0.16 min^{-1}, which is close to that which we previously independently determined (Figure 8). Thus the greater the ease with which a base is exposed, the shorter the interval of time during which the base may undergo the modification reaction before the complex is destroyed, a phenomenon we attribute to the multiple sensitive targets for DEP which are present on the enzyme and on the DNA.

Examination of the kinetics of reactivity for adenines −12 and +4 in the absence of substrates gave curves which exhibited a sigmoidal behavior, the onset of reactivity occurring subsequent to modification of the majority of the more reactive adenines (Figure 5). For this reason bases exhibiting this behavior were excluded from the locally separated region (see Discussion).

The reactivity of adenines to DEP and of cytosines to DMS in an open complex in the presence and absence of substrates ApA and UTP is summarized in Figure 10. In the absence of substrates adenine −5 was most reactive, reactivity decreasing proportionally away from this base in either direction, producing a bell-shaped profile. While there was no change in the intrinsic reactivity of bases from −7 to −1, in the presence of ApA and UTP, the reactivity of adenine −10 to DEP was reduced to a great extent whereas adenines +3 and +4 reacted appreciably more rapidly with the reagent. The kinetics of adenine +4 no longer gave a sigmoidal curve, being instead

hyperbolic. Adenine at position −12 remained unreactive under all the conditions studied. Note that cytosine at position +5 is reactive to DMS in the presence of ApA and UTP but that adenine +6 remains unreactive to DEP. These differences were not observed when ATP and UTP were used as substrates.

Discussion

In order to study more precisely deformable DNA regions in complexes formed between RNA polymerase and promoter sequences, we have used a kinetic approach and introduced the reagent DEP. The use of DEP allows reactive adenines to be identified (whereas before only single-stranded cytosines had been localized by chemical means). An examination of the reaction kinetics for both DEP and DMS on the template strand shows that neither reagent probes fully exposed bases in the locally melted region but rather they report their more or less easy access to a single-stranded conformation. This conclusion was reached by comparison of the dependence of the kinetics, for both DEP and DMS, on the concentration of the reagent in the case of single-stranded oligonucleotides and DNA in open complexes. The amount of product formed per unit time in single-stranded oligomers was base position independent, and the rate of the reaction increased with increasing concentration of the reagent. Such should be the case in an open complex once the DNA around each base is correctly structured to allow the base to become reactive. All the data are interpreted according to this idea.

Adenine and cytosine residues on the upper nontemplate strand of the open complex were refractory to DEP and DMS, respectively. The reactivity of a cytosine on the lower template strand toward DMS was limited by a slow first-order process which is interpreted as representing the rearrangement of the base. The rate is governed by the position of the base within the locally melted region. However when ApA and UTP are added, the cytosine at position +5 becomes accessible.

The reactivity of DEP with adenine residues in an open complex followed the same pattern as DMS. A careful kinetic examination of DEP reactivity confirmed that, as for DMS methylation of cytosines, the reactivity of bases, in this case adenines, was dependent upon the position of the base. Indeed, the analysis of the effects of DEP emphasizes that chemical probes actually report a transient accessible structure which may not be interpreted simply as being "single stranded". It remains to be clarified as to which actual conformations of adenine bases are reacting with DEP. Previous studies on DMS alone relied on a qualitative assessment where cytosines having the N3 position not involved in a hydrogen bond were reactive to DMS and hence by inference were interpreted as being within a single-stranded region. The slightly lower rate of reactivity of DEP than that of DMS with residues in a single-stranded oligomer probably reflects the enhanced steric encumbrance associated with DEP. DEP reports a perturbation in normal base stacking not necessarily interpretable as being due to a complete departure from a double-stranded character. More interesting however is the observation that the limiting rate observed in open complexes is of the same order of magnitude independent of the chemical reagent or its concentration and thus practically rules out the possibility that this corresponds to the chemical modification step.

In an open complex we show two classes of bases reactive to DEP: one containing adenines −10 to +3 which react within the residence time of the modified open complex and another consisting of bases −12 and +4 which do not fall within this category and may be considered to be either outside the putative single-stranded region or within a transition region between the locally separated and normal duplex DNA. The criteria which determine this classification are the rates of the base-specific structural rearrangement and subsequent chemical modification. Only a kinetic study is able to discriminate between these two extremes. Adenines −12 and +4 in an open complex had a negligible initial rate of modification with DEP and became reactive only after adenines from −7 to +3 had already been modified. It is possible that the reactivity of adenines −12 and +4 to DEP in an open complex after 4 min reflects the perturbations induced by modification of the other adenine residues especially in view of the presence of the sterically bulky ethoxyformate groups on adjacent adenines.

The reactivity of adenines −10 to +3 in an open complex and the lack of reactivity of adenine +4 would seem to set the size of the locally melted region to 13 base pairs in length, which is in agreement with that found by Siebenlist and Gilbert (1980) but smaller than that predicted by the observed degree of topological unwinding which if translated as 1.6 × 10.5 would give a length of 17 base pairs (Gamper & Hearst, 1982). The analysis presented here shows that only about two-thirds of the linking deficiency could be stored in the open region. We suggest that the presence of the RNA polymerase acting as a selective modulator determines the minimal size of the separated region, translating only part of the linking deficiency into true strand separation.

The lack of reactivity of adenines in the nontemplate strand and the observation that the overall rate constant is first order with regard to DEP suggest that DEP is probing the accessibility of these residues. Furthermore, the reactivity of each residue toward DEP or DMS gives a bell-shaped profile over the length of the locally separated region which is consistent with the idea that the single strand has to be extracted from its strong interaction with the enzyme prior to reaction with the reagent. This deformation becomes increasingly difficult closer to the extremes, in agreement with the concept of a restructuring of a chain being held firmly at two fixed points.

In the presence of suitable substrates such as ApA and UTP or ATP and UTP for *lac* UV_5, RNA polymerase enters a series of cycles of abortive initiation. It is of great interest to determine whether the locally separated region is translocated or is of constant length during any of these steps. Assuming that ApA formation is rate limiting, then the low turnover number in the presence of ATP and UTP and the fact that cytosines at position +5 and adenines at +4 do not react with DMS and DEP, respectively, suggest that the complex spends most of its time, during an abortive cycle, performing the first phosphodiester linkage. In the presence of ApA and UTP the first phosphodiester bond is preformed, the turnover number of synthesis to +4 increases by an order of magnitude, the reactivity profile of adenines toward DEP shifts downstream, and cytosine +5 reacts with DMS. Adenine at position +6 does not become reactive to DEP. Adenine −10 decreases in reactivity, suggesting only partial closure upstream. These observations are easily accounted for by imagining the locally separated region as oscillating between −10 to +3 and −8 to +5.

If one transposes the same length for this region during the elongation of transcription, synthesis at base $i + 1$ implies that the nascent RNA occupies a maximum of 10 bases, which is equal to or less than one helical turn of B DNA. This would minimize the topological problem for RNA polymerase displacement as outlined in Yager and von Hippel (1987) or in Gamper and Hearst (1982).

Our data are consistent with models like the one proposed by Gamper and Hearst which postulate a very high degree of

order for the template strand during catalysis and a fixed distance between the upstream and downstream boundaries of the melted region and the catalytic site (Gamper & Hearst, 1982). At *lac* UV_5 the closed complex is already appreciably unwound (Amouyal & Buc, 1988). Strand separation and open complex formation could then be viewed as a "melting-in" process occurring between two strong anchoring points of the RNA polymerase with, respectively, the −11 to −12 region and the +3 to +4 region of the promoter sequence.

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Registry No. DMS, 77-78-1; DEP, 1609-47-8; cytosine, 71-30-7; adenine, 73-24-5; guanine, 73-40-5.

REFERENCES

Abdulwajid, A. W., & Wu, F. Y. H. (1986) *Biochemistry 25*, 8167–8172.

Amouyal, M., & Buc, H. (1987) *J. Mol. Biol. 195*, 795–808.

Brenowitz, M., Senear, D. F., Shea, M. A., & Ackers, G. K. (1986) *Methods Enzymol. 130*, 132–181.

Buc, H., & McClure, W. R. (1985) *Biochemistry 24*, 2712–2723.

Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4638.

Carpousis, A. J., & Gralla, J. D. (1985) *J. Mol. Biol. 183*, 165–177.

Drew, H., & Travers, A. A. (1984) *Cell 37*, 491–502.

Furlong, J. C., & Lilley, D. M. J. (1986) *Nucleic Acids Res. 14*, 3995–4007.

Galas, D. J., & Schmidt, A. (1978) *Nucleic Acids Res. 5*, 3157–3170.

Gamper, H. H., & Hearst, J. E. (1982) *Cell 29*, 81–90.

Herr, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8009–8013.

Herr, W., Corbin, V., & Gilbert, W. (1982) *Nucleic Acids Res. 10*, 6931–6944.

Johnsrud, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5314–5318.

Johnston, B. H., & Rich, A. (1985) *Cell 42*, 713–724.

Kirkegaard, K., Buc, H., Spassky, A., & Wang, J. C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2544–2548.

Leonard, J., McDonald, J. J., Henderson, R. E. L., & Reichman, M. E. (1971) *Biochemistry 10*, 3335–3342.

Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344–1352.

Lutter, L. C. (1978) *J. Mol. Biol. 124*, 391–420.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.

McClure, W. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6381–6385.

Mendel, D., & Dervan, P. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 940–914.

Nordheim, A. (1984) in *The Impact of Gene Transfer Techniques in Eukaryotic Cell Biology*, Springer-Verlag, Berlin and Heidelberg.

Reisbig, R. R., Woody, A. Y. M., & Woody, R. W. (1979) *J. Biol. Chem. 254*, 11208–11217.

Roe, J. H., Burgess, R. R., & Record, M. T., Jr. (1984) *J. Mol. Biol. 176*, 495–521.

Runkel, L., & Nordheim, A. (1986) *Nucleic Acids Res. 14*, 7143–7158.

Schaeffer, F., Kolb, A., & Buc, H. (1982) *EMBO J. 1*, 99–105.

Scholten, P. M., & Nordheim, A. (1986) *Nucleic Acids Res. 10*, 3981–3993.

Siebenlist, U., & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 122–126.

Siebenlist, U., Simpson, R. B., & Gilbert, W. (1980) *Cell 20*, 269–281.

Spassky, A. (1986) *J. Mol. Biol. 188*, 99–103.

Spassky, A., Kirkegaard, K., & Buc, H. (1985) *Biochemistry 24*, 2723–2731.

Straney, D. C., & Crothers, D. M. (1987) *Biochemistry 26*, 5063–5070.

Vincze, A., Henderson, R. E. L., McDonald, J. J., & Leonard, N. J. (1973) *J. Am. Chem. Soc. 95*, 2677–2682.

Wang, J. C., Jacobsen, J. H., & Saucier, J. M. (1977) *Nucleic Acids Res. 4*, 1225–1241.

Yager, T. D., & von Hippel, P. H. (1987) in *E. coli and S. typhimurum: Cellular and Molecular Biology* (Neidhart, F. C., Ed.) pp 1241–1275, American Society of Microbiology, Washington, DC.