Binding affinity of *Escherichia coli* RNA polymerase-\(\sigma^{54}\) holoenzyme for the *glnAp2, nifH* and *nifL* promoters

Sabine K. Vogel, Alexandra Schulz and Karsten Rippe

Deutsches Krebsforschungszentrum, Biophysik der Makromoleküle (H0500), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany and 1Deutsches Krebsforschungszentrum, Molekulare Genetik (H0700), Im Neuenheimer Feld 280 and Kirchhoff-Institut für Physik, Physik molekularbiologischer Prozesse, Universität Heidelberg, Schröderstraße 90, D-69120 Heidelberg, Germany

Received February 25, 2002; Revised and Accepted July 22, 2002

**ABSTRACT**

*Escherichia coli* RNA polymerase associated with the \(\sigma^{54}\) factor (RNAP-\(\sigma^{54}\)) is a holoenzyme form that transcribes a special class of promoters not recognized by the standard RNA polymerase-\(\sigma^{70}\) complex. Promoters for RNAP-\(\sigma^{54}\) vary in their overall ‘strength’ and show differences in their response to the presence of DNA curvature between enhancer and promoter. In order to examine whether these effects are related to the promoter affinity, we have determined the equilibrium dissociation constant \(K_d\) for the binding of RNAP-\(\sigma^{54}\) to the three promoters *glnAp2, nifH* and *nifL*. Binding studies were conducted by monitoring the changes in fluorescence anisotropy upon titrating RNAP-\(\sigma^{54}\) to carboxyrhodamine-labeled DNA duplexes. For the *glnAp2* and *nifH* promoters similar values of \(K_d = 0.94 \pm 0.55\) nM and \(K_d = 0.85 \pm 0.30\) nM were determined at physiological ionic strength, while the *nifL* promoter displayed a significantly weaker affinity with \(K_d = 8.5 \pm 1.9\) nM. The logarithmic dependence of \(K_d\) on the ionic strength \(I\) was \(-\Delta \log(K_d)/\Delta \log(I) = 6.1 \pm 0.5\) for the *glnAp2*, \(5.2 \pm 1.2\) for the *nifH* and \(2.1 \pm 0.1\) for the *nifL* promoter. This suggests that the polymerase can form fewer ion pairs with the *nifL* promoter, which would account for its weaker binding affinity.

**INTRODUCTION**

RNA polymerase from *Escherichia coli* complexed with the alternative \(\sigma\) factor \(\sigma^{54}\) (RNAP-\(\sigma^{54}\)) recognizes a specific class of promoters with conserved sequence elements around position -24 and -12 upstream of the transcription start site at +1 (1). RNAP-\(\sigma^{54}\) can bind to the promoter and form a stable closed complex. However, the enzyme is unable to melt the DNA at the transcription start site, i.e. to undergo the transition into the open complex. This process involves specific transcription factors with ATPase activity as for example nitrogen regulatory protein C (NtrC or NRII) or nitrogen fixation protein A (NifA). The binding sites of these proteins are located at upstream enhancer sequences and require looping of the DNA to enable interaction with RNAP-\(\sigma^{54}\) (2–6). The mechanism of transcription activation in this system has been discussed in recent reviews (7–9). Several lines of evidence indicate that the activator protein targets the \(\sigma^{54}\) subunit and works by triggering a conformation change that involves the N-terminus of \(\sigma^{54}\) (10).

In a number of studies differences in the relative strength of RNAP-\(\sigma^{54}\) promoters have been reported with respect to the *in vivo* RNA levels (11), the activity of a \(\beta\)-galactosidase reporter gene (12,13) or by measuring the equilibrium amount of open complexes formed *in vitro* in single round transcription experiments (14–16). Here we define the overall promoter strength as the rate with which the open complex \(R_P\) of RNAP-\(\sigma^{54}\) (R) at a given promoter \(P\) is formed in a multi-step reaction according to equation 1.

\[
\begin{align*}
R + P & \rightarrow RP_c \\
& \rightarrow \cdots \\
& \rightarrow RP_o
\end{align*}
\]

In this equation 1 the forward and backward rate constants for the formation/dissociation of the closed complex \(RP_c\) are given by \(k_1\) and \(k_{-1}\) so that the equilibrium dissociation constant \(K_d = k_{-1}/k_1\) in equation 2 reflects the affinity of the promoter for RNAP-\(\sigma^{54}\).

\[
K_d = k_{-1}/k_1 = ([R][P])/[RP_c]
\]

For the standard *E.coli* RNAP-\(\sigma^{70}\) holoenzyme a number of promoters have been analyzed in detail. It has been demonstrated that both the binding of the RNA polymerase to the promoter as well as the subsequent conversion of the closed complex into the open complex \(RP_o\) can be rate limiting (17). For RNAP-\(\sigma^{54}\) no quantitative analysis of the promoter strength in terms of the relative contributions of the separate
steps has been reported so far. From footprinting experiments a $K_d$ of 3 nM at approximately physiological salt concentrations was estimated for the glnAp2 promoter (18). In contrast, the nifH (19,20) and nifL (21–23) promoters from *Klebsiella pneumoniae* showed no or only a very weak footprint under similar conditions. Accordingly, glnAp2 has been designated as a ‘strong’ promoter as opposed to the ‘weak’ nifH and nifL promoters. However, it should be noted that both glnAp2 and nifH are ‘strong’ promoters in the sense that they can be expressed at high levels under suitable physiological conditions (13,24). In contrast, the nifL promoter displayed an approximately 5-fold lower expression level than the nifH promoter (13). Here we have measured the equilibrium dissociation constant $K_d$ dependence on the ionic strength for oligonucleotide duplexes with sequences from the glnAp2, nifH and nifL promoters. The binding of RNAP-$\sigma^{54}$ to DNA was monitored by fluorescence anisotropy measurements using DNA oligonucleotide duplexes with a carboxyhyd-amine end label.

**MATERIALS AND METHODS**

**DNA and RNAP-$\sigma^{54}$ preparation**

HPLC-purified DNA oligonucleotides were purchased from PE-Applied Biosystems (Weiterstadt, Germany). The fluorescent dye 6-carboxy-X-rhodamine (ROX) was covalently attached to the 5′-end via a C6 linker. The extinction coefficients of the single DNA strands were determined as described previously (25). Equimolar amounts of complementary single strands were mixed in a buffer containing 10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 0.1 mM EDTA and annealed by 2 min heating at 70°C followed by slow cooling to room temperature over several hours. Purification of the resulting DNA duplexes was done by extraction from native polyacrylamide gels according to Rippe et al. (26). From an analysis of various ROX-labeled DNAs of different lengths an average extinction coefficient of ROX attached to DNA of $\varepsilon_{350} = 96\,000\,M^{-1}\,cm^{-1}$ at 25°C was calculated (J.F. Keupert and K.Rippe, unpublished results). This value was used to determine the concentration of the purified ROX-labeled DNA duplex stock solutions. The concentration of the unlabeled promoter duplexes was determined based on an extinction coefficient of $\varepsilon_{260} = 559\,000\,M^{-1}\,cm^{-1}$ at 25°C. The sequences of the three ROX-labeled duplexes correspond to the glnAp2 promoter from *E.coli* and to the nifH and nifL promoters from *K.pneumoniae* (Fig. 1) (1). In the nifH and nifL promoters the 5 bp found adjacent to the ROX label were kept identical to the glnAp2 sequence in order to avoid differences in the spectroscopic properties of the ROX label.

RNA polymerase core enzyme from *E.coli* was purchased from Epicentre Technologies (Madison, WI). It was mixed with $\sigma^{54}$ in a ratio of 1:1.5 to form the RNAP-$\sigma^{54}$ holoenzyme at a stock concentration of ~1 nM. This preparation was stored at −20°C in a buffer containing 50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 50% glycerol. The $\sigma^{54}$ protein does not bind to the wild-type nifH and glnAp2 promoters in the absence of the core RNA polymerase (27,28). Only for a special mutant form of the nifH promoter (−17 to −15 sequence TTTT) has binding of isolated $\sigma^{54}$ ($K_d = 10^{-7}$ M) at moderate ionic strength been demonstrated (27). Thus, the presence of excess $\sigma^{54}$ protein should not affect the association of RNAP-$\sigma^{54}$ with the sequences studied here.

The activity of the purified DNA duplexes and the RNAP-$\sigma^{54}$ holoenzyme was confirmed in native gel electrophoresis. The purified DNA duplexes and ROX-labeled single strands were mixed with varying amounts of RNAP-$\sigma^{54}$ holoenzyme. Complexes and free DNA were separated on a 5% polyacrylamide gel (29:1) and visualized by ethidium bromide staining.

**Fluorescence anisotropy measurements**

Fluorescence anisotropy measurements were performed with a SLM 8100 fluorescence spectrometer (SLM Aminco Inc.) using an L-format setup. The ROX excitation wavelength of 580 nm was selected with a double grated monochromator using an 8 or 16 nm slit width for high intensity. In the emission channel scattered light was suppressed with a 610 nm cut-off filter. Intensity variations of the lamp were corrected by normalization to a reference channel with a rhodamine quantum counter. All measurements were conducted at 25°C in a buffer containing 20 mM HEPES–KOH, pH 8.0, 5 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA, 0.01% NP-40 detergent (Roche Diagnostics, Germany), supplemented with potassium acetate at a concentration from 50 to 350 mM.

According to the Perrin equation (29), the anisotropy of a fluorescent complex increases with its volume and reflects its rotational mobility. The assay used here is based on the rationale that the free DNA has a relatively low fluorescence anisotropy. This signal increases upon protein binding, due to the reduced rotational diffusion time after formation of the protein–DNA complex. The same approach has been used successfully in a number of studies (see for example 25,30–35).

The RNAP-$\sigma^{54}$ DNA binding activity was determined by stoichiometric titrations at a DNA concentration of 10 nM duplex in low salt binding buffer (50 mM potassium acetate) for high affinity binding. From the linear increase at low protein concentration and the plateau region obtained at saturation of the binding sites the equivalence point for the formation of a 1:1 complex was determined. In these experiments a DNA binding activity between 80 and 90% with respect to the core polymerase concentration given by the manufacturer was determined for different RNAP-$\sigma^{54}$ holoenzyme preparations (data not shown).

For the determination of dissociation constants a DNA solution with 25–200 pM duplex in binding buffer was titrated with a RNAP-$\sigma^{54}$ protein solution diluted into the same buffer. After addition of protein the sample was equilibrated for ~3 min before measuring the equilibrium anisotropy value. For each anisotropy value the average of 20 measurements with an integration time of 5 s was determined. To check whether the quantum yield of the ROX dye changed upon binding of the polymerase the fluorescence intensity of the free promoter DNA and the intensity after saturation of the DNA binding sites was recorded under polarization-independent ‘magic angle’ conditions (vertically polarized excitation and emission polarizer oriented at 54.7°) for every titration.
**Data analysis**

Equilibrium binding data were analyzed according to the reaction given in equation 1 for the formation of a 1:1 complex between RNAP-σ54 and the ROX-labeled duplex with the promoter sequence. The DNA duplex concentration \([P]\) in the experiments was chosen so that 10-[\(P\)] \(\leq K_d\). Under these conditions the concentration of free RNAP-σ54 \([R]\) can be approximated by the total polymerase concentration \([P]\), i.e. \([R] = [P] - [P_d]\), and the fractional saturation \(\theta\) of the promoter duplex with RNAP-σ54 is given by

\[
\theta = \frac{[P] - K_d}{[P] + K_d} = \frac{(r - r_p)/(r_p - r_p)}{[R]_\text{tot} + K_d}
\]

In equation 3 \(r\) is the measured anisotropy at a given polymerase concentration. The anisotropy values of the free promoter DNA and that of the \(RP\) complex are given by \(r_p\) and \(r_{RP}\), respectively. Rearrangement of equation 3 leads to equation 4, which was used to determine \(K_d\) from a least squares fit of the binding curve obtained by plotting \(r\) versus the added RNA polymerase concentration \([P]_\text{tot}\) with \(r_p\) and \(r_{RP}\) as additional fit parameters.

\[
r = \frac{(r_p - K_d)[P]_\text{tot} + r_p}{([P]_\text{tot} + K_d)}
\]

The least squares fit was computed with the program Kaleidagraph v.3.5 (Synergy Software, PA). The use of equation 4 is only correct if the quantum yield of the ROX dye does not change upon binding of RNAP-σ54, which was the case in the experiments reported here (see below).

**RESULTS**

**Gel analysis of ROX-labeled promoter DNA**

The oligonucleotide duplexes of 43 bp length shown in Figure 1 were studied with respect to their binding affinity for RNAP-σ54. The sequences correspond to the glmAp2 promoter from \(E.coli\) and the \(nifH\) and \(nifL\) promoters from \(K.pneumoniae\). All duplexes carried the fluorescent dye ROX at the 5'-end. Gel analysis of the purified duplexes is shown in Figure 2A (three left lanes) in comparison to the ROX-labeled single strands (Fig. 2A, three right lanes), which displayed a higher electrophoretic mobility. Both the ROX fluorescence signal as well as ethidium bromide staining of the duplexes revealed only a single band demonstrating that the synthesis and reconstitution of the promoter DNA sequences was successful.

The binding of RNAP-σ54 to these duplexes was qualitatively characterized in an electrophoretic gel mobility shift assay under conditions of stoichiometric binding (Fig. 2B). Both the RNAP-σ54 enzyme and the DNA promoter sequences were active with respect to binding as indicated by the almost fully shifted DNA fraction at an approximate 1:1 ratio of protein and DNA (Fig. 2B, highest protein concentrations).

**Binding affinity of RNAP-σ54 for glmAp2, nifH and nifL promoters**

In order to measure the dissociation constant \(K_d\) of RNAP-σ54 with the three different promoters under true equilibrium conditions and at defined ionic strength and pH the binding of RNAP-σ54 was followed by fluorescence anisotropy measurements. The polymerase was titrated into a solution of the ROX-labeled DNA duplex at a given salt concentration. Protein was added until all binding sites were saturated and the anisotropy reached a plateau value, which reflects the anisotropy of the 1:1 complex of RNAP-σ54 with the DNA. The resulting binding curve was fitted to equation 4 and \(K_d\) as
well as the anisotropies of the free promoter DNA (\(r_p\)) and the protein–DNA complex (\(r_{CP}\)) were obtained. As expected, similar average values of both \(r_p\) (0.165–0.169) and \(r_{CP}\) (0.244–0.266) were obtained for the three promoters indicating similar rotational diffusion times for the free DNA and its complex with the polymerase (Table 1).

The analysis of the binding curve according to equation 4 is only valid if the quantum yield of the ROX dye does not change upon binding of the polymerase (36). To test whether this was the case the fluorescence intensities of each sample before and after the titration were measured. After correction for dilution these intensity ratios corresponded to quenching factors \(q\) of 1.13 ± 0.1 (glnAp2), 1.08 ± 0.1 (nifH) and 1.02 ± 0.1 (nifL) (Table 1). Thus, within the accuracy of the measurements, the binding of RNAP\(\sigma^{54}\) to the promoter DNA did not change the ROX quantum yield so that the analysis of the data according to equation 4 is valid.

Figure 3 displays representative binding curves recorded by titrating the glnAp2, nifH and nifL promoters at an approximately physiologically ionic strength (200 mM potassium acetate and 5 mM magnesium acetate, \(I = 0.229\) M). The data were fitted to equation 4 to obtain \(K_d\), \(r_p\) and \(r_{CP}\) (Fig. 3A). The measured \(r\) values were then converted according to equation 3 into the fractional saturation of the DNA \(\theta\) (Fig. 3B). A good agreement of the measured data according to the 1:1 binding model described by equations 3 and 4 was obtained yielding \(K_d\) values of 0.7 ± 0.1 (glnAp2), 1.2 ± 0.1 (nifH) and 10.1 ± 1.1 nM (nifL) for the titrations shown in Figure 3. While the glnAp2 and nifH promoters displayed a similar affinity at an ionic strength of \(I = 0.229\) M, binding to the nifL sequence was about an order of magnitude weaker. Average values from multiple measurements are summarized in Table 2 with \(K_d\) values of 0.94 ± 0.55 (glnAp2), 0.85 ± 0.30 (nifH) and 8.5 ± 1.9 nM (nifL) at \(I = 0.229\) M.

In addition, whether the ROX-fluorophore affected the differences in binding affinities observed in the experiments described above was tested at the same ionic strength with unlabeled DNA duplexes. A 10 nM solution of preformed RNAP\(\sigma^{54}\) complex with a given ROX-labeled promoter DNA was titrated with unlabeled glnAp2, nifH or nifL duplex. The concentration of the unlabeled DNA which was required to displace 50% of the ROX–DNA from the complex with RNAP\(\sigma^{54}\) was determined. For titrating glnAp2–ROX with nifH and nifH–Rox with glnAp2 this concentration was the same within ~10%. Thus, the unlabeled glnAp2 and nifH promoter fragments displayed essentially the same binding affinities at physiological ionic strength. In contrast, the nifL promoter (nifL–Rox versus glnAp2, nifL–Rox versus nifH and glnAp2–ROX versus nifL) displayed a significantly weaker association with RNAP\(\sigma^{54}\).

**Salt dependence of RNAP\(\sigma^{54}\) promoter binding affinity**

When the experiments were performed at different salt concentrations in the range \(I = 0.079–0.379\) M \(K^+\) equivalents the value of \(K_d\) increased at higher ionic strength. This is due to a weakening of electrostatic interactions between protein and DNA as reviewed in Record et al. (37). An example of this type of experiment is given in Figure 4, which shows binding curves of the nifL promoter in buffer supplemented with

### Table 1. Fluorescence anisotropy parameters for the binding of RNAP\(\sigma^{54}\) to the glnAp2, nifH and nifL promoters

<table>
<thead>
<tr>
<th>Promoter</th>
<th>glnAp2</th>
<th>nifH</th>
<th>nifL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisotropy of free DNA ((r_p^a))</td>
<td>0.165 ± 0.02</td>
<td>0.169 ± 0.01</td>
<td>0.166 ± 0.01</td>
</tr>
<tr>
<td>Anisotropy of complex ((r_{CP}^a))</td>
<td>0.244 ± 0.03</td>
<td>0.260 ± 0.01</td>
<td>0.266 ± 0.02</td>
</tr>
<tr>
<td>Quenching factor (q^b)</td>
<td>1.13 ± 0.1</td>
<td>1.08 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

*aThe anisotropies of free \((r_p)\) and complexed DNA \((r_{CP})\) were derived from fitting the binding curves to equation 4.

*bThe quenching factor \(q\) was determined for every titration under polarization-independent conditions and averaged.
potassium acetate at a concentration of 50 mM ($K_d = 0.43$ nM), 150 mM ($K_d = 5.2$ nM) and 250 mM ($K_d = 22$ nM). A plot of the logarithm of the average $K_d$ values determined at a given salt concentration versus the logarithm of the ionic strength displayed an apparently linear relation (Fig. 5). The slopes of this plot −Δlog($K_d$)/Δlog($I$) were $6.1 \pm 0.5$ (glnAp2), $5.2 \pm 1.2$ (nifH) and 2.1 ± 0.1 (nifL). This means that the dissociation constant became weaker by a factor of $10^{5}$ (glnAp2), $10^{5.2}$ (nifH) and $10^{2.1}$ (nifL) per decade of higher ionic strength. Again, the glnAp2 and nifH promoters were indistinguishable within the accuracy of the measurements, whereas the nifL promoter showed a much weaker salt dependence.

### DISCUSSION

The promoter binding affinity of RNAP-$\sigma^{54}$ as expressed by the dissociation constant $K_d$ is an important parameter for analyzing the strength of different promoters. In addition, other steps involved in the activation pathway leading to the melting of the DNA at the transcription start site (open complex formation) could also be rate limiting (equations 1 and 2). These involve the interaction of the closed complex with the activator protein at the enhancer, the release of the block imposed by $\sigma^{54}$ to open complex formation as well as the kinetics of the isomerization step itself. Here we report data for the binding affinity of RNAP-$\sigma^{54}$ to the E.coli glnAp2 promoter and the two promoters nifH and nifL from *K.pneumoniae* (Fig. 1). Promoters for RNAP-$\sigma^{54}$ are characterized by the −24 consensus sequence 5′-(T/C)TGGCACG-3′ (~27 to −20) and a conserved 5′-TTGC(A/T)-3′ motif (~15 to −0) at position −12 (1). All three promoters have conserved residues from −27 to −20 and −13 to −12 residues, changes of which have been shown to strongly reduce binding of RNAP-$\sigma^{54}$ (11,27). The glnAp2 promoter most closely resembles the above consensus sequence (two deviations at −20 and at −14) whereas nifH (−23, −21 and −15) and nifL (−26, −22 and −20) have differences in three positions. A change of the G–C base pair at position −22 in the nifL sequence into the consensus A–T increased the expression level more than 2-fold (38). However, the simple assumption that the consensus sequence with conserved residues from −27 to −20 and −15 to −11 provides the highest rate of transcription initiation appears not

### Table 2. Dissociation constants $K_d$ for the binding of RNAP-$\sigma^{54}$ to the glnAp2, nifH and nifL promoters as determined by fluorescence anisotropy measurements

<table>
<thead>
<tr>
<th>Salt concentration</th>
<th>$K_d$ (nM) glnAp2</th>
<th>$K_d$ (nM) nifH</th>
<th>$K_d$ (nM) nifL</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM potassium acetate $I = 0.079$ M</td>
<td>0.76 ± 0.56 (5)</td>
<td>3.2 ± 1.7 (4)</td>
<td>4.6 ± 0.9 (2)</td>
</tr>
<tr>
<td>100 mM potassium acetate $I = 0.129$ M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mM potassium acetate $I = 0.179$ M</td>
<td>0.40 ± 0.04 (4)</td>
<td>0.50 ± 0.36 (3)</td>
<td>4.6 ± 0.9 (2)</td>
</tr>
<tr>
<td>200 mM potassium acetate $I = 0.229$ M</td>
<td>0.94 ± 0.55 (6)</td>
<td>0.85 ± 0.30 (3)</td>
<td>8.5 ± 1.9 (3)</td>
</tr>
<tr>
<td>250 mM potassium acetate $I = 0.279$ M</td>
<td>5.1 ± 2.3 (13)</td>
<td>2.4 ± 0.7 (4)</td>
<td>10 ± 8 (7)</td>
</tr>
<tr>
<td>300 mM potassium acetate $I = 0.329$ M</td>
<td>11 ± 9 (6)</td>
<td>16 ± 10 (3)</td>
<td>18 ± 13 (3)</td>
</tr>
<tr>
<td>350 mM potassium acetate $I = 0.379$ M</td>
<td>15 ± 11 (8)</td>
<td>40 ± 11 (3)</td>
<td></td>
</tr>
</tbody>
</table>

Average values for $K_d$ and corresponding standard deviations are given in nanomolar concentrations and were determined in binding buffer (20 mM HEPES–KOH, pH 8.0, 5 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA, 0.01% NP-40), supplemented with the indicated potassium acetate concentrations, yielding the indicated ionic strength $I$. The number in parentheses after the value of the dissociation constant refers to the number of experiments averaged.

### Figure 4. Representative binding curves of nifL at different ionic strength.

Data are shown for 50 mM (filled squares), 150 mM (open squares) and 250 mM (filled triangles) potassium acetate buffer. The concentration of DNA in the given buffer was 25 pM for the 50 mM potassium acetate buffer and 200 pM for the higher salt concentrations.

### Figure 5. Effect of ionic strength on binding affinity. All $K_d$ values for the glnAp2 (filled triangles), nifH (open squares) and nifL (filled squares) promoters are displayed in a double-logarithmic plot against the ionic strength $I$ (see Table 1). The lines correspond to linear regressions according to $\log(K_d) = -5.6 + 5.2 \log(I)$ (glnAp2), $\log(K_d) = -5.0 + 6.1 \log(I)$ (nifH) and $\log(K_d) = -6.8 + 2.1 \log(I)$ (nifL).
to be correct, as deduced from an in vivo comparison of 17 promoter sequences (11). In this context it is noteworthy that the glnAp2 promoter has a consecutive tract of seven A-T base pairs from −5 to +2 whereas for the nifL and nifH promoter only two or three A-T base pairs are found in this region (Fig. 1). This might lead to differences in the kinetics of strand separation during open complex formation.

It has been shown for RNAP-σ70 holoenzyme that the promoter affinity is strongly dependent on the ionic strength (39,40). Accordingly, the $K_d$ for RNAP-σ54 was quantitated at different salt concentrations. The ionic strength in E.coli varies between 0.17 and 0.3 M K⁺ equivalents (41,42) and an in vivo activity of divalent cations such as Mg$^{2+}$ between 1 and 10 mM has been estimated (43). Since Mg$^{2+}$ is also essential which is indicated in Figure 5. At this ionic strength average ~9 Å length from the RNAP´ length) separates the dye attached to a flexible alkyl linker of binding affinity as compared to the unlabeled DNA. For the large range of solution conditions (25,30±35). A potential binding measurements. Typical physiological conditions should correspond to an ionic strength around $\approx 0.23$ M, which is indicated in Figure 5. At this ionic strength average $K_d$ values of 0.94 ± 0.55 and 0.85 ± 0.3 nM for the nifH and glnAp2 promoters and 8.5 ± 1.9 nM for the nifL promoter were measured (Table 2). Thus, the nifL promoter displayed an about 10-fold lower binding affinity as compared to the two other promoters. The results for the glnAp2 and nifL promoters are in good agreement with previous footprinting experiments (18,21–23). However, the nifH promoter showed an unexpectedly high affinity under these conditions very similar to that of glnAp2 (Table 2). This is in contrast to the footprinting data, which revealed a much lower occupancy of the nifH promoter as compared to the glnAp2 promoter (19,20). Furthermore, a mutation of the CCC residues in the nifH promoter from −15 to −17 to TTT as in the glnAp2 sequence (Fig. 1) increased the footprinting protection (19). Mutations of C to T at −15 and −16 enhanced integration host factor (IHF)-independent gene expression in an in vitro transcription/translation assay (12) indicating that the binding affinity is also related to the promoter strength. In the fluorescence anisotropy binding experiments described here the glnAp2 and nifH promoters showed comparable $K_d$ values over the whole range of salt concentrations whereas the nifL promoter displayed a much weaker salt dependence. This excludes the possibility that the apparently very similar binding affinities of the glnAp2 and nifH promoters is restricted to a certain ionic strength, and it is unclear how the differences between our results and those of the footprinting experiments described in Morret and Buck (19) and Buck and Cannon (20) can be explained. The determination of binding affinities by monitoring changes in the fluorescence anisotropy of dye-labeled DNA duplexes as it has been used here is a true equilibrium method. It is applicable for accurate determinations of $K_d$ values over a large range of solution conditions (25,30–35). A potential source of errors could be an effect of the fluorescent dye on the binding affinity as compared to the unlabeled DNA. For the present set of duplexes a spacer sequence of 5 bp (~17 Å length) separates the dye attached to a flexible alkyl linker of ~9 Å length from the RNAP-σ54 binding region as determined by footprinting (44,45). Accordingly, a direct interaction between dye and polymerase appears unlikely but cannot be excluded, since the high resolution structure of the RNAP-σ54 closed complex is presently unknown. However, when preformed complexes of the ROX-labeled promoters with RNAP-σ54 were titrated with unlabeled glnAp2 and nifH duplexes no significant differences in their affinities were observed. Thus, we infer that the dye label had no effect on the relative binding affinities. It is also conceivable that residues outside the region protected in the footprinting experiments affect the binding affinity. In addition, the apparent contradiction between the fluorescence anisotropy and footprinting analysis of binding to the nifH promoter could be explained by a different mode of binding to nifH as opposed to glnAp2, which could lead to a change in the protection pattern.

A characteristic feature of protein–nucleic acid interactions is the strong dependence of $K_d$ on the ion concentration. The slope of the regression line $-\Delta \log(K_d)/\Delta \log(I)$ in the double logarithmic plot with $\log(K_d)$ versus the log of the salt concentration (Fig. 5) can be used to calculate the number of salt bridges between a protein and its DNA binding site in the absence of divalent ions (37,39,40,46,47). For the RNAP-σ70 closed complex at 0°C a value of 10.5 ± 1.5 has been determined with the T7 A1 promoter, which corresponds to about 12 salt bridges formed between polymerase and DNA (39). If Mg$^{2+}$ is present in addition to monovalent ions, it acts as a competitor with the negatively charged phosphate groups of the DNA backbone. This leads to a reduction in the apparent slope and some curvature in the log-log plot, especially at higher Mg$^{2+}$ concentrations (39,40). This effect is relevant for the binding studies described here, which were conducted in the presence of 5 mM MgCl₂. Since our data set did not display a significant curvature in the range of salt concentration studied the data were fitted with a linear regression line. Values of the slope of 6.1 ± 0.5 (glnAp2) and 5.2 ± 1.2 (nifH) were obtained (Table 1). From the data reported in Strauss et al. (39) and Shaner et al. (40) a value of $-\Delta \log(K_d)/\Delta \log(I) = 6$ in the range 0.1–0.4 M NaCl is estimated for RNAP-σ70 for a concentration of 5 mM Mg$^{2+}$. This suggests that similar numbers of salt bridges are formed in the closed complex of RNAP-σ54 with the glnAp2 and nifH promoters as compared to the RNAP-σ70 T7 A1 promoter. In contrast, the slope of the salt dependence of the nifL promoter was clearly reduced and a value of 2.1 ± 0.1 was measured (Fig. 5 and Table 1). This demonstrates that the number of ion pairs between RNAP-σ54 and the nifL sequence is significantly smaller as compared to the two other promoters. We conclude that the reduced strength of the nifL promoter can be explained by its weak binding affinity due to a reduced number of electrostatic interactions with this promoter DNA.

Apart from overall strength, the three promoters for RNAP-σ54 are also different in their response to intrinsic DNA curvature and bending induced by binding of IHF. On the basis of the available data it appears that transcription activation of a ‘strong’ promoter like glnAp2 by NtrC or NifA on superhelical templates is not facilitated by DNA curvature (48). In contrast, the nifH and nifL promoters showed a 3- to 20-fold increase in the equilibrium amount of open complexes in single round transcription experiments if a curved sequence or IHF-induced bending was present between enhancer and promoter (12,49–51). This observation can be explained by a model in which the strong glnAp2 promoter has a higher affinity than the nifH and nifL promoters (48), which is supported by footprinting experiments (18–23). The $K_d$ measurements reported here clearly demonstrate that the glnAp2 promoter has a significantly higher binding affinity
than the nifL promoter. However, the differences in promoter occupation reported previously between the glnAp2 and nifH promoters were not detected in our experiments, as discussed above. The about 20-fold stimulation of open complex formation by HIF or by an intrinsically curved DNA sequence at the nifH promoter on supercoiled DNA is not observed with the glnAp2 promoter (12,48,49,51). If the two promoters indeed have very similar binding affinities for RNAP\(\sigma^4\) other steps in the activation reaction that leads to open complex formation must be responsible for the observed differences with respect to promoter strength and the effect of DNA bending.

ACKNOWLEDGEMENTS

We acknowledge the support of Jörg Langowski. Part of the work by K.R. was done at the division Biophysik der Makromoleküle of the Deutsches Krebsforschungszentrum. The project was funded by the DFG (grant Ri 828-1) and by Makromoleküle of the Deutsches Krebsforschungszentrum.

REFERENCES

3. Reiter,L.J. and Magasanik,B. (1986) Transcription of glnA in E. coli is stimulated by activator bound to sites far from the promoter. Cell, 55, 785–792.


