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Changes in the DNA Structure of the *lac* UV5 Promoter during Formation of an Open Complex with *Escherichia coli* RNA Polymerase†

A. Spassky,‡ K. Kirkegaard,§ and H. Buc*,‡

Département de Biologie Moléculaire, Institut Pasteur, 75724 Paris Cedex 15, France, and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT: By chemical and enzymatic methods, two stable complexes between *Escherichia coli* RNA polymerase and a linear DNA fragment carrying the *lac* UV5 promoter have been identified. In these binary complexes, DNA can adopt two alternate conformations as a function of temperature. Contacts between RNA polymerase and the DNA phosphate backbone are indistinguishable in these two forms, as revealed by probing with pancreatic DNase I. Protection of enhancement of the reactivity of the bases toward $(CH_3)_2SO_4$ occurs, however, only in the form that predominates above 22 °C, RP_o. The form stable at low temperature, RP_i, is a "closed" complex since no single-stranded region is detectable in the DNA. The strong temperature dependence of the equilibrium constant, the midpoint value of the transition, and the rate of conversion between these two forms are in close agreement with a series of measurements performed by using a transcriptional assay and reported in the preceding paper [Buc, H., & McClure, W. R. (1985) *Biochemistry* (preceding paper in this issue)]. These data further support the postulated mechanism of open complex formation involving three sequential steps: $R + P \rightleftarrows RP_c \rightleftarrows RP_i \rightleftarrows RP_o$. The binary complex RP_c, which accumulates transiently at 37 °C before the isomerization leading to open complex formation, is not significantly protected against enzymatic cleavage or chemical modification and is therefore distinct from RP_i and RP_o. When considered in conjunction with the results of the kinetic studies, these characteristics of the three binary complexes, RP_c, RP_i, and RP_o, imply that the second step ($RP_c \rightarrow RP_i$) corresponds to the acquisition of a stable footprint and the last isomerization to the formation of single-stranded regions. The DNA structure of the UV5 promoter is further modified when substrates are added, following formation of the open complex: although the DNase I footprint is only slightly modified during the synthesis of the 5′-terminal tetranucleotide ApApUpU, the single-stranded region revealed by dimethyl sulfate reactivity moves downstream, and the methylation of a distal guanine at position −32 is affected.

The rate of formation of an active complex between *Escherichia coli* RNA polymerase and a DNA fragment containing a promoter sequence governs the frequency of initiation of synthesis of the corresponding messenger. This frequency defines the strength of the promoter (Seeburg et al., 1977; von Gabain & Bujard, 1977, 1979). Extensive studies have been performed to characterize the structure of the DNA in the active complex. One of the best known examples is the *lac* UV5 promoter [cf. Galas & Schmitz (1978), Johnsrud (1978), Simpson (1979), Siebenlist et al. (1980), and Chenchick et al. (1981)]. In this case, we know that the active complex RP_o is "open" because a well-defined region downstream of the Pribnow box is single stranded (Siebenlist et al., 1980; Kirkegaard et al., 1983) and because the promoter is topologically unwound.

In the preceding paper (Buc & McClure, 1985), we have explored the use of the abortive initiation assay devised by Johnston & McClure (1976) to investigate the kinetics of open complex formation. These studies led us to propose this multistep scheme:

$$R + P \rightleftarrows RP_c \rightleftarrows RP_i \rightleftarrows RP_o$$

The existence of a new species, RP_i, was postulated because the kinetically competent species can be rapidly and reversibly inactivated by lowering the temperature; this new binary complex, unlike RP_c, remains resistant to poly(deoxyadenylate-thymidylate) [poly[d(A-T)]] challenge at these lower temperatures. The location of RP_i in the pathway accounts well for the observed temperature dependence of all the kinetic constants.

In the work presented here, we have tried to characterize the reactivity of the promoter DNA in these different binary complexes as well as in ternary complexes formed after addition of the two substrates ApA and uridine 5′-triphosphate (UTP). First, independent support for the existence of the additional complex RP_i was obtained by monitoring the change in DNA reactivity in the temperature range where the two conformations RP_i and RP_o are postulated to occur. Second, we adapted our methods to kinetic studies and looked for structural changes occurring before the establishment of the

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‡ Institut Pasteur.
§ Massachusetts Institute of Technology.

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open complex at 37 °C, under conditions where the closed complex RP_c was predominant. Last, we studied the effect of substrate addition on the structure of the active complex at 37 °C.

Materials and Methods

E. coli RNA polymerase was prepared according to the procedure of Burgess & Jendrisak (1975) modified as indicated by Lowe et al. (1979). The 203-bp[1] *lac* UV5 fragment containing the *lac* UV5 promoter sequence from position −140 to position +63 was isolated from a pBR322 derivative (Schaeffer et al., 1982). DNA fragments were isolated by electroelution and labeled by ^{32}P at the 3′ or the 5′ end according to Maxam & Gilbert (1980). Singly end-labeled promoter DNA was prepared by digesting the ^{32}P-end-labeled 203-bp fragment with *Pvu*II. The 16-bp *Eco*RI–*Pvu*II fragment was not removed from the mixture.

DNase I "footprinting" was performed according to Galas & Schmitz (1978), and "G" dimethyl sulfate reactions were performed as described by Maxam & Gilbert (1980). In each case, preliminary assays were carried out to ascertain that the experiments were performed under conditions where single-hit attack of the fragment prevails (more that 63% of the fragment being intact). Moreover, several reagent concentrations or several reaction times were used. Experiments to detect single-stranded cytosines have been described by Kirkegaard et al. (1983).

Experiments performed at equilibrium were conducted as follows: 1–4 nM labeled UV5 fragment (20 000–40 000 cpm) was incubated in 25 mM Hepes, pH 8, 100 mM KCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 100 μg/mL BSA; 180 nM RNA polymerase was added. The mixture was incubated for 15 min in a water bath regulated within ±0.3 °C. At the required time the reagent was added, under gentle stirring. These conditions are strictly similar to the ones used for enzymatic assays and are reported in Buc & McClure (1985). The temperature range was from 37 to 14 °C. Below 14 °C the kinetics are complex due to the occurrence of slow rearrangements. Care was also taken to avoid long incubation times between RNA polymerase and DNA in the low-temperature range.

Usually, nucleolytic attacks were carried out with 75 ng/mL DNase I (for 18 s at 37 °C or 45 s at 14 °C). G reactions were performed with 50–200 mM dimethyl sulfate for 1 min. Single-stranded cytosines were best detected after reaction with 200–400 mM dimethyl sulfate, depending on the temperature, for a reaction time of 2 min. Experiments involving the substrates were performed in the presence of 500 μM ApA and/or 50 μM UTP. It was checked that, under our experimental conditions, $(CH_3)_2SO_4$ attack did not appreciably affect the stability of the complex: After methylation at 37 or 19 °C, the complex was probed by pancreatic DNase. In these cases, the usual pattern of protection and enhancement against the second reagent was observed. Electrophoresis through sequencing gels was performed as described by Maxam & Gilbert (1980). The gels were then autoradiographed, and the autoradiograms were analyzed by densitometry (cf. below).

Kinetic Experiments at 37 °C. At time zero, RNA polymerase and DNA were mixed in the Hepes buffer described above (final volume, 20 μL). After a given time of incubation, 2 μL of reagent [either $(CH_3)_2SO_4$ or DNase] was added and the incubation allowed to proceed for 5 s more. Then, the reactions were stopped with 200 μL of the appropriate quenching solution, and 4 s later, 200 μL of redistilled phenol was added. For the footprinting experiments, two concentrations of DNase I were used (1.5 and 6.0 μg/mL), and the reactions were stopped by adding 200 μL of 2.5 M ammonium acetate, 20 mM ethylenediaminetetraacetic acid (EDTA), and 10 μg/mL DNA. For the $(CH_3)_2SO_4$ reactions, two concentrations of dimethyl sulfate (50 and 200 mM final) were applied, the quenching mixture in this case being composed of 0.7 M sodium acetate, 0.5 M 2-mercaptoethanol, and 50 μg/mL tRNA. DNA samples were then processed as described above. Essentially the same procedure was used for the temperature-shift experiments from 37 to 19 °C.

Quantitative Evaluation of the Reactivity of Different Residues. Films corresponding to the various assays were scanned on a Vernon photodensitometer. Scans fulfilling the conditions of single-hit kinetics were selected. Whenever the migration was not exactly the same in each lane, a linear interpolation was applied to put all the bands in each lane in register. Intensities of the different bands were then compared. In the case of DNase attack, the area associated with unperturbed regions of the DNA upstream of position −60 or downstream of position +20 was integrated, averaged, and compared from one lane to the next. The same proportionality factor was then applied to the area of all the peaks present in a given lane yielding profiles analogous to the ones given in Figure 5. For samples reacted with dimethyl sulfate, the process was simplified since few bands show a differential reactivity in the presence of RNA polymerase (Johnsrud, 1978). The reactivity of guanine and cytosine residues in the region between positions −10 and +10 was evaluated, taking as reference unperturbed bands corresponding to guanines −20, −19, −16, and +10. Similarly, guanine −30 was used as a reference to evaluate the reactivity of guanine −32.

Sequences are numbered from the transcription start point of the promoter. Sequences downstream of this point are labeled with a "+" prefix, and sequences upstream are labeled with a "−" prefix.

Results

Phosphate Backbone: Protection against DNase Attack at Various Temperatures. A 203-bp fragment containing the *lac* UV5 promoter was labeled either at its 5′ end or at its 3′ end and cut by *Pvu*II restriction enzyme. The labeled fragment was incubated at concentrations of 1–4 nM with a large excess of *E. coli* RNA polymerase in the temperature range relevant to kinetic results and then digested with pancreatic DNase I under conditions yielding single-hit kinetics. Experiments presented in Figure 1 indicate that the pattern of protection did not qualitatively change as the temperature was lowered from 37 to 14 °C. Regions corresponding to an enhancement of reactivity due to RNA polymerase binding were also conserved. This similarity of pattern has been confirmed by densitometric scans of these data. The only significant effect due to incubation at low temperature was that the concentration of RNA polymerase required to reach full protection was increased, a result expected from the temperature dependence of the association constant between RNA polymerase and *lac* UV5 [cf. Buc & McClure (1985)]. Footprint analysis with pancreatic DNase I thus indicates that RNA polymerase, bound to the UV5 promoter, remains positioned in the same manner with respect to the phosphate backbone of the DNA as the temperature is lowered from 37 to 14 °C.

[1] Abbreviations: bp, base pair; BSA, bovine serum albumin, DNase I, pancreatic deoxyribonuclease I; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

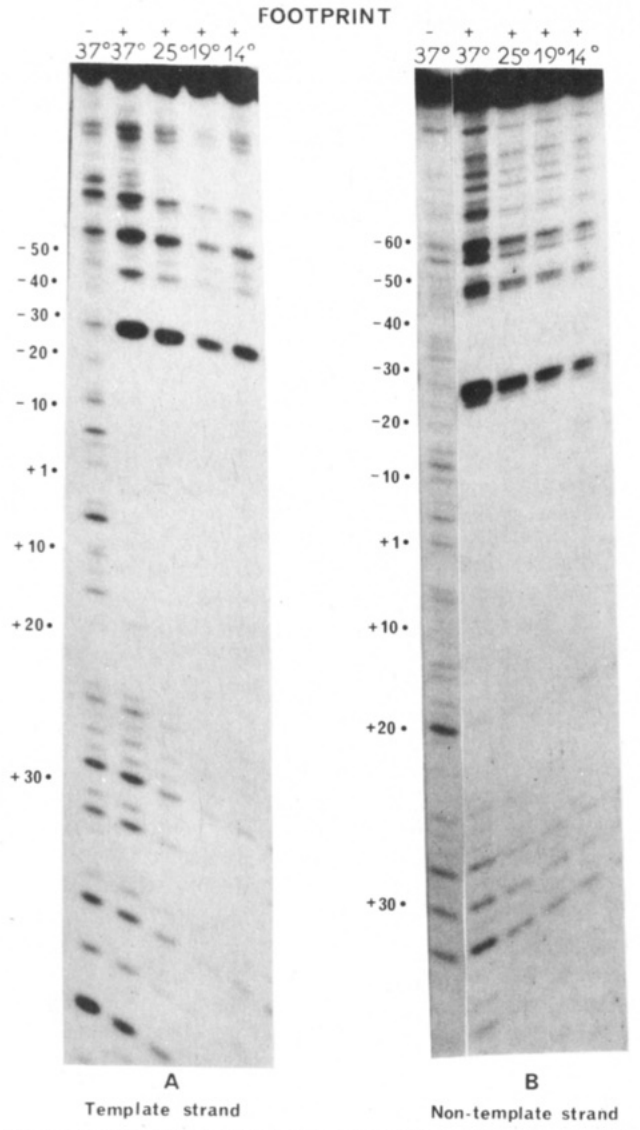

FIGURE 1: Protection by RNA polymerase of the UV5 lactose control region against DNase I attack at various temperatures. Conditions are as described under Materials and Methods (4 nM labeled UV5 fragment, 180 nM RNA polymerase). (Panel A) Template strand labeled at its 5′ end by polynucleotide kinase and [γ-^{32}P]ATP; (panel B) nontemplate strand labeled at its 3′ end by the Klenow fragment and [α-^{32}P]dATP. In each panel, the first lane corresponds to the attack of an unprotected fragment at 37 °C. No significant difference in this control is seen when the attack is performed at lower temperatures.

Contact with Bases: Protection against $(CH_3)_2SO_4$ Attack at Various Temperatures. At 37 or 29 °C, binding of RNA polymerase at the UV5 promoter resulted in well-defined perturbations of the reactivities of guanine and adenine residues with dimethyl sulfate [cf. Johnsrud (1978)]. On the nontemplate strand, the reactivity of the N7 groups of guanines −1 and −17 (and possibly −38) was specifically enhanced. N3 adenine residues at positions −26 and −33 also have their reactivity increased. Change in the reactivity pattern in the template strand is also evident: increased reactivity of guanine −14 and decreased reactivity of guanine −32 (cf. Figure 2). Several other changes reported by Johnsrud using a slightly different technique and another buffer were not found to occur reproducibly.

When parallel experiments were performed at 19 or 14 °C, the perturbations observed at 37 °C were not observed, even though different dimethyl sulfate concentrations (50–200 mM) or reaction times (1 or 2 min) were used. The loss of differential reactivity of dimethyl sulfate for its targets was not due to a failure of the reagent to probe the DNA–protein complex at low temperature: we found that the "tac" promoter, a hybrid promoter that melts 3 °C below the UV5 promoter in the presence of RNA polymerase, exhibited a differential reactivity with respect to dimethyl sulfate at temperatures as low as 17 °C [cf. Kirkegaard et al. (1983)].

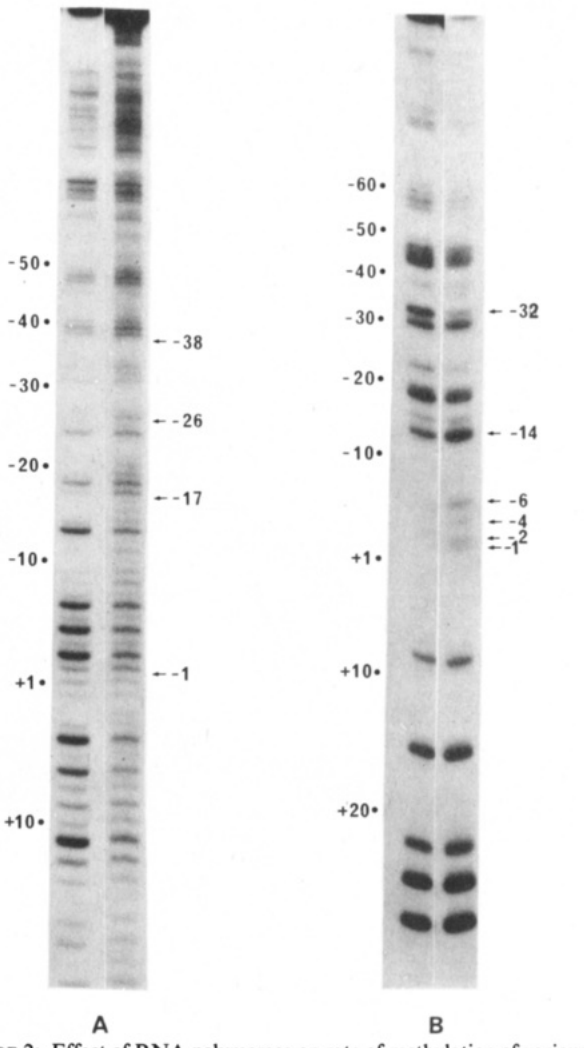

FIGURE 2: Effect of RNA polymerase on rate of methylation of various bases of the UV5 lactose control region by dimethyl sulfate at 37 °C. (A) Nontemplate strand; (B) template strand. Lanes 1 and 3 correspond to uncomplexed DNA fragments. When the temperature is decreased to 14 °C, fragments complexed with RNA polymerase exhibit a reactivity pattern undistinguishable from an unprotected fragment (which behaves as in lane 1 or in lane 3) (see Figure 3).

We reasoned therefore that the loss in the differential reactivity of the methylating reagent for DNA reflected a conformational change in the structure of the *lac* UV5 RNA polymerase complex. In order to strengthen this point, we examined the effect of temperature on the exposure of the N7 residue of guanine −14, the protection of the same group at guanine −32, and the increased reactivities of cytosines −6, −4, −2, and −1 on the template strand of the complex. As explained under Materials and Methods and in the legend of Figure 3, the extent of methylation of those residues was calibrated by comparison with the reactiviy of other residues of the *lac* UV5 promoter sequence that are unaffected by the binding of RNA polymerase. Figure 3 shows the experimental results (full protection corresponds to a value of 0 and maximal reactivity to a value of 1 on the ordinate). In each case, the loss of differential reactivity occurred in the same narrow temperature range (14–29 °C). These results were compared

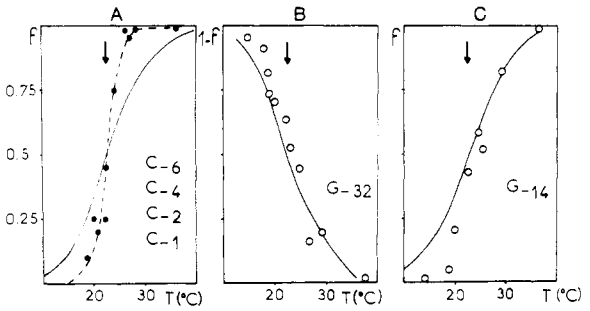

FIGURE 3: Loss of differential reactivity of various bases of the template strand toward methylation as temperature is lowered. The lactose UV5 control region was complexed with RNA polymerase at various temperatures. The reactivity of bases −6, −4, −2, −1, −14, and −30 was monitored in the presence and in the absence of RNA polymerase. (Panel A) The reactivity of cytosines −6, −4, −2, and −1 is compared to the rate of methylation of guanine residues −16 and +10 taken as internal standards. Above 30 °C this ratio reaches a plateau. By convention, the fraction of reactive cytosine f_{max} is equal to 1 under these conditions. This proportionality factor then allows definition of the fraction f of reactive cytosines at each temperature (●). The dashed line corresponds to the predicted value of f for an equilibrium between two forms of the complex (RP_i having no reactive cytosines and RP_o containing the fully reactive cytosines) with T_m = 22.5 °C and ΔH = +120 kcal/mol. The solid line corresponds to the fraction f of active complex deduced from the enzymatic assay [cf. Buc & McClure (1985)] with T_m = 22.5 °C and ΔH = +41 kcal/mol. (Panel B) The reactivity of guanine −32 is compared to the reactivity of guanine −30 at each temperature. In the unprotected fragment the ratio of the area of the corresponding bands is temperature independent and equal to 1.12, a value found also at 15 °C in the presence of RNA polymerase. For the other temperatures, the corresponding ratio r is lower. At 42 °C, guanine −32 is fully protected. $r/1.12$ was taken as a measure of the probability for guanine −32 to be exposed to methylation. This quantity is plotted as a function of temperature (○). The solid line corresponds to the fraction $1-f$ of the RP_i complex present in this temperature range, as deduced from the enzymatic assay (cf. above). (Panel C) The reactivity of guanine −14 is compared to those of guanines −20, −19, −16, and +10, when complexed by RNA polymerase at 37 °C. A procedure similar to the ones described above was used to deduce, from the reactivity of these bases, the probability for guanine −14 to be hyperreactive at each temperature (○). As in (B), the curve represents the fraction f of complex active in the transcription assay.

with the data obtained from the enzymatic assay, which provides the fraction f of active complex RP_o among the stable species (RP_i + RP_o) (Buc & McClure, 1985). It can be seen in Figure 3 that these data are internally consistent (the solid lines drawn on each panel represent the results of the enzymatic assay; the points correspond to the present data). In particular, the midpoint of the transition was consistently 22–23 °C.

We conclude that an equilibrium between two states, RP_i and RP_o, accounts for the present results. These two complexes display identical reactivity to DNase I but different reactivities to dimethyl sulfate, significant enhancements or protections against methylation being observed in the open complex only. Formation of the transcriptionally active binary complex, RP_o, occurs over a very narrow temperature range and is accompanied by changes in the DNA structure of the promoter extending from position −32 to position −1.

Kinetic Assay: Shift from 37 to 19 °C. The static assays reported above differentiate the species RP_i and RP_o by the reactivity of specific bases in the promoter region. The two species RP_i and RP_o appear to be "closed" and "open", respectively, according to the reactivity of the N3 positions of cytosines −6, −4, −2, and −1. In the preceding paper (Buc & McClure, 1985), a major argument to distinguish this closed species RP_i from the transient intermediate RP_c and to order the pathway was that the rate of conversion from RP_o to RP_i is relatively fast at 19 °C (about 1 min^{-1}) whereas dissociation of RP_o or RP_i into RP_c takes place in the hour range. The kinetics of the conformational interconversion were further examined in the present study by using DNase I and $(CH_3)_2SO_4$. RNA polymerase was first incubated with the *lac* UV5 promoter at 37 °C for a time sufficiently long to form an open complex. This mixture was then quickly diluted into a solution thermostated at 19 °C, and pancreatic DNase I or $(CH_3)_2SO_4$ was added at different times after the temperature shift.

The experiment in Figure 4 shows that the DNase footprint remained the same in a time interval ranging from 30 s to 30 min after the temperature perturbation (cf. Figure 4A). However, the four crucial cytosines became protected against $(CH_3)_2SO_4$ attack at times too short to be determined by our method (dead time, 3 min; results not shown). The change in the reactivity of guanine residues −14 and −32 was less rapid; however, the kinetic process could be recorded only at the end and with a poor accuracy (Figure 4B). This rate of conversion is compatible with a time constant on the order of 1 min. The transition from RP_o to RP_i thus takes place in the time range expected from the enzymatic measurements.

Search for an Intermediate at 37 °C. As shown in the preceding paper, the pathway leading to formation of the open complex, RP_o, is simpler at 37 °C and can be expressed as

$$R + P \underset{}{\overset{K_1}{\rightleftharpoons}} RP_c \xrightarrow{k_2} RP_o$$

When the concentration of RNA polymerase is large enough, the first equilibrium is displaced toward the formation of RP_c. Under these conditions, one follows the isomerization of RP_c into RP_o (time constant, k_2^{-1}). The values of K_1 and k_2, as measured with the abortive initiation assay, are 160 μM^{-1} and 0.1 s^{-1}, respectively; the mixing of 100 nM RNA polymerase and 4 nM DNA fragment would thus result in the immediate formation of the species RP_c. This species, which should initially represent 95% of the total promoter concentration, is then irreversibly converted within 40 s into the active species RP_o.

The results presented in Figure 5A compare the DNase protection patterns obtained for the free promoter, for the final open complex, and for a sample taken after a short incubation (5 s, followed by 5 s of nuclease attack). The short incubation yielded a pattern that was intermediate between free promoter and final complex for all the phosphodiester bonds examined. From the intensities of the bands in the footprint patterns, it is possible to estimate the frequency of DNase cleavage at each phosphodiester bond, under the three experimental conditions. Within experimental error one finds that the patterns observed are those anticipated from a 2:1 mixture of open complex relative to free DNA. This result was confirmed by comparing the intermediate pattern with the scan of a reconstituted 2:1 mixture (Figure 5B). The two scans were identical, within experimental error, except perhaps between base pairs −45 and −52. It can be concluded from these data that no region of the promoter DNA is strongly protected against or exposed to DNase attack in the RP_c complex. If this were the case, the corresponding footprint would be present at the early times and the intermediate tracing given on Figure 5A would be distinguishable from a mixture of free DNA and promoter DNA engaged in an open complex with RNA polymerase.

Those results can also be compared with those expected from the enzymatic studies. From the corresponding kinetic constants one can calculate the amount of open complex, closed complex, and free promoter that would be present under conditions corresponding to the intermediate pattern. This

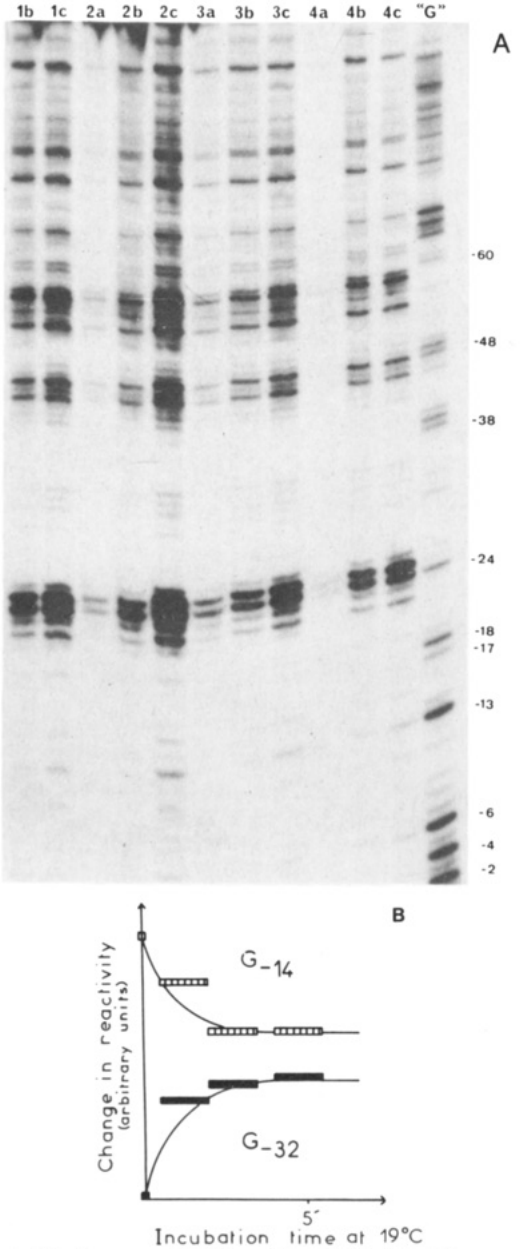

FIGURE 4: Kinetic assay: shift from 37 to 19 °C of the binary complex between RNA polymerase and the *lac* UV5 promoter. (Panel A) Absence of a transient change in the protection pattern against DNase I during the shift. DNA was labeled on the nontemplate strand; RNA polymerase (1.2 μM) was incubated with 40 nM UV5 promoter at 37 °C for 5 min. At time zero this mixture was quickly diluted in a 10-fold excess of buffer preequilibrated at 19 °C. After incubation for a time t, an aliquot of the mixture was attacked by DNase I for 6 s, the temperature being maintained at 19 °C. Incubation times were respectively 5 s (samples 1), 30 s (samples 2), 1 min (samples 3), or 5 min (samples 4). a, b, and c correspond to three different DNase concentrations. The left lane is a sequencing "G" assay. After scanning, all the footprints appear identical. In an independent transcription assay the interconversion rate constant between the active and the inactive form of the complex was found equal to 1 min^{-1}. (Panel B) Disappearance of the pattern of differential reactivity of the bases. DNA was labeled on the template strand. After formation of the open complex at 37 °C with 200 nM RNA polymerase, the complex was brought to 19 °C, incubated for a time t, and labeled for 90 s with $(CH_3)_2SO_4$. Reactivities of guanine residues −14 and −32 were measured as explained in the legend of Figure 3. The reactivity of guanine −32 relative to guanine −30, r, increases and reaches the equilibrium value given in Figure 3B, 0.86, while the relative reactivity of guanine −14 decreases from 1 to 0.70, in agreement with the results shown in Figure 3. The curve is the theoretical time course expected for an interconversion rate constant of 1 min^{-1} obtained from an independent measurement (burst experiment performed under similar conditions; Buc & McClure, 1985).

calculation yields 55% open complex, 42% closed complex, and 3% free DNA. We thus conclude that the digestion pattern of the closed complex is not appreciably different from that given by free promoter, differences observed in the intermediate scan being wholly accounted for by the amount of open complex present. Chemical attacks performed with dimethyl sulfate under analogous conditions failed also to reveal a pattern of reactivity for the closed complex distinct from free promoter (data not shown).

In summary, chemical and enzymatic attacks of the RNA polymerase *lac* UV5 complex performed at 37 °C during the period of latency for production of the abortive transcript reveal the formation of the open complex. The different regions of the promoter evolve at indistinguishable rates, the overall rate being that predicted from the enzymatic assay. Within the limited accuracy of those methods, the intermediate complex RP_c cannot be distinguished from free promoter by these two probes. It is clearly distinct from RP_i.

Effect of Substrate Addition on the Structure of Promoter in the Open Complex. At the *lac* UV5 promoter the 5′ end of the message is ApApUpU; formation of ternary complexes by substrate addition can thus be described by the scheme:

where RP_{1-2} refers to the complex formed with ApA and RP_{1-4} to the complex that produces the abortive transcript ApApUpU.

Contacts of RNA polymerase with the phosphodiester backbone were probed by DNase treatment at 37 °C in the presence of either ApA or UTP, or both, at concentrations of 500 and 50 μM, respectively. ApA and UTP addition produced a slight modification of the footprint (data not shown). The reactivity of the bases, however, was drastically modified when ApA and UTP were added together (generating RP_{1-4}) (cf. Figure 6). A new cytosine residue was now present in the single-stranded region, cytosine +5, located 1 bp downstream of the region of the template that is effectively transcribed. In addition, the protection of guanine −32 due to RNA polymerase binding was less marked than in the absence of substrate addition. This last feature could reflect a greater flexibility of the DNA template when translocation takes place.

Discussion

The conclusions of this study arise from the combined use of two reagents, dimethyl sulfate and DNase I, as probes for the DNA structure of various RNA polymerase–*lac* UV5 promoter complexes. This methodology relies heavily on previous enzymatic studies, performed under the same conditions. The results of the abortive transcription assays allowed us to define the appropriate range of RNA polymerase and substrate concentrations, as well as the temperature and the time scales, to be employed for the structural analyses.

Our basic findings are as follows: (a) At 37 °C, only one binary complex is clearly detectable by deoxyribonuclease or dimethyl sulfate probing. The rate of accumulation of this complex corresponds to the rate of formation of kinetically competent species at this temperature. This is the case even when the RNA polymerase concentration initially added to the DNA fragment was sufficient to cause an initial accumulation of the closed complex RP_c. The formation of this binary complex does not lead to a footprint clearly distinct from the pattern observed with free DNA or to marked changes in the reactivity of bases. (However, we cannot rule

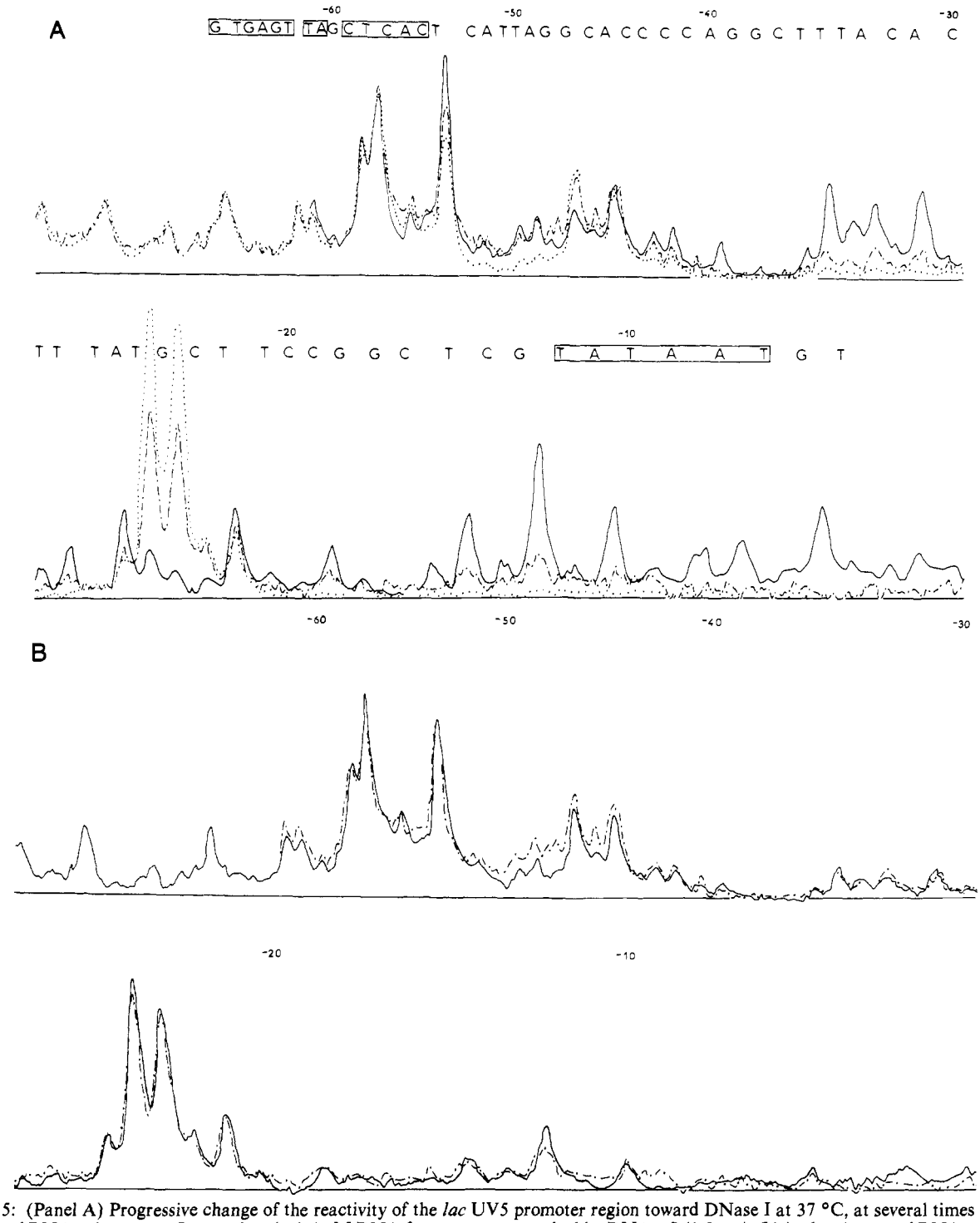

FIGURE 5: (Panel A) Progressive change of the reactivity of the *lac* UV5 promoter region toward DNase I at 37 °C, at several times following addition of RNA polymerase. In sample a (—) 4 nM DNA fragment was attacked by DNAse I (1.5 μg/mL) in the absence of RNA polymerase for 5 s. In sample b (···) 4 nM DNA fragment was mixed for 6 min with 100 nM RNA polymerase and then exposed to DNase I for 5 s. In sample c (–·–) the conditions were similar to sample b except that the incubation time with RNA polymerase was 5 s followed by 5 s of exposure to DNase I. These samples as well as others where the concentration of DNase I was increased to 6 μg/mL were then submitted to electrophoresis and autoradiographed. The films were scanned and the data processed as explained under Materials and Methods. The DNA sequence corresponding to the strand that was labeled has been put in register with the pattern. (Panel B) Comparison of the scan corresponding to sample c above (–·–) with a scan of a 2:1 mixture of samples b and a above (—).

out that very minor enhancements in nucleolytic attack could take place around positions −45 and −52.) This negative finding is consistent with results obtained in similar experiments performed with other promoters for which the rate of open complex formation is slower and where it is therefore easier to probe the transient phase preceding open complex formation. For example, analogous studies performed with wild-type lactose and galactose promoters, in the presence or in the absence of the complex formed between cAMP and its receptor protein (CRP), have shown that the establishment of a characteristic footprint parallels the formation of species that are heparin resistant and catalytically active (Spassky et al., 1984).

(b) At lower temperature, a complex different from RP_o or RP_c is present. This species differs sharply from the open complex. Cytosine residues, present in the single-stranded region, are inaccessible to the dimethyl sulfate reagent, presumably because the corresponding DNA segment has rean-

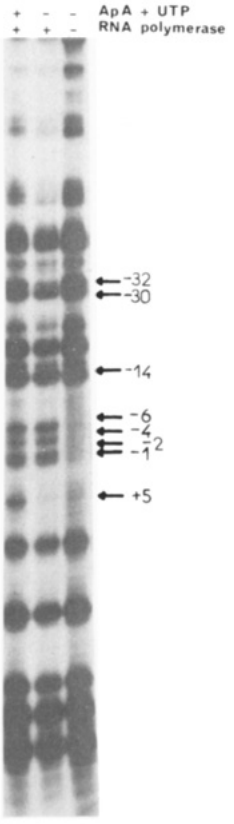

FIGURE 6: Effect of substrate addition. Comparison of the reactivity of G and C bases with respect to dimethyl sulfate at 37 °C. (Right lane) DNA alone; (middle lane) DNA (1 nM) + RNA polymerase (150 nM); (left lane) DNA (1 nM) + RNA polymerase (150 nM) + ApA (500 μM) + UTP (50 μM).

nealed. The pattern of differential reactivity of all the guanine and adenine residues examined is also lost in the same narrow temperature interval. However, the positioning of RNA polymerase with respect to the DNA backbone, as monitored by the DNase I pattern, remains unchanged. This species RP_i has been shown to be inactive in the enzymatic assay and to be resistant to poly[d(A-T)] challenge [cf. Buc & McClure (1985)]. We conclude that the different protection patterns reflect two alternate modes of binding of RNA polymerase to the same DNA region of the promoter.

The new species, RP_i, appears also to be distinct from RP_c: RP_i gives rise to marked footprint and is resistant to poly[d(A-T)] challenge, properties not exhibited by RP_c. RP_i cannot be a binary complex basically identical with RP_c but "frozen" by the change in temperature at which it is monitored (19 °C instead of 37 °C). This conclusion arises from the very different rates at which the active complex RP_o can be converted into the two closed complexes at 19 °C. At 19 °C the transition from RP_o to RP_i is fast (cf. Kinetic Assay: Shift from 37 to 19 °C under Results) while the formation of poly[d(A-T)]-sensitive species is slow. These experiments thus support the conclusion drawn in the previous paper that three binary complexes, RP_c, RP_i, and RP_o, should be taken into account to explain the kinetics of open complex formation at the *lac* UV5 promoter in this temperature range. However, the sequential order that has been proposed still relies entirely on kinetic arguments since the present experiments could not distinguish the scheme compatible with the enzymatic results

$$RP_c \rightarrow RP_i \rightleftarrows RP_o$$

from the alternate hypothesis

$$RP_c \rightarrow RP_o \rightleftarrows RP_i$$

Results in fair agreement with ours have been reported by Becker & Wang (1984). These authors probed RNA polymerase *lac* UV5 complexes by photofootprinting at 16 and 32 °C, under conditions identical with ours. They found that binding of RNA polymerase resulted in a similar reduction of photoproduct formation at both temperatures, with the exception of five photoproducts that were specifically enhanced at 32 °C. These products were shown to arise from bases located in the single-stranded region of the RP_o complex.

(c) The structure of the open complex is further modified when substrates are added. In agreement with Carpousis (1983), we have found that the initiation complex and the original open complex have analogous footprints, demonstrating that the contacts with the phosphate backbone are maintained. The use of dimethyl sulfate reveals however that guanine and cytosine contacts are markedly affected. Specifically, the addition of two uridine residues to the nascent RNA transcript at positions +3 and +4 triggers the opening at position +5. During this process, RNA polymerase, while maintaining a close contact with the DNA duplex in the −40 region, exposes guanine residue −32 to methylation. This method thus offers the opportunity to detect the stepwise unwinding of G-C base pairs as transcription proceeds and to correlate the results with unwinding measurements [cf. Gumper & Hearst (1982)]. It is clear from the present findings that a significant translocation of the enzyme on this template takes place during the first elongation steps (Figure 7).

Three general features of the interaction leading to the formation of an open complex can be deduced from these studies. First, among the structural changes that characterize the formation of an open complex at the *lac* UV5 promoter, we have been able to distinguish those that correspond to a correct positioning of the enzyme on the DNA template (the DNase I footprint) from those that are associated with formation of a catalytically active complex [presence of a single-stranded region or differential reactivity of other base residues with respect to $(CH_3)_2SO_4$]. The pattern obtained at low temperature indicates that a correct positioning can be observed in a stable binary complex that lacks activity and that does not exhibit a reactivity to $(CH_3)_2SO_4$. Second, the equilibrium between the two forms is very temperature dependent. The formation of RP_o is endothermic (+41 kcal/mol), and the transition from the inactive to the active binary complex must therefore be driven by entropy. Third, the process is cooperative: the whole DNA region that interacts with the enzyme in both complexes is coordinately affected in the same narrow temperature interval. These three features suggest that localized strand separation must be coupled with a marked transconformation of the enzyme–DNA binary complex.

The methodology employed in these experiments provided a very useful adjunct to the enzymatic studies. In addition to permitting independent verification of the kinetic scheme, the structural analyses allow the detection of stepwise changes in the DNA structure of the promoter during formation of the initiation complex. On the basis of results obtained with this approach, we have elaborated a mechanism leading to open complex formation. This model is shown in Figure 8. We have adopted the sequential scheme proposed in the introduction, and we have included relevant observations published elsewhere. Nothing is known about the closed complex RP_c except that in this complex the DNA is already partially unwound. This is indicated by the strong dependence of the corresponding association constant, K_1, on the superhelical

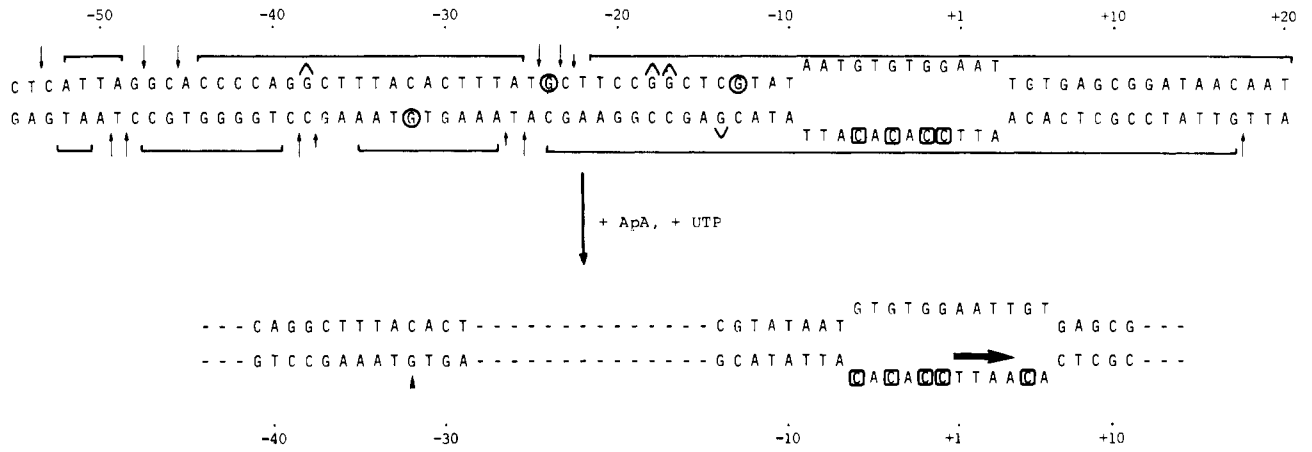

FIGURE 7: RNA polymerase contacts with the *lac* UV5 promoter in the open complex and in the initiation complex. DNase I sensitivity: Arrows indicate an increased sensitivity of the phosphodiester bond to DNase I, and a straight line indicates a marked protection. Dimethyl sulfate attack: A circle or a caret indicates a purine that the polymerase protects from or enhances to dimethyl sulfate attack, respectively; a box indicates a cytosine that becomes reactive after polymerase binding. (Top) The open complex before substrate addition. The location of the single-stranded region is the one given by Siebenlist et al. (Bottom) The initiation complex after addition of ApA and UTP (same symbols as above). The horizontal arrow represents the product of the abortive initiation assay, ApApUpU. The vertical arrow points to the guanine that recovers the same reactivity as in unprotected DNA, though the DNase footprint is maintained. We have assumed that the size of the unwound region is maintained [cf. Gamper & Hearst (1982)] and translocated the single-stranded region accordingly.

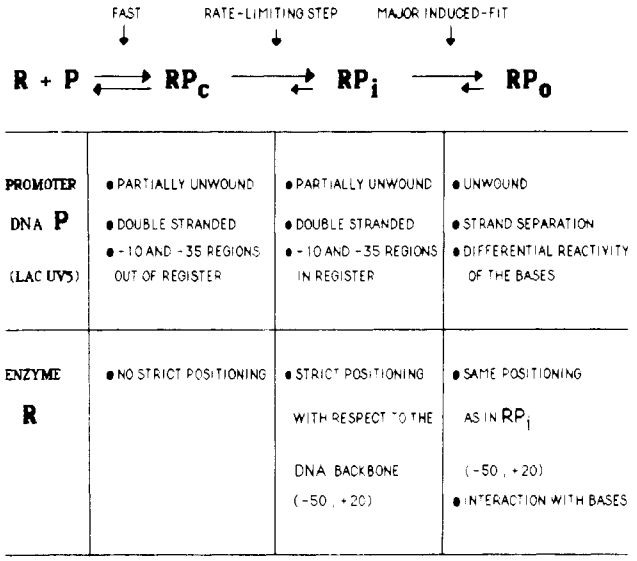

FIGURE 8: A plausible scheme for open complex formation in the case of the linear UV5 *lac* promoter (see text).

density of the template [cf. Malan et al. (1984)]. As seen above, this mode of binding of RNA polymerase does not cause a marked localized footprint. We propose that the interaction between the promoter DNA and the different protein domains of the enzyme is not yet optimized. The second step leads to a permanent and tight interaction between the promoter DNA and the enzyme. RNA polymerase is then stably positioned with respect to the DNA sequence. As the footprint against DNase, in the RP_i complex, extends from position −50 to position +20, we assume that this step serves essentially to bring the −35 and −10 regions into register. Interactions between amino acid side chains and base residues present in the major groove are not optimal in this complex. The last isomerization is fast at 37 °C. The passage from RP_i to RP_o implies a cooperative transconformation of the enzyme; the DNA grooves are penetrated by the protein, and the double helix is affected at least from position −38 to position +4. In particular, strand separation takes place around position +1, yielding an active complex. We have argued in the preceding paper that the rate-limiting step corresponds to the passage from RP_c to RP_i. We now assign this step to a strict positioning of the −35 and −10 regions with respect to each other. If this is the case, *lac* promoters should be very sensitive to alterations in spacing between these regions, particularly since the distance between the two canonical regions is 18 bp instead of the 17 bp found in most other promoters. Indeed, Stefano & Gralla (1982) have shown that the strength of the parent *lac* p^s promoter is strongly affected when the distance between these regions is changed, and they proposed an explanation similar to the one given here. Also, an increase in the superhelical density of the template diminishes the strength of the *lac* UV5 promoter in vivo (Sanzey, 1979) and decreases the rate of isomerization k_2 in vitro (Malan et al., 1984; Buc & McClure, 1985). This unusual dependence of promoter strength on negative supercoiling could be accounted for by a similar mechanism since the crucial distance between the −35 and the −10 regions is also increased when the detorsion of the DNA double helix is increased.

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Effects of Ca^{2+} and Mg^{2+} on the Actomyosin Adenosine-5′-triphosphatase of Stably Phosphorylated Gizzard Myosin†

Richard J. Heaslip and Samuel Chacko*
Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104
Received May 29, 1984; Revised Manuscript Received October 5, 1984

ABSTRACT: There are conflicting reports on the effect of Ca^{2+} on actin activation of myosin adenosinetriphosphatase (ATPase) once the light chain is fully phosphorylated by a calcium calmodulin dependent kinase. Using thiophosphorylated gizzard myosin, Sherry et al. [Sherry, J. M. F., Gorecka, A., Aksoy, M. O., Dabrowska, R., & Hartshorne, D. J. (1978) *Biochemistry 17*, 4417–4418] observed that the actin activation of ATPase was not inhibited by the removal of Ca^{2+}. Hence, it was suggested that the regulation of actomyosin ATPase activity of gizzard myosin by calcium occurs only via phosphorylation. In the present study, phosphorylated and thiophosphorylated myosins were prepared free of kinase and phosphatase activity; hence, the ATPase activity could be measured at various concentrations of Ca^{2+} and Mg^{2+} without affecting the level of phosphorylation. The ATPase activity of myosin was activated either by skeletal muscle or by gizzard actin at various concentrations of Mg^{2+} and either at pCa 5 or at pCa 8. The activation was sensitive to Ca^{2+} at low Mg^{2+} concentrations with both actins. Tropomyosin potentiated the actin-activated ATPase activity at all Mg^{2+} and Ca^{2+} concentrations. The calcium sensitivity of phosphorylated and thiophosphorylated myosin reconstituted with actin and tropomyosin was most pronounced at a free Mg^{2+} concentration of about 3 mM. The binding of ^{125}I-tropomyosin to actin showed that the calcium sensitivity of ATPase observed at low Mg^{2+} concentration is not due to a calcium-mediated binding of tropomyosin to F-actin. The actin activation of both myosins was insensitive to Ca^{2+} when the Mg^{2+} concentration was increased above 5 mM. The data indicate that, at low Mg^{2+} concentrations, Ca^{2+} regulates the actomyosin ATPase not only by its effect on the phosphorylation of light chain by calcium calmodulin dependent kinase but also by a direct effect on the actin-activated ATP hydrolysis.

Phosphorylation of the 20 000-dalton (Da)[1] light chain of smooth muscle myosin is associated with severalfold increase in its actin-activated ATPase activity (Gorecka et al., 1976; Sobieszek, 1977; Chacko et al., 1977; Ikebe et al., 1978; Rees & Frederiksen, 1981). Phosphorylation of the light chain is catalyzed by a calcium calmodulin dependent kinase called myosin light chain kinase (Frearson & Perry, 1975; Dabrowska et al., 1978; Adelstein et al., 1978). Hence, calcium activates the actomyosin ATPase activity via its effect on the phosphorylation of the myosin light chain. Using myosin isolated from vas deferens, Chacko et al. (1977) reported that calcium regulates the actomyosin ATPase not only through its effect on myosin light chain kinase but also through a direct effect on actin-activated ATP hydrolysis. This direct effect on the actomyosin ATPase activity by Ca^{2+} was shown only for phosphorylated myosin. Subsequent studies utilizing myosins isolated from aorta (Rees & Frederiksen, 1981), pulmonary artery (Chacko & Rosenfeld, 1982) and gizzard (Nag &

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); P_i, inorganic phosphate; Da, dalton.

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