Kinetics of the specific binding of a second RNA polymerase to the standard bacterial-transposon-Tn3 *bla* promoter complex

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It was shown previously, that at moderate excess of RNA polymerase (RNAP) relative to DNA, the complex of the *bla* promoter from bacterial transposon Tn3 with RNAP is locked in an inactive, heparin-resistant, isomeric state which is not that of the 'open' complex. This 1:1 isomer can accommodate a second RNAP, which becomes tightly and specifically bound just upstream of the first RNAP [Duval-Valentin & Reiss (1990) Mol. Microbiol. 4, 1465–1475]. Both the resulting 2:1 complex and its antecedent 1:1 complex formed at excess of RNAP are immediately and permanently inhibited for transcription initiation. Using the gel-retardation technique, we investigate here the kinetics of formation and decay of the 2:1 complex under various experimental conditions. The data are consistent with pseudo-first-order kinetics at moderate excess of RNAP. The salt-dependence of rate and equilibrium constants has been analysed within the framework of the theoretical model described by Lohman, Dehaseth & Record [(1978) Biophys. Chem. 8, 281–294]. It was found that the salt-dependence is consistent with the existence of a transient intermediate during formation of the 2:1 complex, which forms rapidly on the time scale of its isomerization to the final 2:1 complex. The intermediate is characterized by the release of about seven cations from the 1:1 complex, one additional cation being released upon its final isomerization. Formation of the 2:1 complex at high excess of RNAP becomes inhibited, probably as a result of a 'bumping' effect of the complex by the enzyme, also observed with several other promoters. We conclude that formation of the 2:1 complex closely mimics that of the standard 1:1 complex, except that the final isomerization step to an 'open' complex is lacking. A mechanism of the formation of the 2:1 complex and of its role in transcription regulation of constitutive promoter by RNAP is proposed.

INTRODUCTION

A constitutive promoter is defined as a DNA sequence enabling transcription of the gene located downstream by RNA polymerase (RNAP) without any further assistance, i.e. promoter sequence and RNAP fully specify the transcription initiation process. In a given species, the level of gene expression from constitutive promoters may vary by several orders or magnitude. In *Escherichia coli*, for instance, stable RNAs are transcribed from constitutive promoters at a rate which may approach one copy/s under favourable growth conditions; by contrast, *lac* i mRNA, also under control of a constitutive promoter, is transcribed at a rate of one copy/cell generation time (20–30 min) only, three orders of magnitude below that of stable RNA.

Results from our laboratory show that there are at least two ways of specifying the efficiency of a constitutive promoter in *E. coli*. Efficiency may be set permanently by appropriate choices, in the promoter sequence, of the contexts of the –10 and –35 consensus sequences and, presumably, the latter themselves. Direct sequence–efficiency links of this kind have been demonstrated *in vivo* by the use of synthetic constitutive promoters (see [1,2]). A second possibility for setting promoter efficiency would be to use a passive RNAP particle as a special sort of effector. The promoter sequence would again be involved, but indirectly, providing, for instance, a RNAP particle with a specific binding site. This RNAP particle would not become transcriptionally active at this site (no open-complex formation), but would enhance or inhibit transcription from a nearby open complex involving another RNAP particle [3–5]. In this case, efficiency of the promoter comes under control of RNAP availability, thus enabling, paradoxically, regulation of transcription from a true constitutive promoter.

An illustration of a 'regulated' constitutive promoter is

provided by the promoter of the β -lactamase (*bla*) gene of bacterial transposon Tn3, carried by plasmid pBR322. Studies *in vitro* show that, for a specified amount of promoter DNA fragment (nanomolar range), the amount of *bla* mRNA increases with RNAP concentration until a critical ratio of RNAP to promoter is reached, above which transcription of the gene becomes inhibited [3–5]. The onset of inhibition correlates closely with the binding of a second RNAP, as seen by gel-retardation experiments or revealed directly by electron microscopy.

This second RNAP binds to the promoter sequence in a highly specific manner: (1) the interaction is of the 'tight-binding' type [it withstands polyanionic (heparin) challenge]; (2) the second RNAP footprints distinctly the promoter from –60 to –100 (+1 is the transcription start address), i.e. upstream of the sequence footprinted in the 1:1 complex (+20 to –55); (3) close contact points between DNA and the second RNAP are revealed by chemical probing; the complex between the promoter sequence and this RNAP shares these three characteristics with the conventional (1:1) complexes, but is at variance with the latter; (4) no transcript starts in the region footprinted by the second RNAP; (5) no DNA unwinding can be detected in this region; (6) RNAP is unable to bind (as specified above) to the –15 to –120 promoter fragment; (7) the second RNAP can bind specifically only to the pre-existing 1:1 complex and (8) does so only if the latter is in some stable isomeric state.

We took advantage of these characteristics of the *bla* promoter, first to gain some insight into the mechanisms by which the 2:1 complex forms and dissociates and, hence, possibly regulates transcription of the *bla* gene. Because binding of the second RNAP displays all the usual characteristics of promoter–RNAP complexation, except that it does not enter the step leading to open complex formation, the *bla* system also offers an opportunity to investigate specific interactions of RNAP with DNA

in the absence of the DNA-opening step. The system is therefore a valuable model, closely resembling the isomers found as transient intermediates preceding the formation of the activated RNAP-promoter complex.

We find that formation and decay of the 2:1 complex closely follows the minimal two-step reaction scheme proposed by McClure [6] for the 1:1 complex, at moderate excess of RNAP. The regulation of the kinetics of the interaction of RNAP with the 1:1 complex by salt concentration [7] is consistent with this scheme and predicts that, upon formation of the 2:1 complex, about eight anionic charges are neutralized by RNAP, among which seven charges are neutralized upon formation of a transient intermediate during 2:1-complex formation. At larger excess of RNAP (corresponding to the upper limit of physiological values), formation of the 2:1 complex becomes increasingly inhibited, indicating that the simple minimal reaction scheme observed at moderate excess of the enzyme is no longer valid.

MATERIALS AND METHODS

The 267-bp *Hph*I-*Eco*R1 fragment from plasmid pBR322, isolated by standard procedures and end-labelled with ^{32}P , contains the *bla* promoter and the 94-bp sequence of the *bla* gene first transcribed. The fragment is kept in buffer B [10 mM-Tris/HCl (pH 8)/1 mM-DTT/1 mM-EDTA]. *E. coli* RNAP, prepared as described in [8], was estimated to be about 50% active by the abortive-initiation assay [9]. The enzyme was stored at -20°C in buffer C [10 mM-Tris/HCl (pH 8)/0.1 M-NaCl/0.1 mM-EDTA/50% (v/v) glycerol].

RNAP concentrations

At the moderate-to-high RNAP concentrations used in the present study, the holoenzyme can aggregate, the aggregation equilibrium depending on the NaCl and MgCl_2 concentrations. Shaner *et al.* [10] showed that dimerization of RNAP becomes significant as the Cl^- concentration is reduced below 200 mM (Na^+ and Mg^{2+} have no specific effect on dimerization).

On the other hand, Shaner *et al.* [11] reported that RNAP complexes rapidly, and with high affinity, to the ends of DNA fragments, but the resulting complexes are not of the tight-binding type (they dissociate rapidly upon polyanion challenge). To estimate the amount of active RNAP available for formation of the 2:1 complexes, we must therefore subtract from the total amount of RNAP (active and non-active) the amount involved in aggregation at the specified $[\text{Cl}^-]$ and the amount bound to the ends of the DNA fragments. The remaining amount of RNAP should be divided by 2 to account for the activated fraction of RNAP, and the amount of RNAP bound by the promoter to form the 1:1 complex subtracted. The remaining amount of RNAP is then available for formation of the 2:1 complexes and is termed 'available RNAP' (B_0) below, to be distinguished from 'total RNAP' (R_T), which refers to the total amount of RNAP added.

Kinetics of association and dissociation

An 8 μl portion of DNA solution (4–5.5 nM) and 2 μl of RNAP solution of appropriate concentration were incubated together for times as indicated; the final glycerol concentration was kept at 10% (v/v) in all cases. Non-specifically bound RNAP was removed by a short (less than 5 min) heparin challenge (1 μl ; 100 $\mu\text{g}/\text{ml}$). By contrast, for kinetic dissociation experiments, we challenged with 1 μl of poly[d(A-T)]·poly[d(A-T)] (100 $\mu\text{g}/\text{ml}$); the reason for this is that, because of the long exposures, the challenge by heparin might dissociate to some extent specific complexes as well, whereas poly[d(A-T)]·poly[d(A-T)] would not, or much less so. After 30 min of incubation, the

2:1 complex was challenged by poly[d(A-T)]·poly[d(A-T)] and the slope of the logarithm of its decay versus time taken as k_d (in s^{-1}). We are confident that the challenge by poly[d(A-T)]·poly[d(A-T)] does not noticeably participate in the dissociation of the 2:1 complex.

Gel-retardation electrophoresis was carried out in 1.2% agarose gel, in TBE buffer [90 mM-Tris/HCl (pH 8)/90 mM- H_3BO_3 /2.5 mM EDTA] at 5 V/cm, 30°C . Electrophoresis lasted for about 90 min. The gels were exposed to Fuji RX film, with and without an intensification screen, and scanned with a Shimadzu CS 130 densitometer. The amount of species in each lane was taken as being proportional to the integrated absorbance of the corresponding band and was expressed as percentage of the total intensity of the bands in a given lane.

The differences in the radioactivity between the lanes does not deviate from the mean by more than 10%. Each lane was normalized to 100%. Even under conditions where all the DNA should be complexed, a small quantity of free DNA of the order of 5% is always observed, whatever the DNA and RNAP preparations used. We have no explanation for this observation; however, analogous observations in filter-retention experiments have been reported from many laboratories (see [7]). A set of typical gel-retardation pictures is shown in Fig. 1.

A MINIMAL SCHEME FOR THE 2:1-COMPLEX FORMATION

The interaction of *E. coli* RNAP with a promoter containing DNA goes through successive and coupled steps, including non-specific DNA-RNAP interaction, promoter recognition by RNAP and formation of the closed, then open, complex. This is summarized in the classical, minimal scheme [6]:

where R is RNAP, P is promoter, PR_c stands for a (closed) intermediate and PR_o is the open complex capable of initiating transcription (see [12] for a review).

For the *bla* promoter and with an excess concentration of RNAP over promoter, it was reported [5] that, after specific binding of a first RNAP particle, at the usual position of the promoter, and isomerization of the 1:1 complex to some intermediate state PR_x , a second RNAP can bind upstream of the 1:1 complex, displaying the main characteristics of the 1:1 complex (heparin resistance, footprinting, close contacts) except DNA opening. The kinetic experiments reported below show that the rate of formation of the 2:1 complex (PR_2) is very much dependent upon NaCl and/or MgCl_2 concentration. This leads one to postulate a mechanism with at least two steps, one of which is characterized by an association rate constant depending strongly on ionic concentration [13]. Therefore, as a reasonable hypothesis, we assume that, at excess RNAP, PR_2 forms at the expense of PR_x according to a scheme analogous to that usually assumed for the 1:1 complex formed at low RNAP concentration:

Scheme 1

RNAP (R) is assumed to be in large excess over PR_x . Here, we do not further characterize PR_x , which is described in more detail in [5], except to state that PR_x is not the open complex PR_o and is permanently devoid of detectable transcription activity. It was shown [5] that the kinetics of formation of PR_x exceeds by an order of magnitude at least that of $(\text{PR}_2)^*$ or PR_2 .

Fig. 1. Autoradiographs of retardation gels showing association kinetics of RNAP ($R_T = 66$ nM) with DNA fragments bearing the *bla* promoter (4 nM)

From (a) to (f) NaCl concentration increases from 100 mM to 200 mM in 20 mM steps. Lane 1, no RNAP; lanes 2–10, incubation times of 1, 2, 3, 4, 5, 10, 20, 30 and 60 min respectively.

As for the minimal scheme, where $(PR)_c$ is in rapid equilibrium with the reactants, we assume that $(PR_2)^*$ is an intermediate 2:1 complex also in rapid equilibrium with PR_x , on the time scale leading to the stable final complex, PR_2 . The assumptions made allow one to state that formation of PR_2 conforms to standard pseudo-first-order kinetics (see the Appendix).

RESULTS

Scheme 1 fits the kinetic data for 2:1 complex formation at moderate excess of RNAP over DNA

In order to test the validity of the model and the simplifying assumptions made, we checked whether the kinetic data obtained

Fig. 2. Association kinetics of RNAP with complex 1:1 at increasing NaCl concentrations

D , PR_2 ; D_∞ , is the plateau value of D . The total RNAP concentration (R_T was 66 nM, [DNA] was 4 nM and the buffer was at pH 8 and 37 °C.

for different buffers and variable, but moderate, excesses of RNAP concentration can be fitted by the model.

(1) RNAP (33 nM) was incubated with 4 nM-promoter fragment at 37 °C, in buffers of 100, 120, 140, 160, 180 and 200 mM-NaCl-containing buffers (no $MgCl_2$). After the selected incubation time, complex formation was stopped by heparin challenge and the mixture was analysed by the gel-retardation method (see Fig. 1), and the constituents quantified as described in the Materials and methods section.

In Fig. 2, the relative amounts of 2:1 complex formed after a given time of incubation are plotted versus time (semi-logarithmic plot), for the selected values of NaCl concentration. It can be seen that, for a given NaCl concentration, the experimental points indeed fit rather satisfactorily a single exponential.

The slopes of the straight lines, obtained by regression analysis, and the plateau values of 1:1 and 2:1 complexes (their amounts at equilibrium (60 min, A_∞ and D_∞ respectively) allow one to compute the dissociation rate constant, k_4 (see the Appendix) and $K_{obs} = D_\infty/A_\infty \cdot B_0$ [B_0 is the (excess) concentration of RNAP actually available for the formation of the 2:1 complex; see the Materials and methods section and below]. These constants are given in Table 1 (data derived from Fig. 2), together with the calculated apparent association constant,

Table 1. Equilibrium and rate constants as a function of NaCl concentration

Constants were derived from association and dissociation kinetics of RNAP and the 1:1 complex PR_x . Total RNAP concentration (R_T) was 66 nM; B_0 is the RNAP concentration actually available for the formation of the 2:1 complex (see the text). The DNA concentration was 4 nM. The numbers of independent measurements are given in parentheses (see Table 2). The buffer was at pH 8 and 37 °C; no $MgCl_2$ was present. Abbreviations: nd, not determined; –, not calculated.

Parameter	[NaCl] (mM)...	Value					
		100 (4)	120 (3)	140 (2)	160	180	200
B_0 (nM)		18	20	22	23	24	25
$r = B_0/[DNA]$		4.5	5.0	5.5	5.6	5.8	6.2
$10^{-8} \times K_{obs}$ (M^{-1})		4.8	3.4	1.1	0.5	0.17	0.06
$10^4 \times k_4$ (s^{-1})		3.7	5.7	6.7	6.7	6.9	7.0
$10^{-5} \times k_{app}$ ($M^{-1} \cdot s^{-1}$)		1.8	1.9	0.74	0.33	0.12	0.04
$10^4 \times k_d$ (s^{-1})		2	2.3	3.3	3.2	nd	nd
$10^{-5} \times k'_{app}$ ($M^{-1} \cdot s^{-1}$)		0.96	0.78	0.36	0.16	–	–

Table 2. Equilibrium and rate constants as a function of RNAP concentration

No MgCl_2 was present. See Table 1 for other conditions.

Parameter	R_T (nm)...	Value						
		[NaCl] (mm)...				120		
		2	8	16	33	8	16	33
B_0 (nm)		1.6	5.7	10	18	6	11	20
[DNA] (nm)		0.2	1	2	4	1	2	4
$r = B_0 / [\text{DNA}]$		8	6	5	4.5	6	5.5	5
$10^4 \times k_4$ (s^{-1})		3	2.8	3	5.9	7	5	5
$10^{-8} \times K_{\text{obs.}}$ (M^{-1})		4.6	4.4	5.4	4.8	3.2	3.8	3.2
$10^{-5} \times k_{\text{app.}}$ (s^{-1})		1.4	1.2	1.6	2.8	2.2	1.9	1.6
$k_{\text{ass.}} = 2.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$		$k_{\text{ass.}} = 2.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$						
$k_3 = 4 \times 10^{-3} \text{ s}^{-1}$		$k_3 = 3.3 \times 10^{-3} \text{ s}^{-1}$						
$K_{1,2} = 6.4 \times 10^7 \text{ M}^{-1}$		$K_{1,2} = 7.2 \times 10^7 \text{ M}^{-1}$						
$K = 5.7 \times 10^8 \text{ M}^{-1}$		$K = 3.7 \times 10^8 \text{ M}^{-1}$						
(using $k_4 = 3.7 \times 10^{-4} \text{ s}^{-1}$)		(using $k_4 = 5.7 \times 10^{-4} \text{ s}^{-1}$)						

$k_{\text{app}} = K_{\text{obs.}} \cdot k_4$, and the apparent dissociation rate constant k_d measured directly as described above, for the salt concentration assayed; we also computed $k'_{\text{app.}} = K_{\text{obs.}} \cdot k_d$.

(2) At 100 and 120 mM-NaCl, with no MgCl_2 , the kinetics of formation of the 2:1 complex were monitored for various RNAP and DNA concentrations. In each case, the pseudo-first-order approximation accounts satisfactorily for the results (not shown). The variations of the initial rate of formation of the 2:1 complex and of D_∞ with B_0^{-1} , yield $K = k_1 \cdot k_3 \cdot (k_2 \cdot k_4)^{-1}$, $K_{3,4} = k_3 \cdot k_4^{-1}$, $K_{1,2} = k_1 \cdot k_2^{-1}$, k_3 and $k_{\text{ass.}} = k_1 \cdot k_3 \cdot k_2^{-1}$ (see the Appendix). The values of these constants are given in Table 2 (bottom), together with $K_{\text{obs.}}$, k_4 and $k_{\text{app.}}$, derived from individual experiments performed for each value of B_0 .

Table 1 displays a very strong dependence of $K_{\text{obs.}}$ and $k_{\text{app.}}$, as well as $k'_{\text{app.}}$, on NaCl concentration, whereas k_4 and k_d show a much weaker salt-dependence. Table 2 shows that k_3 is about an order of magnitude above k_4 and, consistently, that the main contribution to K comes from $K_{1,2}$.

The data displayed indicate that the accuracy with which rate and equilibrium constants can be quantified by the gel-retardation method is not very high; it is, however, good enough to ascertain that Scheme 1 and the assumption made account satisfactorily for the formation of the 2:1 complex under the experimental conditions used.

For the present analysis of the data, we have assumed that the intermediate species, $(\text{PR}_2)^*$, when challenged by the polyanion, loses the second RNAP bound and hence appears in the gel-retardation experiment together with RP_x , not RP_2 . This assumption is justified *a posteriori*; indeed, using the data in Table 2 for 100 mM-NaCl ($K = 5.7 \times 10^8$ and $K_{1,2} = 6.4 \times 10^7$), under the hypothesis made for Scheme 1, it is easy to show (see the Appendix) that $A_\infty/A_0 \sim (K \cdot B_0 + 1)^{-1}$ is about 0.04, $C_\infty/A_0 \sim K_{1,2} \cdot B_0 \cdot A_\infty/A_0$ is about 0.006 and $D_\infty/A_0 (= 1 - A_\infty/A_0 - C_\infty/A_0)$ is about 0.9 [0 and ∞ stand for the initial and plateau values, and A , C and D are concentrations of RP_x , $(\text{PR}_2)^*$ and PR_2 respectively]. Since the observed values of $(D_\infty/D_0)_{\text{obs.}}$ and $(A_\infty/A_0)_{\text{obs.}}$ are 0.85 and 0.12 respectively, the latter most probably includes C_∞/C_0 , since $(A_\infty + C_\infty) \cdot A_0^{-1}$ is about 0.10.

Salt-dependence of the 2:1 complex is consistent with the existence of the intermediate $(\text{PR}_2)^*$

(a) **Univalent ions.** The existence of intermediates during formation of the open 1:1 complex between a standard promoter and RNAP is an important characteristic of the activation pro-

cess. It is therefore of importance to ascertain the existence of the intermediate $(\text{PR}_2)^*$ postulated in Scheme 1, in order to lend support to the assumption of promoter-like binding characteristics leading to formation of PR_2 .

The intermediates of the active 1:1 complexes for standard promoters have been characterized directly, either kinetically (promoter 'opening', tested chemically, for instance) or functionally (abortive-initiation assay). Since PR_2 exhibits no DNA unwinding, is unable to sustain transcription initiation and would not distinguish from PR_x in a filter-binding assay, we must depend on indirect methods to test for the existence of $(\text{PR}_2)^*$ and gain information on some of its characteristics.

In a series of publications by Record and co-workers (see [14] for a recent review), it was demonstrated that salt concentration could be a powerful probe for the formation of intermediates in protein-DNA complexes in general.

The formation of the non-covalent 2:1 complex from its 1:1 antecedent in a buffer containing NaCl is likely to involve the release of ions and water, according to the global scheme:

Scheme 2

where a , b and c are respectively the sum of the number of cations, anions and water molecules initially attached to the 1:1 complex and/or to RNAP and released or excluded upon formation of the 2:1 complex. Ions a and b , thermodynamically bound to the reactants, can be classified according to [13] into ions condensed to charges carried by the reactants (a_c and b_c) and a class of more mobile ions which screen interactions between the un-neutralized charges of each reactant (a_s and b_s). For DNA, it was shown that, over a large range of NaCl concentration (0.5 to 500 mM), on average three out of four anions are condensed by Na^+ , but only one out of ten is screened by a counter-ion.

The observed equilibrium constant, $K_{\text{obs.}}$, depends of course on experimental conditions, in particular ionic strength of the reaction buffer. If the ionic strength is shifted, $K_{\text{obs.}}$ is expected to change such that $SK_{\text{obs.}} \sim -(a+b)$ (where $SX = d \log X / d \log [\text{NaCl}]$); the contribution of water release to $SK_{\text{obs.}}$ is neglected here, a reasonable assumption for the present experiments, where [NaCl] only varies from 100 mM to 200 mM.

In Fig. 3, the experimental values of $K_{\text{obs.}}$ (Table 1) are plotted as $\log K_{\text{obs.}}$ versus $\log [\text{NaCl}]$; we found $SK_{\text{obs.}}$ to be about -8 ; this is the total amount of ions (cations and anions) released

Fig. 3. Dependence of the apparent association (RNAP and PR_2) constant upon NaCl concentration in the presence (□) of $MgCl_2$ (5 mM) or in its absence (■)

See Tables 1 and 3 for experimental details.

upon formation of a 2:1 complex from the 1:1 complex under the present conditions. The negative slope indicates that increasing the salt concentration of the buffer drives the reaction towards the reactants, i.e. the 1:1 complex and RNAP.

Certain aspects of the mechanism underlying reactions of the kind displayed by Scheme 2, in particular the occurrence of an intermediate, can be elucidated by analysing the salt-dependence of the apparent forward (k'_{app}) and backward (k_d) rate constants, $K_{obs.} = k'_{app}/k_d$.

If the bimolecular reaction (Scheme 2) is assumed to occur in a single step, the reactants would transiently combine as a collisional complex where they have mutually penetrated the ion atmospheres surrounding them, but in which the condensed ions, a_c and b_c , have not yet been released. The rate of formation of the collisional complex depends on the coulombic interactions between the reactants, which in turn are related to the screening of the reactants by the cloud of mobile ions surrounding them. This screening is directly governed by the salt concentration of the solution, in contrast with the subsequent step (formation of RP_2), where the release of condensed ions ($a_c + b_c$) would be almost independent of salt concentration. Under reasonable simplifying assumptions (similar to those discussed by Lohman *et al.* [13]), it can then be shown that $SK'_{app} = -(a_s + b_s) < 0$ and consequently $Sk_d \sim a_c + b_c > 0$. The salt-dependence of k'_{app}

involves mainly the amount of screening ions, that of k_d depending on the amount of condensed ions.

If, by contrast, Scheme 2 involves an intermediate (PR_2)* in which a' and b' of the thermodynamically bound ions a and b have already been released:

Scheme 2'

and if this equilibrium is assumed to be established rapidly on the time scale of the conversion of (PR_2)* into PR_2 , then the rate constant of the latter is essentially independent of salt concentration: $SK'_{app} \sim -(a' + b') < 0$ and consequently $Sk_d \sim (a + b) - (a' + b') > 0$.

$Sk_d > 0$ indicates that cations recondense on the reactants upon their dissociation from the complex; the transfer of these ions from the dilute solution to the dissociating species involves a change in entropy.

The screening ions are usually a small fraction of the total (thermodynamically) bound ions, especially for DNA, as seen above. Therefore, if Sk_d is of the order of $SK_{obs.}$, Scheme 2 without an intermediate is likely, but if Sk_d is small compared with $SK_{obs.}$, an intermediate is to be expected. From the results in Table 1 we obtain a Sk_d value of about 0.5. This result is in favour of an intermediate in Scheme 2 and justifies Scheme 1. We conclude that the NaCl-dependence of the apparent rate and equilibrium constants is consistent with that predicted if an intermediate (PR_2)* is present, with $(a' + b') \sim 7$ and $(a + b) \sim 8$. Seven out of the eight ions released upon formation of PR_2 are already released upon the fast formation of the intermediate, (PR_2)*.

(b) Mixed univalent/bivalent ions. Testing the activity of RNAP requires the presence of bivalent ions. We therefore have to check whether Scheme 1 can account for formation kinetics of the 2:1 complex in the presence of Mg^{2+} , at moderate excess of RNAP. On the other hand, further confirmation of the existence of the intermediate, (PR_2)*, can be obtained by measuring salt-dependence of $K_{obs.}$ and k'_{app} in mixed Na^+/Mg^{2+} buffers.

The experimental data of the association kinetics of 2:1 complex (4 nm-promoter; 33 nm-RNAP) in 100 mM-NaCl/10 mM- $MgCl_2$, conform satisfactorily to pseudo-first-order kinetics as predicted by Scheme 1 (results not shown), yielding the rate and equilibrium constants given in Table 3.

The observations made for Tables 1 and 2 can be repeated for Table 3. In particular, we notice again the strong reduction of $k_{app.}$ for a modest increase in $[MgCl_2]$; $k_{app.}$ decreases from 2×10^5

Table 3. Equilibrium and rate constants as a function of NaCl concentration in the presence of magnesium

Total RNAP concentration (R_T) was 78 nM; [DNA] was 5 nM, except for the last column, where R_T was 66 nM and [DNA] was 4 nM. The numbers of independent measurements are given in parentheses. See Table 1 for other explanations.

Parameter	[MgCl ₂] (mM) ... [NaCl] (mM) ...	Value						
		5	5	5	5	5	5	10
$10^9 \times B_0$ (nm)		15	22	24	26	28	28	23
$r = B_0/[DNA]$		3	4.4	5	5	5.6	5.6	5.7
$10^{-8} \times K_{obs.}$ (M ⁻¹)		8.6	3.7	1.9	0.9	0.3	0.09	0.9
$10^4 \times k_4$ (s ⁻¹)		2.4	2.4	5.8	6.0	7	8.7	3.4
$10^{-5} \times k'_{app.}$ (M ⁻¹ ·s ⁻¹)		2.1	0.9	1.1	0.5	0.2	0.08	0.3
$10^4 \times k_d$ (s ⁻¹)		1.3	2.2	3.0*	4.2	9.5	nd	nd
$10^{-5} \times k'_{app.}$ (M ⁻¹ ·s ⁻¹)		1.1	0.8	0.6	0.4	0.3	—	—

Table 4. [MgCl₂]-dependence of the apparent equilibrium constant upon titration of 5 nM-DNA with 312 nM-RNAP (R_T)

B_0 is the RNAP concentration available for formation of PR_2 from PR_x (see the text). The buffer was at pH 8.0 and 37 °C; 50 mM-NaCl was present, as was MgCl₂ at the indicated concentrations.

Parameter	Value					
[MgCl ₂] (mM)...	8	10	15	20	25	30
B_0 (nM)	28	34	49	65	80	94
$r = B_0/[DNA]$	5.6	6.7	9.8	13	16	19
$10^{-7} \times K_{obs.}$ (M ⁻¹)	5.1	3.1	1.2	0.87	0.25	0.15

to $3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ as [MgCl₂] increases from 0 to 10 mM, somewhat larger than would be expected from the corresponding increase in ionic strength (see Table 1; 120 and 140 mM-NaCl).

To evaluate the total number of anions neutralized when RNAP binds to the 1:1 complex in a mixed Na⁺/Mg²⁺ buffer, we again rely on the variation of $K_{obs.}$ with [NaCl] for [MgCl₂] = 5 mM. The results, given in Table 3, are displayed as a log-log plot in Fig. 3. It can be seen that the dependence of $\log K_{obs.}$ is a linear function of $\log[NaCl]$ for 100 mM $\leq$ NaCl $\leq$ 175 mM; indeed, according to Lohman *et al.* [13], the Na⁺ release upon RNAP binding can be deduced from:

$$(d \log K_{obs.} / d \log [NaCl])_{Mg^{2+}} = -(a+b)(1-2\theta)$$

where the corrective term 2θ depends on [NaCl], [MgCl₂] and the equilibrium constant for Mg²⁺ binding to DNA. For [NaCl] $\geq$ 125 mM, the correction term becomes small ($\leq$ 0.1) because the effect of Mg²⁺ is 'masked' by that of Na⁺; then $(d \log K_{obs.} / d \log [NaCl])_{Mg^{2+}}$ is ~ -6.5 , consistent with the value found previously in pure NaCl buffer. Some curvature in the plot is visible for NaCl < 100 mM, as predicted by theory [13], but as it is also detected in absence of MgCl₂ over the same NaCl range, this interpretation is questionable. It is possible that some kind of inhibition may be more effective at relatively low NaCl concentrations (see below).

From Table 3 we obtain $Sk_d \approx 3$. This value is again consistent with the existence of an intermediate species during formation of the 2:1 complex from its 1:1 antecedent, just as observed in the absence of Mg²⁺ ions.

Finally, we also performed a series of equilibrium experiments complementary to the preceding one, in which we varied [Mg²⁺] from 8 to 30 mM, for [NaCl] = 50 mM. The results are given in Table 4.

The data align well on a $\log K_{obs.}$ -versus- $\log[MgCl_2]$ plot (not shown), with slope $SK_{obs.} = -2.5$. This result is consistent with the much higher affinity of Mg²⁺ ions, compared with univalent ions, for DNA. The data for MgCl₂ > 15 mM should, however, be considered with caution, as the excess of RNAP is beyond the limit of what we consider as 'modest' excess ($r = [B_0]/[DNA] \leq 10$) (see below).

2:1-Complex dissociation

The experimentally determined dissociation rate constant, k_d , and the rate constant k_4 computed from the kinetic data plotted according to Scheme 1 and the underlying assumption, differ consistently by a factor of about 2, both in the absence of MgCl₂ (Table 1) and in the presence of 5 mM-MgCl₂ (Table 3).

This discrepancy could be due to aggressive challenging of PR_2 by the polyanion, speeding up its dissociation, hence increasing k_d . We favour another mechanism, namely one in which RNAP itself would dissociate PR_2 , when present at large excess relative

to PR_x (see the following section). Indeed, k_d is measured after removal of unbound and unspecifically bound RNAP particles, but k_4 is evaluated with the enzyme present; k_4 would then be affected by dissociation mediated by excess RNAP, but k_d would not.

Formation of the 2:1 complex and RNAP concentration

It was shown by several groups of workers that for certain promoters, complexation *in vitro* by RNAP can be inhibited by excess concentration of the enzyme [15], a phenomenon also observed for the formation of a 2:1 complex of the *bla* promoter, [4,5]. On the other hand, it is well established also that RNAP (holoenzyme) can dimerize at low anionic strength; the dimerization equilibrium constant, K_2 , was indeed shown by Shaner *et al.* [10] to depend strongly on [Cl⁻]:

$$\log [K_2 (\text{M}^{-1})] = (0.7 \pm 0.6) - \{(5.6 \pm 0.9) \log [\text{Cl}^-]\}$$

for 60 mM < [Cl⁻] < 190 mM and 10 mM-MgCl₂. These observations prompted us to investigate the dependence of equilibrium and rate constants of 2:1-complex formation over a large range of enzyme concentrations, in buffers where copious formation of RNAP dimers can take place.

(a) **Inhibition of 2:1-complex formation due to substrate shortage at low ionic strength.** In the Material and methods section, we briefly indicated how we compute B_0 , the amount of RNAP actually expected to be available for 2:1-complex formation; the total amount of RNAP added to the promoter solution, concentration R_T , is corrected (i) for the amount of dimers formed at the specified anionic strength [10] (this assumes dimers unable to bind DNA specifically); (ii) for the amount of RNAP binding to DNA ends, as it was shown [11] that the enzyme binds the latter at a rate and with an affinity comparable with those applying for promoters, that is, much larger than for a 1:1 complex; (iii) the remaining amount of RNAP is divided by 2 to account for the 50 % activity of our RNAP preparations, and the RNAP bound to the promoter is then subtracted. These corrections yield B_0 .

As an example, in a buffer of 75 mM-NaCl/5 mM-MgCl₂, starting with $R_T = 39$ nM, we compute that 4.5 nM-RNAP dimerizes, 11 nM binds to DNA ends, leaving 9.5 nM active RNAP and $B_0 = 4$ nM, but for $R_T = 78$ nM in the same buffer, we obtain $B_0 = 15$ nM (15 nM dimerizes, 11 nM binds to DNA ends and 20 nM active RNAP is left for binding specifically to the promoter and to PR_x).

Because of these large corrections of RNAP concentrations, especially at low ionic strength, the assumptions made, namely (1) that dimer is unable to bind DNA specifically and (2) that the affinities of RNAP for DNA ends and promoter are much larger than for PR_x , to which only active RNAP can bind, need to be substantiated. Fig. 4 shows retardation gels corresponding to titration experiments performed at the RNAP concentrations and the buffer conditions just given in the above example ([DNA] = 5.5 nM). For the experiment starting with $R_T = 39$ nM, we observed that about one-third of the DNA remains free; by contrast, for $R_T = 156$ nM, only the (usually observed) small amount (less than 10 %) of DNA remains free. We believe this is direct evidence for RNAP shortage in the former experiment, which shifts the global reaction leading to PR_2 towards the reactants. Actually, the corrections we apply to R_T may be insufficient, especially at low ionic strength, since in the former experiment even the active RNAP concentration is too small to allow full titration of the promoter to form the 1:1 complex. Because of the consistency of the rate and equilibrium constants given in Tables 1–5, we are confident that the corrections are essentially valid at ionic strength $\geq$ 100 mM.

Fig. 4. Late kinetics of 2:1-complex formation in 75 mM-NaCl/5 mM-MgCl₂/5 nM-DNA

(a) $R_T = 39$ nM; (b) $R_T = 156$ nM. Lanes 1, 2 and 3 for (a), 30, 45 and 60 min, respectively, and for (b), 15, 20 and 30 min respectively, after the start of the reaction.

(b) Inhibition of 2:1-complex formation by large excess of substrate. On the other hand, it can also be seen in Table 5 that, as B_0 increases relative to [DNA] ($r = B_0/[DNA] \geq \sim 5$), $K_{obs.}$ decreases strongly. Thus, 2:1-complex formation is increasingly inhibited as the excess of available RNAP (B_0) goes from moderate ($r \sim 5$) to high ($r \sim \geq 10$), due either to a decrease of $k_{app.}$ or an increase in k_4 ($K_{obs.} = k_{app.} \cdot k_4^{-1}$). Kinetic experiments done in 100 mM-NaCl/5 (or 10) mM-MgCl₂ buffer clearly indicate that most, if not all, of the decrease in $K_{obs.}$ is due to the concomitant increase in k_4 (Table 5). Thus at excess RNAP, the dissociation of the 2:1 complex is strongly favoured. Clearly the simple scheme, Scheme 1, no longer holds at large excesses of RNAP, and some additional mechanism must be invoked to account for this inhibition effect. We also observe that the latter is enhanced as the ionic strength of the buffer is lowered.

Temperature-dependence of $K_{obs.}$ and $k_{app.}$

The formation of PR_2 is very dependent upon the incubation temperature, as has been noted for tight binding sites in general [16]. We measured the equilibrium concentration of 2:1 and 1:1 complexes at 25, 30 and 37 °C in 100 mM-NaCl/10 mM-MgCl₂ buffer; the DNA concentration was 5 nM and R_T was 312 nM. We assume that the B_0 value derived from R_T ($B_0 = 107$ nM) does not change with temperature, nor does the inhibition effect (although $r = 21$). From the data we computed the van't Hoff enthalpy change (ΔH) to be ~ 155 kJ (37 kcal)/mol and the activation energy E_a to be about 121 kJ (29 kcal)/mol. These values are close to those obtained for the λP_r promoter [17]. In the latter

case, the interpretation of the high activation energy is an RNAP transconformation (burying of hydrophobic groups) accompanying the nucleation step that precedes helix opening. For the 2:1 complex, helix opening is, however, not observed, but the burial of hydrophobic groups of RNAP seems nevertheless to take place when it binds to RP_x .

DISCUSSION AND CONCLUSIONS

The analytical portrayal associated with Scheme 1 accounts for 2:1-complex formation

Our experimental results show that the process of binding of a second RNAP to the specific 1:1 complex of RNAP and the *bla* promoter is satisfactorily accounted for by Scheme 1, at moderate excess of RNAP ($r < 5$). Binding of the second RNAP to the 1:1 complex resembles closely that of RNAP to a standard promoter. In addition to the structural characteristics (heparin-resistant binding, footprinting and formation of close contacts between RNAP and the sequence from -60 to -100 ; see [3]), the thermodynamic characteristics are also similar: binding of the second RNAP occurs via an intermediate, with the release of about seven univalent ions; this step is followed by isomerization of this intermediate complex to the final 2:1 complex, with the additional release of about one univalent ion. The thermodynamic parameters associated with the binding of the second RNAP are similar to those which have been obtained for RNAP binding to standard promoters, and they support the conclusion that the binding process of the second RNAP is mainly entropy-driven.

However, in contrast with the 1:1 complex involving standard promoters, binding of the second site does not result in DNA opening; no obvious consensus sequences are found in the region footprinted by it, and its binding is subjected to the presence of the 1:1 complex in its activated state. Furthermore, the footprint of the second RNAP has about half the size (40 bp) of that observed in the 1:1 complex (~ 75 bp). The interaction of the second RNAP with the 1:1 complex offers, then, the opportunity to study a specific binding of the enzyme to DNA in the absence of the DNA unwinding process (always observed upon complexation of standard promoters). Our results suggest that many of the characteristic physical data obtained for the latter are only slightly affected by the unwinding process. This observation lends support to the hypothesis [2] that the promoter sequence carries two kinds of instructions: structural instructions, which enable RNAP to dock on to the promoter sequences (specific sequence recognition, complex-formation, footprinting

Table 5. Equilibrium and rate constants for formation of PR_2 from PR_x at large ($r \geq 10$) and moderate ($r \leq 6$) relative excess of RNAP

The buffer was at pH 8 and at 37 °C. Abbreviation: n.d., not determined.

Parameter	Ionic conditions...	Value								
		{ 75 mM-NaCl* 5 mM-MgCl ₂			100 mM-NaCl 5 mM-MgCl ₂			100 mM-NaCl 10 mM-MgCl ₂		
R_T		78	156	312	66	78	132	66	132	312
B_0 (nM)		16	32	57	21	22	45	23	49	107
[DNA] (nM)		5.5	5.5	5.5	4	5.5	4	4	4	5
$r = B_0/[DNA]$		2.9	5.9	10	5.3	4.1	11	5.8	12	21
$10^{-8} \times K_{obs.}$ (M ⁻¹ ·s ⁻¹)		8.6	6.5	0.66	3.7	3.6	0.88	0.9	0.31	0.23
$10^4 \times k_4$ (s ⁻¹)		nd	nd	nd	2.4	nd	5.5	3.1	6.1	8.9

* Buffer at 30 °C.

and formation of close contacts), and executive instructions, which control promoter activation, the rate of activation and selection of transcription start site(s). The *bla* site binding the second RNAP appears to lack the latter set of instructions.

The *bla* promoter is clearly distinct from the tandem promoter sometimes found on chromosomal genes, first, because the promoters in tandem may each become active under specific conditions, and also because they are usually unable to bind RNAP simultaneously.

The 2:1-complex formation shares with many standard promoters an inhibition effect in the presence of excess RNAP (RNAP concentrations towards the upper limit of the physiological value). The (phenomenologically) suggested mechanism ($PR_2 + R \rightarrow PR_3$) reminds one of the 'bumping' effect, which was assumed by Shanblatt & Revzin [15] to account for inhibition of 1:1-complex formation of certain promoters by excess RNAP; briefly, while the 1:1 complex is undergoing the activation process, bumping by RNAP particles (for instance, sliding along the DNA) could remove RNAP from the complex. In the present case, bumping would remove one RNAP from the 2:1 complex and thus account for the inhibition process we observe.

Incidentally, footprinting experiments of the 1:1 'like' complex revealed [5] that RNAP resides from +20 to -55, just as in the usual 1:1 complex formed at low RNAP concentration; hence, bumping 'kicks' the second RNAP, which footprints from -60 to -100.

A possible mechanism leading to the 2:1 complex

Biochemical data obtained in our laboratory permit us to propose a four-step model to account for the mechanism by which the *bla* promoter can successively and specifically accommodate two RNAPs.

The promoter sequence, -41 to -56, already footprinted in the 1:1 complex, bears a dense set of contacts with RNAP, revealed in the minor groove of the template and involving a row of adenine residues [3]. This might indicate a firm orientation (bending?) of the upstream promoter sequence (bp-60 to -100 and beyond), relative to the 1:1 complex.

The lack of specific RNAP complexation activity of the isolated DNA fragment, bp-15 to -123, of the *bla* promoter [5] is taken as an indication that the primary binding of the second RNAP to the pre-existing 1:1 complex requires a protein-protein interaction. It was indeed shown by Shaner *et al.* [10] that RNAP can di- or even multi-merize, involving, in part at least, ionic

interaction. The data presented above suggest that this protein-protein-interaction step coincides with, or accompanies, the formation of the transient intermediate, $(PR_2)^*$. The release of seven ions, observed as this intermediate forms, corresponds to both protein-protein and protein-DNA interactions.

In a final step, $(PR_2)^*$ isomerizes to PR_3 , involving binding to RNAP of about one additional anion from the firmly oriented DNA segment, -60 to -100; reclining of the latter over RNAP is indicated by the 10 bp periodicity of sites hypersensitive to DNAase I displayed by this sequence [3].

The present study illustrates the usefulness of combining simple physical and biochemical studies for deepening our insight into the structure and function of specific nucleoprotein complexes.

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APPENDIX

We rewrite Scheme 1 in the main paper as:

We assume the first step is fast, the second slow, and that B (RNAP) is in large excess, i.e. initial concentration $A_0 \leq B_0 \approx B$. We will investigate the slow step:

$$dD/dt = -k_4D + k_3C$$

Because the first step is fast on the time scale of the second step:

$$dA/dt = dB/dt = -k_1AB + k_2C \sim 0$$

$$A = K_{2,1}C/B \text{ with } K_{2,1} = k_2/k_1$$

since

$$C + D = A_0 - A + B_0 - B \sim A_0 - A, C = (A_0 - D)/(1 + K_{2,1}/B_0)$$

Then:

$$dD/dt = -k_4(1 + L^{-1})D + k_4A_0L^{-1}$$

where

$$L = K_{4,3}(1 + K_{2,1}/B_0) \text{ and } K_{4,3} = k_4/k_3$$

Upon integration, we obtain the percentage of 2:1 complex formed at the expense of the 1:1 complex:

$$y = D/A_0 = (1 + L)^{-1} \{1 - \exp[-k_4(L^{-1} + 1)t]\}$$

At the plateau ($t \rightarrow \infty$), $y_\infty = (D/A_0)_\infty = (1 + L)^{-1}$; the initial slope, $y'_0 = [d(D/A_0)/dt]_0 = k_4L^{-1}$.

Introducing y'_0 and y_∞ into the expression for y yields, of course:

$$y = y_\infty [1 - \exp(-y'_0 \cdot t / y_\infty)]$$

or

$$\ln(y_\infty) - \ln(y_\infty - y) = (y'_0 / y_\infty) t$$

From the values of y_∞ and y'_0 we deduce:

$$k_4 = y'_0 (1 / y_\infty - 1)$$

$$1 / y'_0 = (K_{2,1} / k_3) \cdot 1 / B_0 + 1 / k_3 = (1 / k_{\text{app.}}) \cdot 1 / B_0 + 1 / k_3$$

$$1 / y_\infty = (1 / K_{\text{obs.}}) \cdot 1 / B_0 + (K_{4,3} + 1)$$

The plot of $1 / y'_0$ versus $1 / B_0$ (analogous to the classical 'tau plot') yields k_3 , $K_{2,1}$ and $k_{\text{app.}}$. The plot of $1 / y_\infty$ versus $1 / B_0$ yields $K_{\text{obs.}} = K_{1,2} \cdot K_{3,4}$.